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The Effect of Chronic Ethanol Administration on Organelles Involved in RNA/Protein Synthesis in Mature and Immature Hamster Neurons

Carole R. Dunmire-Graff
Loyola University Chicago

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THE EFFECT OF CHRONIC ETHANOL ADMINISTRATION
ON ORGANELLES INVOLVED IN RNA/PROTEIN SYNTHESIS
IN MATURE AND IMMATURE HAMSTER NEURONS

by

Carole R. Dunmire-Graff

A Dissertation Submitted to the Faculty
of the Graduate School of Loyola University of Chicago
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy

December, 1981
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To my mother, Jean Dunmire, and most of all to my father, Dr. Burt Dunmire, who continually encouraged me to strive for the Doctor of Philosophy degree. Without their faith in me and their loving support, this dissertation would never have seen fruition.
VITA

The author, Carole R. Dunmire-Graff, was born in Kittanning, Pennsylvania on May 8, 1951. In 1969, she graduated from Penn Manor High School in Millersville, Pennsylvania. She attended Randolph-Macon Woman's College in Lynchburg, Virginia, taking part in their Junior Year Abroad program at the University of Reading, England in 1971-1972. She was inducted into Phi Beta Kappa and graduated, magna cum laude, with a Bachelor of Arts degree in Biology in the spring of 1973.

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LaVelle and LaVelle (1975) have described in hamster facial motor neurons an intranucleolar body (INB) which holds promise of serving as an indicator of levels of RNA/protein synthesis in nerve cells. Light microscopic evidence has shown the INB to be a rounded, RNA-containing mass approximately $1.5\mu\text{m}$ in diameter and localized within the nucleolus of various types of adult hamster neurons (LaVelle and LaVelle, 1975; LaVelle, 1976). With electron microscopy this body appears as a tight aggregation of granules the size of ribonucleoprotein particles (Kinderman and LaVelle, 1976).

In these studies the labile nature of the INB has been demonstrated during normal postnatal development and during neuronal response to axotomy. Thus, it is known that the intranucleolar body is absent in rapidly growing cells and in cells undergoing the retrograde reaction; both are times of heightened RNA/protein synthesis and utilization. Only when the cells reach a "stable, mature state" does the INB appear, suggesting greater reserves of RNA/protein in this body. The consistent presence of a prominent INB in hamster neurons is unusual among laboratory animals (Jacob and LaVelle, 1977) and is postulated to represent a species difference in the balance between protein production and utilization in nerve cells.

The present study sought to determine whether INB structure in the adult neuron and general nucleolar development in the immature
neuron might be altered by exogenous chemical means. Ethanol was chosen as the chemical agent for two reasons: 1) biochemical evidence for its ability to alter RNA/protein metabolism in the brain has been demonstrated (Noble and Tewari, 1975; Tewari et al., 1975; Loh et al., 1977; Tewari et al., 1980), and 2) there has been renewed interest in determining the effects of alcohol on the human central nervous system, with particular emphasis on the relations between long-term ethanol use and brain damage in the adult and between chronic maternal alcoholism and neurological deficits seen in the fetal alcohol syndrome.

In this investigation, the effects of chronic ethanol administration have been studied cytologically in two neuronal populations of the adult and 20-day-old hamster. Counts and measurements aimed at assessing nucleolar changes at the population level were performed with light microscopy. A qualitative electron microscopic study was also undertaken in an attempt to correlate any observed histological changes with possible alterations in the subcellular structures known to be involved in RNA/protein synthetic activity.

The golden hamster, Mesocricetus auratus, was the animal of choice in these investigations because of the detailed knowledge of its normal nucleolar configurations (LaVelle and LaVelle, 1958a, 1959, 1975), its unique possession of a prominent intranucleolar body, and its preference for ingestion of alcohol solutions over water (Emerson et al., 1952; Arvola and Forsander, 1961, 1963). In addition, the hamster is particularly useful in developmental studies because of
its breeding facility, its short (16-day) gestation period (Boyer, 1953), the relative immaturity of its offspring at birth (LaVelle and LaVelle, 1959; Daly, 1976), and the rapid postnatal development of its central nervous system (Boyer, 1953; Lakars and Herring, 1980). (See Appendix A for numbers of animals utilized in the various segments of this study.)
REVIEW OF RELATED LITERATURE

THE NUCLEOLUS AND RNA/PROTEIN SYNTHESIS IN THE CELL

Role of nucleolus in RNA/RNP formation

The nucleolus is known to be responsible for the production of ribosomal ribonucleic acid (rRNA) and ribonucleoprotein (RNP), the bulk of which eventually pass from the nucleus into the cytoplasm as ribosomal components for utilization in protein synthesis (Busch et al., 1963; Busch and Smetana, 1970; Dalgarno and Shine, 1973; DeRobertis and DeRobertis, 1980). Indeed, a change in nucleolar size and/or organization appears to reflect a change in RNP production and protein synthetic activity (Stowell, 1963; Smetana and Busch, 1974; Adamstone and Taylor, 1979; Luetzeler et al., 1979). Most of the work in this field has been done by cytologists and molecular biologists using cultures of normal or malignant cells (frequently fibroblasts or leukocytes). (As examples, see Granboulan and Granboulan, 1965; Geuskens and Bernhard, 1966; Das et al., 1970; Royal and Simard, 1975; Hernandez-Verdun and Bouteille, 1979. See also, review by Bouteille et al., 1974.)

The nucleolar changes observed by these many investigators occur not only during normal growth and differentiation but also as a result of exposure to chemical agents known to inhibit or otherwise alter the synthesis and/or use of rRNA/protein by the cell. Among the agents
used have been actinomycin D (Connan et al., 1980; Magalhães and Magalhães, 1980; Quadagno et al., 1980), thioacetamide (Busch et al., 1963), α-amanitin (Barsotti et al., 1980; Connan et al., 1980), cycloheximide (Craig and Perry, 1970; Smetana et al., 1980), and a variety of carcinogens and antibiotics (Simard et al., 1974; Goessens, 1978; Calin and Dragomir, 1980).

Nucleolar nomenclature has been continuously modified as knowledge about the organization and function of the nucleolus increased (Swift et al., 1966; Jordan and Loening, 1977). It is generally agreed that the nucleolus consists of 1) a pars fibrosa containing 45S pre-rRNA molecules, 2) a pars granulosa containing 28S RNA and intermediary products of rRNA processing, 3) nucleolar "spaces" with no definitive structure, and 4) a pars amorpha or fibrillar center containing rDNA.

Ultrastructural localization of RNA transcription has occurred largely through the use of electron microscopic autoradiography of \(^{3}\text{H}\) uridine labeling of RNA (see recent review by Fakan and Puvion, 1980). It is now generally believed that the term "nucleolar fibrillar center" can be equated with nucleolar organizing region, although the transcription of its rDNA actually occurs in the dense fibrillar area just peripheral to the fibrillar center (Goessens and Lepoint, 1979). As precursor RNA is broken down and its products combine with protein to form RNP particles resembling cytoplasmic ribosomes, \(^{3}\text{H}\) uridine label shifts from the pars fibrosa to the pars granulosa region of the nucleolus. It is believed that the nuclear pores may be the sites of
final processing and assembly in ribosome formation (Franke and Scheer, 1974). Other work has indicated that the nucleolus is involved not only in the formation of ribosomal components but also may play a role in the transport of nucleolar RNA (rRNA) as well as in the transport of RNA made elsewhere in the nucleus (mRNA, tRNA) (Sidebottom and Harris, 1969; Jordan and Loening, 1977). Ribosomes in the cytoplasm are responsible for the synthesis of proteins, whether structural, enzymatic, or for purposes of export.

RNA and protein metabolism in the brain

The mechanisms of RNA-protein synthesis are generally believed to be similar among animals of different species, as well as in different organs of the same animal. However, there is increasing evidence that the specialized and complex functioning of the brain is closely tied to a certain uniqueness or complexity in its DNA, RNA, and protein content (Zomzely-Neurath and Roberts, 1972; Ozawa et al., 1980). RNA and protein turnover rates in the brain are quite rapid. Half-life values from 26 hours to almost 15 days have been reported for the turnover of the different classes of RNA (Bondy, 1966; Dawson, 1967), while half-life values for brain protein turnover range from 15 hours for some protein fractions to almost 20 days for others (Austin et al., 1972; Lajtha et al., 1976). In developing brain, RNA and protein metabolism appears to be even more rapid (Soto et al., 1974; Lajtha et al., 1979). Superimposed upon a maturational change in brain (and cellular) RNA
content are dynamic, reversible fluctuations in RNA levels that may occur as a result of functional demands placed upon a single nerve cell or a group of neurons (Hydén, 1967).

With regard to its protein synthetic capacity, brain tissue is not strictly thought of as being secretory in nature. In support of this is the fact that only approximately 15% of ribosomes in (rat) brain are membrane-bound (Merits et al., 1969). Furthermore there is increasing evidence that not all intracellular proteins are exclusively synthesized by free ribosomes. Tata (1973), working with rat cerebral cortex, demonstrated that a small proportion of its membrane-bound ribosomes differed in their mode of discharge of newly synthesized proteins. These ribosomes released their proteins directly into the cytosol for intracellular use rather than into the cisternae of the endoplasmic reticulum for export out of the cell. What significance this may have on the dynamic functioning of the brain during growth and following maturation remains to be seen.

Nucleolar development in the neuron

The complex interrelationships among the neuronal nucleus, nucleolus, and Nissl substance (rough endoplasmic reticulum), which culminate in protein synthesis and utilization, are only now beginning to be discussed in terms of molecular biology. Several years ago LaVelle (1951, 1956) became the first investigator to correlate convincingly nucleolar structure with the amount and form of Nissl
substance in the neuronal cytoplasm and also to catalogue the pro-
gression of cytologic changes which occurs in the nucleus, nucleolus,
and cytoplasm of a nerve cell as it matures. Large multipolar neurons,
such as those in the facial motor nucleus, undergo steplike changes in
nucleolar development. The cells initially contain multiple, homo-
geneous "chromatin bodies" located, for the most part, along the
periphery of the nucleus (LaVelle, 1956). With maturation, these DNA-
containing bodies became indented, and then fragmented, by non-DNA
material (RNA). In the large, adult motor neuron, there is usually
only a single, centrally-located nucleolus that is capped or ringed
by perinucleolar chromatin. Accompanying this maturation process in
the nucleus is one in the cytoplasm which involves the initial
appearance of Nissl substance as a fine basophilia (chiefly free
ribosomes) and its subsequent increase to form "clumps" of Nissl
material (stacks of rough endoplasmic reticulum).

LaVelle first detailed this sequence of developmental changes in
nerve cells of the fetal guinea pig and later reported the same
progression of changes in neurons of the early postnatal hamster
(LaVelle and LaVelle, 1958a, 1958b). Of interest is the fact that
stages of cellular (facial motor neuron) differentiation which occur
between days 28 and 47 gestation age in the guinea pig take place
between postnatal days 0 and 7 in the hamster. Cytological examina-
tion of many different adult neuronal cell populations led these
investigators to conclude that the final size and appearance of the
nucleolus, together with the quantity and distribution of Nissl substance, differ in various cell types. Facial motor neurons and other large multipolar cells display the most cytologically "advanced" nucleolar configurations and the largest amounts of Nissl substance, while many other mature cell populations (cortical pyramidal cells, cerebellar Purkinje cells, primary sensory neurons) possess less coarsely clumped Nissl material and, concomitantly, nucleolar configurations which are identical to the less advanced stages seen in developing facial neurons. At least in a cytological sense, therefore, all nerve cells do not express developmental potentiality to the same degree.

Subsequent studies on facial motor neurons in both developing and adult hamsters led to the hypothesis that successive stages in neuronal maturation can be correlated with the way in which the neurons react to injury (LaVelle and LaVelle, 1958a, 1958b; 1959; LaVelle and Smoller, 1960; LaVelle, 1963). The changing responses of these nerve cells to axotomy in utero and in various stages of postnatal life led LaVelle (1964) to conclude that such changes are based on "... a changing intracellular metabolism paralleling maturation ..." (p. 96). It is clear that the demands placed upon the protein-synthesizing organelles of a neuron change as the cell enlarges, spins out its dendritic tree, establishes synaptic contacts, and finally settles down to its adult needs for transmission and maintenance.

Apparent cytological maturity somewhat precedes the ability of a neuron to respond to injury in a completely mature manner. Thus,
LaVelle and LaVelle (1958b) observed that while axotomy of the facial nerve at 20 days postnatal age resulted in an "adult" type of response involving diffuse chromatolysis accompanied by nucleolar, nuclear, and somal swelling, axotomy at 15 days resulted in only limited chromatolysis without associated cell swelling. Yet the size and cytological appearance of 15-day and 20-day cells seemingly reflected a similar stage of maturity. In subsequent years, members of this laboratory accumulated further evidence with respect to developmental changes in cytoplasmic organelles (LaVelle, 1963; LaVelle and Sechrist, 1970), electrophoretic protein patterns (Griffith and LaVelle, 1971), and {\( ^{3}\text{H} \)} leucine incorporation into proteins (Griffith and LaVelle, 1971). These studies demonstrated that the final stage of maturation in hamster facial motor neurons involves a fundamental change in intracellular metabolism which is initiated during the critical period between 15 and 20 days postnatally.

**The intranucleolar body: An indicator of neuronal protein metabolism**

More recently LaVelle and LaVelle (1975) have described within hamster facial motor neurons an intranucleolar body (INB) which promises to be an even more sensitive index of both nerve cell maturity and reactive intracellular metabolism than previously documented cytological phenomena. The changes in the INB during cellular differentiation and following axotomy further support the hypothesis that the differential reaction to injury at critical developmental phases reflects different levels of neuronal metabolic capability.
At the light microscopic level the INB appears as a rounded, basophilic mass approximately 1.5μm in diameter and is localized centrally within the nucleolus of a variety of adult hamster neurons. The intranucleolar body was found to be Feulgen-negative but to stain with thionin, a nucleic acid stain. After extraction of RNA with perchloric acid this body was no longer visible, although perinucleolar chromatin (DNA) was still evident. After removal of all nucleic acid, the INB stained intensely with mercuric bromphenol blue, a non-specific protein stain. It was thus concluded that the INB is rich in RNA and protein but contains little or no DNA. With electron microscopy this body appears as a tight aggregation of granules which are of a size comparable to ribonucleoprotein particles (Kinderman and LaVelle, 1976). The composition and appearance of this body, therefore, differs from the "intranucleolar bodies" described by other investigators, notably Dutta et al. (1963); these latter bodies are eccentrically located and contain lipid and protein but no RNA. Moreover, this INB is only occasionally seen in other species thus far investigated: rat, mouse, guinea pig, dog, ground squirrel (Jacob and LaVelle, 1977; LaVelle, unpublished observations).

The labile nature of the intranucleolar body has been demonstrated during normal postnatal development and during neuronal response to axotomy. Developmentally, as described in the hamster facial motor nucleus, the INB begins to appear only after the major portion of the cells' growth has been completed (at approximately 20 days postnatal age). In adult neurons which have undergone axotomy, the INB disappears
during chromatolysis and reappears only after reconstitution of the Nissl substance (LaVelle and LaVelle, 1975; Kinderman and LaVelle, 1976). Accordingly, the INB is believed absent during intense metabolic activity such as the synthesis and utilization of proteins for growth and repair, and it appears during periods when there is less apparent need for concentrated production and use of protein. In effect, it may be a storage reserve of early precursors in the protein synthetic chain. Measurements of \([H^3]\) uridine and \([H^3]\) leucine uptake in developing and mature facial motor neurons after axotomy provide further evidence that the absence of an INB reflects a high rate of turnover of nucleic acid and protein precursors, with the nerve cell operating at maximal synthetic capacity (Kinderman and LaVelle, 1977; McLoon and LaVelle, 1981a, 1981b). Likewise, the presence of an intranucleolar body seems to indicate that a cell is functioning at a level below its maximal protein synthetic capacity and thus is able to sequester a portion of its nucleolar RNA/RNP for use at another time.

The cytological work previously done by the LaVelles on neuronal nucleolar development finds parallels with ultrastructural and biochemical studies on the role of the nucleolus in RNA/RNP production and subsequent protein synthesis. Integrating this information has led to the hypothesis that there is a definite correlation between the appearance of the nucleolus/INB and the balance of synthesis/utilization of protein within the cell. The fact that the intranucleolar body is a
constant feature of many different types of cells in the hamster brain, coupled with the evidence for its quite labile nature, suggest its significance as a cytomorphological indicator of alterations in nucleolar, and therefore cellular, RNA/protein metabolism.

METABOLIC EFFECTS OF ETHANOL CONSUMPTION ON THE ADULT BRAIN

Within the last ten years, a number of investigators have shown that the rate and extent of protein synthesis in brains of adult animals can be altered through the acute and/or chronic administration of ethanol. Since protein production is of considerable significance in neuronal metabolism, the effects of alcohol become a matter of clinical importance as well. Nerve cells not only engage in standard maintenance of their soma and processes and all related membranes but are also involved in the synthesis of neurotransmitters, the conduction of nerve impulses, and synaptic transmission. Moreover, ribonucleic acids and proteins are increasingly thought to have distinctive roles in the functions of learning and memory.

Metabolism of ethanol in the body

In the United States at least half of the adult population consumes alcohol, while an estimated 10 million Americans are considered to be alcoholics (Halsted, 1980). Directly or indirectly, ethanol is known to affect almost every body organ except the ear and the joints (Edmondson, 1980). After ethanol is orally ingested, it is readily
absorbed into the bloodstream by the stomach and intestine. Since a mere 2-10% is eliminated through the kidneys, skin, and lungs, the remainder is oxidized in the body, principally in the liver (Lieber, 1977). The alcohol molecule is highly water soluble and relatively lipid soluble, and thus it is able to distribute itself freely throughout the body water and to diffuse easily across biological membranes (Wallgren, 1970). In an organ with a rich blood supply and high rate of blood flow (such as the brain), equilibrium is rapidly established between alcohol in the blood and alcohol in the tissue.

The toxic effects of alcohol are extremely variable and differ from individual to individual, organ to organ, and cell to cell. The mechanisms by which alcohol exerts its damage are not well understood. It has been postulated to exert a dual mode of action on the body: 1) as a central nervous system depressant, acting much like a general anesthetic, and 2) as an "aberrant nutrient" which, by its rapid oxidation, can disrupt a variety of normal metabolic processes (Dietrich, 1975; Majchrowicz, 1975). These two roles are, without question, inextricably related.

Since the liver plays the primary role in the metabolism of ethanol, it serves as a major target for ethanol's effects, particularly its long-term effects. The liver cell has three main pathways for ethanol metabolism: the aldehyde dehydrogenase (ADH) pathway of the cell cytosol, the microsomal ethanol oxidizing system of the endoplasmic reticulum, and the catalase pathway of the cell's peroxisomes (Plapp,
The ADH pathway is by far the major route for ethanol elimination and involves the oxidation of ethanol to acetaldehyde and the subsequent oxidation of acetaldehyde to acetate. Most of the acetate is then released for extrahepatic metabolism to carbon dioxide and water. The oxidation of ethanol not only upsets the ratio between nicotinamide adenine dinucleotide (NAD) and reduced NAD (NADH) in the liver, but it also causes an increase in acetaldehyde and acetate levels. Consequently energy metabolism, as well as a number of other metabolic pathways, are affected. In other words, "... the oxidation of ethanol is not regulated and [it] monopolizes metabolism" (Plapp, 1975, p. 79).

Metabolism of ethanol in the brain

Brain ADH activity has been shown to be 1/4000 of that in the liver, and the role of other enzymes in brain ethanol metabolism have not been conclusively established. Therefore, it is expected that metabolic effects of ethanol on the brain and liver are quite different (Noble and Tewari, 1977). If any redox changes occur in the brain following ethanol administration, they are exceedingly minor as compared to those occurring in the liver. It is known that the brain has the capacity to oxidize the ethanol metabolite acetaldehyde, although the amount of acetaldehyde that reaches the brain after ethanol consumption is believed to be small (Tabakoff and Gelpke, 1975). Moreover, the effects of acetaldehyde on the brain are just beginning to be examined, and the effects of increased acetate levels on the brain are virtually
unknown. Evidence for the metabolism of ethanol in the brain and for the functional significance of such metabolism is slight (Cohen et al., 1980). What appears to be a more likely possibility is that ethanol may produce discrete but significant metabolic changes in particular brain regions, cell types, or specific organelles.

**Effects of ethanol on the brain**

Studies dealing with the effects of alcohol on the brain are impeded by the extreme heterogeneity and complexity of this organ. Biochemists often must treat the brain as a homogeneous tissue or must assume that it is composed of different regions which contain the same cellular constituents. The results obtained using such approaches can mask any significant effect that ethanol may exert on small, discrete brain regions or cell types. On the other hand, physiological and cytological studies may be more specific in their design, yet they can become so specific as to ignore crucial factors that are interacting to produce the eventual results that are obtained. A coalescence of various disciplines and an awareness of the complexities involved are essential to investigations in this field.

Most studies concerned with the effects of ethanol on the brain have been carried out on rodents and have dealt with energy metabolism, neuroreceptor-neurotransmitter systems, electrical activity, physiological responses, endocrine effects, alterations in carbohydrate and lipid metabolism, cyclic nucleotide levels, ion concentrations, prostaglandin metabolism, and specific morphological changes. (For reviews see
Kalant, 1970; Schenker, 1970; Kalant, 1975; Rawat, 1975a; Gordon and Southren, 1977; Noble and Tewari, 1977; Ciofalo, 1980; Horrobin, 1980; Karwacka, 1980a, 1980b; Karwacka et al., 1980; Van Thiel, 1980.) They have also been concerned, to a certain extent, with alterations in amino acid and/or RNA/protein metabolism.

In view of the multiple actions of ethanol on brain metabolism and function, it seems increasingly unlikely that a single target site for ethanol will be found. The most likely common locus in the brain may involve neuronal membrane structure and function (Wallgren, 1970; Majchrowicz, 1975; Rawat, 1975a). Alcohol, like many other anesthetics, appears to increase the fluidity or disorder of the membrane and thereby affect its lipid and/or protein components. Various theories have been proposed as to mode of action of the ethanol molecule at the membrane level (Trudell, 1977; Roth, 1979). Gruber et al. (1977) have, in fact, shown that chronic ethanol consumption results in a conformational change in microsomal membranes isolated from rat brain. However, the means by which the action of ethanol on neuronal membranes could account for some or all of the alterations listed above remains to be shown.

A variety of neuropsychological tests have been used on alcoholics and have yielded similar findings of deficits in abstract conceptualization, memory disturbances, motor deficits, and visual-spatial deficits (Chmielewski and Golden, 1980; Hill, 1980; Wilkinson and Carlen, 1980). The results of these tests have been utilized as a basis for defining possible areas of brain damage due to chronic alcoholism. With the
advent of computerized axial tomography (CAT), it has been possible to obtain much more conclusive evidence of brain damage in alcoholics. In particular, CAT scans have shown that alcoholism results in cerebral atrophy (most frequently involving the frontal lobes) and cerebellar atrophy (primarily affecting the superior vermis), although it may cause atrophy in other brain areas as well (Haubek and Lee, 1979; Cala and Mastaglia, 1980; Edmondson, 1980; Lusins et al., 1980; Carlen et al., 1981).

The neuropathology of alcoholism in man includes Wernicke-Korsakoff's syndrome, among other organic brain conditions (see review by Lishman, 1981). Specific anatomical lesions have been found in the mamillary bodies, cerebellar vermis, dorsal and medial thalamus, hypothalamic nuclei, cerebral cortex, optic nerves, spinal cord, basal ganglia, corpus callosum, vestibular and oculomotor nerve nuclei, hippocampus, and amygdala (Goodwin et al., 1975; Bennett, 1977; Alling and Boström, 1980; Edmondson, 1980; Oscar-Berman, 1980; Sato et al., 1981). Even the peripheral nervous system is affected by chronic alcohol ingestion, the most serious occurrence being vagal neuropathy (Duncan et al., 1980; Turnbull et al., 1980).

Animal studies have also pinpointed areas of the brain where ethanol may have a more selective effect. Fabre et al. (1973) administered an intravenous dose of $[^{14}\text{C}]$ ethanol into mice and monkeys and found that ethanol accumulated in various brain regions to different extents, being most concentrated in the cerebellum (including cerebellar nuclei), hippocampus, putamen and caudate nuclei, gray matter of the
cerebral cortex, nuclei of the pons, lateral geniculate nucleus, pineal, and pituitary gland. Ethanol has been shown to affect the spontaneous discharge of several groups of neurons, including cerebellar Purkinje cells and neurons of the lateral vestibular nucleus in the cat, rat, and mouse (Eidelberg et al., 1971; Ikeda et al., 1980; Rogers et al., 1980; Sinclair et al., 1980; Sorensen et al., 1980), as well as cells in the dorsal hippocampus in the rat (Grupp and Perlanski, 1979), the posterior parietal association cortex of the monkey (Hyvärinen et al., 1978), the locus coeruleus in the rat (Pohorecky and Brick, 1977), and the cochlear nucleus and auditory cortex of the cat (Sutko and Weinberger, 1979). The effect seen is usually one of inhibition, although some investigators report excitation, particularly with the administration of lower doses of ethanol.

Reports of morphological alterations in specific brain regions after ethanol consumption are sparse. Riley and Walker (1978) utilized a modified Golgi-Kopsch technique to show that long-term ethanol consumption in mice results in a 50-60% loss of dendritic spines on hippocampal pyramidal cells and dentate gyrus granule cells, as well as a marked decrease in the dendritic arborization of Purkinje cells. Yet it should be noted that Lee et al. (1981), performing both quantitative electron microscopic and electrophysiological analyses, were unable to confirm these findings in the rat hippocampus. Walker et al. (1980) subsequently carried out a quantitative light microscopic study of cresyl violet- and hematoxylin and eosin-stained tissue from rats which
had undergone chronic ethanol ingestion and found approximately a 16% loss of hippocampal pyramidal cells and a 20% loss of dentate gyrus granule cells. From comparison of the findings in human studies with those involving animal experimentation, it can be concluded that at least some regions of the brain exhibit a heightened sensitivity to the presence of ethanol.

Biochemical evidence for the effects of ethanol on brain RNA/protein synthesis

Virtually all of the studies concerned with the effects of ethanol on the synthesis of RNA/protein in adult brain have been biochemical in nature, and the results have been contradictory. Much of the variability in these data is due to differences in 1) the species or strain studied, 2) age of the animal, 3) nutritional status, 4) method of ethanol administration, 5) amount, duration, and continuity of ethanol administration, 6) area(s) of brain investigated, and 7) cellular and subcellular components examined. Moreover, the multitude of factors which participate in RNA and protein metabolism, coupled with the inherent heterogeneity of the brain, make determination of the effects of ethanol even more difficult (Loh et al., 1977).

The effect of acute ethanol administration on brain protein synthesis has not been sufficiently investigated to permit any definite conclusions. Using in vitro preparations, Renis and his co-workers (1975) found no significant change in rat brain protein synthesis,
while Lamar (1972) and Kuriyama et al. (1971) found significant decreases in protein synthesis in rats and mice, respectively. Clearly, additional studies are needed in this area.

The effect of chronic ingestion of ethanol on protein synthesis has been examined to a greater extent. Although Kuriyama et al. (1971) found increased ribosomal protein synthesis in mouse brain following chronic ethanol consumption for one or two weeks, the majority of experimental evidence points to a depression of brain protein synthesis when ethanol is chronically administered to mice or rats for periods of ten days to eight months (Tewari and Noble, 1971; Jarlstedt, 1972; Noble and Tewari, 1973; Renis et al., 1975; Morland and Sjetnan, 1976; Tewari et al., 1977; Tewari et al., 1980). These studies have examined the incorporation of labeled amino acids into both free and membrane-bound ribosomal proteins and have yielded contradictory results. Khawaja et al. (1978a) and Lindholm and Khawaja (1979) reported decreased incorporation into cerebral membrane-bound ribosomes and an increased incorporation into cerebral free ribosomes after ingestion of ethanol by rats for two weeks. On the other hand, Noble and Tewari (1975) found that chronic ingestion for seven weeks by mice resulted in decreased incorporation of protein into both free and membrane-bound brain ribosomes, with free ribosomes showing the greater decrease.

Tewari and Noble and their co-workers have performed a number of detailed biochemical studies over the last ten years in an effort to determine the possible mechanism(s) of the inhibitory effect
of ethanol on brain protein synthesis. For the most part, they have administered a 10% (v/v) ethanol solution as the sole fluid to C57 mice for periods ranging from 10 to 81 days, with removal of alcohol 24 hours before sacrifice. Mice on this diet gained weight normally, exhibited no gross nutritional deficiencies, and showed no evidence of withdrawal symptoms after ethanol removal. In 1971 Tewari and Noble reported, on the basis of cross-over experiments performed among control and alcohol pH5 enzyme and ribosomal fractions, that the major inhibitory effect on the brain protein synthesizing system was located in the pH5 enzymes fraction (source of tRNA, activating enzymes, unlabeled amino acids, etc.). However, they have also found evidence of an inhibitory effect on ribosomes, showing in 1975 that free as compared to membrane-bound ribosomes were more adversely affected. In 1973 they reported that aminoacyl tRNA synthetase function was also altered.

Noble and Tewari performed many of these same experiments with rats made physically dependent on the alcohol contained within a purely liquid diet. Again they found evidence of an inhibition of protein synthesis and defective ribosomal function after chronic ethanol consumption (Noble and Tewari, 1975; Tewari et al., 1977; Tewari et al., 1980). Their work has demonstrated not only that brain protein synthesis is inhibited after chronic ethanol ingestion but also that ethanol has multiple detrimental effects on this synthetic process.

The only work that has been conducted with regard to the effects of ethanol on brain RNA synthesis has been that of Noble and Tewari on
mice and rats chronically ingesting ethanol solutions (Noble and Tewari, 1973, 1975; Tewari et al., 1975; Tewari and Noble, 1977; Tewari et al., 1978). Noble and Tewari (1973) injected [5-3H] orotic acid intraven­tricularly into control and chronic alcohol-imbibing mice and subsequently examined subcellular fractions of brain tissue for in vivo incorporation of label into RNA. They found that ethanol inhibited labeling of RNA in all fractions (nuclear, cytoplasmic, ribosomal, polysomal, and pH5 enzymes). With the nuclear fraction, however, they discovered a biphasic response, i.e., an initial marked increase followed by an eventual decrease in incorporation of label into RNA. This finding led them to suggest that there may be a defect in the nuclear membrane and/or in other related transport mechanisms such that newly synthesized RNA is unable to pass out of the nucleus into the cytoplasm, thus leading to an initial accumulation of (labeled) RNA within the nucleus. However, with time and in the presence of an ethanol-induced decrease in nuclear and/or nucleolar RNA synthesis, this condition could be reversed.

Further work (Noble and Tewari, 1975) demonstrated that chronic alcohol consumption in mice resulted in a decreased in vitro incorporation of [5-3H] uridine into RNA of free polysomes and an increased incorporation into RNA of membrane-bound ribosomes. Consideration of the different half-lives of the various classes of RNA led them to conclude that their results reflected incorporation of label into rRNA. Since free ribosomes comprise the major portion of brain ribosomal populations, they reasoned that the combined effect of free and membrane-
bound ribosomes should result in a decrease in RNA synthetic capacity of ethanol-treated brains. Moreover, this differential effect on brain ribosomal components could affect the synthesis of certain proteins that play specific roles in brain function. Tewari et al. (1975) demonstrated that the availability or synthesis of nucleotides was not responsible for ethanol's effects on RNA metabolism. Rather, ethanol appears to have a direct effect on the transcription and/or subsequent processing of RNA in the nucleus.

In 1977, Tewari and Noble were able to monitor the in vivo incorporation of [5-3H] orotic acid into RNA of the soluble nuclear (mRNA or HnRNA) and particulate nuclear (rRNA) fractions. They showed that both nuclear subfractions exhibited a decreased ability to incorporate label into protein when they were isolated from brains of chronic alcohol-ingesting animals. They also found evidence of decreased formation of polyadenylic acid, poly (A) being an essential component of mRNA.

Finally, Tewari et al. (1978) demonstrated that chronic ethanol consumption inhibits the incorporation of [5-3H] orotic acid into brain mitochondrial RNA, specifically tRNA and rRNA located within the inner membranes of brain mitochondria. It appears, then, that chronic ingestion of alcohol by rodents alters the transcription, transport, and/or overall metabolism of all three classes of RNA in the brain, viz. mRNA, tRNA, and rRNA. As a consequence, the capacity of brain tissue to synthesize protein appears to be diminished. Whether such
alterations occur in all nerve cells in all areas of the brain cannot be determined from biochemical studies which utilize whole brain. Furthermore, such studies do not differentiate between neuronal, glial, and endothelial components of the tissue; work by Pevzner (1972, 1981) has indicated that in at least some brain regions, neurons and glia do differ in their RNA and protein metabolic responses to stress.

Morphological evidence for the effects of ethanol on brain RNA/protein synthesis

Histological or ultrastructural evidence for the effect(s) of ethanol on RNA/protein synthesis in the adult brain is extremely sparse. As a supplement to his biochemical studies, Jarlstedt (1972) examined methylene blue- or cresyl violet-stained sections of cerebral cortex and cerebellum from rats which had ingested a 15% ethanol solution for eight months. He found no evidence of neuronal or glial cell degeneration nor any indication of abnormal histology, even though biochemical analysis of the same tissue revealed a depression of protein synthesis. Light and electron microscopic investigations of spinal ganglia and parietal cortex tissue from rats which had consumed 15% ethanol for six months revealed an increase of intranuclear "lattice-like" inclusions, lipofuscin, and nematosomes in various neurons (Volk, 1978, 1980). Volk also observed that some nerve cell somas contained a filamentous type of material which at the ultrastructural level was found to represent collections of paired helical filaments. He concluded that the nuclear
inclusions and paired filaments could be morphological evidence of an impaired cellular metabolism resulting from chronic ethanol ingestion; in particular, altered neuronal protein synthesis was mentioned.

Karwacka (1980b) administered increasing concentrations of ethanol (2.5-25%) to rats for periods of four, eight, or twelve weeks and subsequently examined the frontal and temporal lobe cortices ultrastructurally. Degeneration of cells in the cerebral cortex was not seen, although organellar structure was altered in many instances. The extent of damage increased with the dose and duration of ethanol consumption. A decrease in the number of ribosomes and polysomes, particularly in the regions of the cell nucleus, was reported along with some reduction in the amount of rough endoplasmic reticulum. A proliferation of the smooth endoplasmic reticulum and an expansion of the cisternae of both smooth and rough endoplasmic reticulum were also found. The most striking changes described, however, were in the mitochondria. These organelles greatly increased in size, assumed bizarre shapes, possessed fewer cristae, and contained myelin-like "whorls". Such alterations could provide morphological evidence for disturbances in neuronal energy metabolism and protein synthesis. To date, no other investigations of this nature have been published.

MATERNAL ETHANOL CONSUMPTION AND ITS EFFECT ON THE BRAINS OF OFFSPRING

Historical perspective: Clinical recognition of the fetal alcohol syndrome (FAS)

Awareness of the adverse effects of maternal ethanol ingestion on
offspring viability and development dates back at least until the time of the Greek and Roman civilizations (see Newman and Correy, 1980). Yet not until the period of England's "gin epidemic" (1720-1750) did any actual observations of the effects of ethanol consumption on pregnancy and its outcome survive (Warner and Rosett, 1975). In America, such a topic was not even mentioned in the literature until the nineteenth century. Although many of these early findings were inconclusive, medical and experimental studies examining ethanol's effects on the fetus slowly accumulated until 1920 and Prohibition. Then, for the next 40 years, there was not only a striking drop in the reporting of prenatal influences of alcohol but also a discounting of the findings from earlier investigations. The decade of the 1960's finally brought with it a gradual "rediscovery" of the detrimental effects of alcohol on the fetus and neonate, yet the actual number of published studies on the subject remained small until the 1970's.

The work which provided the real impetus for research in this field was that of Jones et al. in 1973. Eight clinical cases were presented in an effort to establish convincingly the association between maternal alcoholism and abnormal offspring. Jones and Smith (1973) provided three more clinical cases along with the first post-mortem examination of an infant whose mother had been a chronic alcoholic. Autopsy of this infant yielded evidence of aberrant brain development, including altered neuronal migration, and this appeared to offer at least a partial explanation for the growth retardation and developmental
delay that are now recognized to be so marked in these children. Jones and Smith (1973) became the first individuals to use the term 'fetal alcohol syndrome' or 'FAS'. As Warner and Rosett (1975) stated, it was approximately at this time that a study done in France in 1968 by Lemoine et al. was uncovered. This group had studied 127 offspring of chronic alcoholic mothers and had found an increased incidence of growth retardation, psychological and motor disturbances, and various malformations. The 1968 and 1973 studies had been carried out independently of each other, yet their findings were notably similar. The import of such work is attested to by the voluminous number of clinical and experimental investigations that have appeared in the literature since 1973 and which have unequivocally established the concept of a fetal alcohol syndrome.

Maternal consumption of alcohol has been found to result in an entire spectrum of perinatal effects, ranging from mild to quite severe, in addition to a great variety of malformations. Alcohol appears to have the potential to cause a wider range of adverse effects on the fetus than any other commonly used drug. Certain characteristic features are seen sufficiently often with FAS, however, that they can be grouped into four categories and used for diagnosis of this condition: 1) central nervous system involvement, 2) pre- and/or postnatal growth retardation, 3) a characteristic facial dysmorphology, and 4) variable major and minor congenital malformations involving almost any organ system (Clarren and Smith, 1978).
The incidence of FAS is currently believed to be in the range of 1:600 to 1:1500 live births, with risk to the unborn fetus of a chronic alcoholic mother being anywhere from 30-50% (Rosett et al., 1981). The National Institute on Alcohol Abuse and Alcoholism has projected that FAS is the third leading cause of birth defects with associated mental retardation in the United States (Small, 1979).

Currently it is not known 1) whether ethanol itself or one of its metabolites is the teratogenic agent, 2) how much of a role (if any) nutritional deficiency plays in manifestation of this syndrome, 3) whether ethanol's effects are potentiated by other drugs or additives present in the alcoholic beverage(s) consumed, 4) which organ systems in which stages of pregnancy may be the most sensitive to ethanol insult, or 5) whether there is some type of genetic predisposition for development of FAS.

It is known, however, that alcoholic women have a higher risk of spontaneous abortion (Sokol, 1980), have increased chances of infection and premature placental separation during labor (Rosett, 1980; Sokol et al., 1980), and have a higher percentage of pre-term deliveries (Olegård et al., 1979). Children born to these mothers show increased evidence of neonatal depression and have a higher mortality rate, decreased birth weight, increased congenital anomalies, and even display dermatoglyphic abnormalities (Olegård et al., 1979; Qazi et al., 1980; Sokol et al., 1980). Such features appear to occur independently of medical care, nutritional status, smoking, parity, age, and other such variables. Alcoholic women who
either significantly decrease their alcohol intake or abstain from alcohol during pregnancy give birth to infants with less growth retardation, although almost always there is still evidence of impaired brain development even with maternal abstention (Olegård et al., 1979; Little et al., 1980; Rosett et al., 1980).

What should be of critical importance to any pregnant woman is that no absolutely safe level of ethanol ingestion during pregnancy has been established. There is increasing evidence that even moderate or so-called "social drinking" can have adverse effects on a mother's unborn child, manifesting itself at parturition in decreased birth weight and length, decreased head circumference, increased major and minor malformations, deficits in mental and motor development, and decreased sucking frequency and sucking pressure (Little, 1977; Hanson et al., 1978; Martin et al., 1979; Streissguth et al., 1980). Such clinical studies suggest that there may be a continuum of effects over the range of maternal ethanol consumption patterns, with minor growth disturbances in the infant at one end of the spectrum and clinical diagnosis of complete FAS expression at the other. In order to be safe, the Food and Drug Administration has advised that a pregnant woman should not consume more than two drinks on any single occasion (Small, 1979).

Experimental (animal) evidence of the fetal alcohol syndrome

Animal models of the fetal alcohol syndrome are particularly
useful since they permit control of variables that are difficult to isolate in human studies. Such variables include the dosage and timing of ethanol exposure, nutritional status of mother and offspring, postnatal environmental influences, genetic differences in susceptibility to teratogenic actions of ethanol, and the effects of other drugs and smoking. Perhaps most important is the fact that animal studies can help define the critical developmental periods for, and specific mechanisms behind, ethanol-induced teratogenesis.

Pregnant, as compared to non-pregnant, women have lower peak blood alcohol levels after alcohol ingestion, yet these levels are maintained longer due to delayed stomach emptying and decreased intestinal motility during pregnancy (Newman and Correy, 1980). Since alcohol distributes itself according to the water content of tissues, the effects of alcohol on the fetus vary according to changes in water content throughout pregnancy. The highest fetal water concentrations are in early gestation (Newman and Correy, 1980). It has been shown in several species (including man) that ethanol readily diffuses across the placenta from mother to fetus and results in rapid equilibration of ethanol between maternal and fetal blood (Baker, 1960; Mann et al., 1975; Ayromlooi et al., 1979). Thus, so to speak, if the mother is drunk, so is the fetus. It has also been shown that the infant receives almost the same ethanol concentration through its mother's milk as is found circulating in her blood, at least at lower blood levels of alcohol (Kesäniemi, 1974).
Obe and Ristow (1979) stated that alcohol dehydrogenase (ADH) activity in human fetuses is 3-4% that of an adult's and only begins to rise in later pregnancy. Likewise in rats it has been shown that ADH and ALDH (aldehyde dehydrogenase) activities only begin to be evident at approximately 17 or 18 days gestation and do not reach adult levels until two to three months after birth (Rawat, 1976; Horton and Mills, 1979). As a consequence, fetal and early neonatal animals, although exposed to the same blood ethanol levels as their alcoholic mothers, are not able to metabolize this ethanol to any sufficient degree. The potential for tissue damage is correspondingly much greater in these developing animals.

It has been stated that C.F. Hodge in 1903 became the first "English-speaking scientist" to attempt to utilize an animal model (the cocker spaniel) to examine the effects of maternal alcohol consumption on the ensuing progeny (see Warner and Rosett, 1975). Since this time, results from studies using a variety of animal species have provided support for the belief that ethanol does adversely affect the fetus. The primary species utilized have been the rat (Tze and Lee, 1975; Rosman and Malone, 1976; Henderson and Schenker, 1977; Volk, 1977; Abel and Greizerstein, 1979; Brown et al., 1979; Jacobson et al., 1979; Kornguth et al., 1979; Leichter and Lee, 1979; Woodson and Ritchey, 1979; Osborne et al., 1980; Sherwin et al., 1980; Uttrich and Dietzmann, 1980) and the mouse (Chernoff, 1975, 1977, 1980; Kronick, 1976; Boggan and Randall, 1979; Randall and Taylor, 1979; Yanai and Ginsburg, 1979;
Rasmussen and Christensen, 1980; Fish et al., 1981). Others have used the sheep (Potter et al., 1980), miniature swine (Dexter et al., 1980), and the beagle dog (Ellis and Pick, 1980) as well as the chicken, the guinea pig, the rabbit, and the pigtail macaque (Randall, 1979; Newman and Correy, 1980; Streissguth et al., 1980).

The effects obtained in these studies can be grouped into six categories: 1) reduction in maternal reproductive capacity (decreased number of conceptions, increased numbers of resorptions and stillbirths, lengthening of gestation period, decreased litter size, decreased numbers of male offspring), 2) congenital malformations (eye-lid and other facial dysmorphism, soft tissue and skeletal abnormalities, lung irregularities, and neural, cardiac, and urogenital deformities), 3) growth retardation and developmental delay (decreased birth weight and length, decreased postnatal weight gain, impaired suckling capability, decreased postnatal viability, increased variability in pup size with majority of "runts", delay in attainment of developmental parameters), 4) changes in fetal-neonatal metabolic parameters (increased fetal body water, decreased fetal lipid free solids, decreased or highly variable total body DNA, RNA, and protein contents, alterations in body levels of trace elements, changes in postnatal liver metabolism), 5) behavioral modifications (hyperactivity, increased emotionality, enhanced reactivity and seizure susceptibility), and 6) evidence of learning/memory deficits.

Results obtained from animal studies have also demonstrated that maternal ethanol consumption during the suckling period alone can cause
postnatal growth retardation and maldevelopment in mice (Swanberg and Wilson, 1979) and in rats (Pilström and Kiessling, 1967; Bauer-Moffett and Altman, 1975, 1977; Martin et al., 1977; Anderson and Sides, 1979; Antal et al., 1979; Rider, 1979, 1980; DaSilva et al., 1980; Diaz and Samson, 1980).

Although the majority of animal research confirms the human studies as to the teratogenic effects of alcohol, there are a number of investigators who have published negative findings (particularly when ethanol is administered in "moderate" amounts) on subsequent offspring development (Pilström and Kiessling, 1967; Collard and Chen, 1973; Øisund et al., 1978; Schwetz et al., 1978; Abel and York, 1979; Varma and Persaud, 1979; DaSilva et al., 1980; Lindenschmidt and Persaud, 1980; Mendelson and Huber, 1980; Sonderegger et al., 1980).

With animal investigations in particular, caution must be exercised in the interpretation of the effects assumed to be produced by ethanol. Methodological differences as to maternal diet, timing and dose of ethanol used, route of ethanol administration, and strain and species of animal chosen make it difficult to reproduce results from one experiment to another. Furthermore, it is virtually impossible to account for all extraneous variables such as nutritional status of mother and offspring, environmental conditions, and maternal behavior in any single experimental paradigm.

The mechanisms through which ethanol exerts its effects remain unclear. Whether ethanol and/or its metabolites are the primary
teratogenic agents continues unanswered, particularly since acetaldehyde (the primary metabolite of ethanol) has been shown to be teratogenic (O'Shea and Kaufman, 1979). It has also been demonstrated, primarily through studies utilizing rats and rabbits, that ethanol in higher concentrations inhibits the release of oxytocin (Fuchs and Wagner, 1963; Fuchs, 1966, 1969). This suggests that at least some of the growth retardation and developmental delay observed with postnatal ethanol administration is a secondary alcohol effect due to insufficient milk supply (neonatal malnutrition). Direct maternal influences must also be taken into account, since it has been shown that both the mother's ability to metabolize ethanol and her care of and behavior toward her offspring can affect their growth and maturation (Abel, 1978; Swanberg and Wilson, 1979; Chernoff, 1980; DaSilva et al., 1980).

Maternal ingestion of alcohol and its effects on the developing central nervous system

Warner and Rosett (1975), in their historical survey of the effects of maternal alcohol ingestion on human offspring, stated that in 1813 the Englishman Thomas Trotter first proposed the idea that maternal alcohol drinking could cause mentally defective progeny. Yet it was not until 1973 and the work of Jones and his collaborators that neonatal brain damage and chronic maternal alcoholism were conclusively linked clinically. Jones et al. (1973) and Jones and Smith (1973) reported microcephaly, incomplete development of the cerebral cortex, agenesis
of the corpus callosum, disorientation of neuronal and glial cells, and evidence of "aberrant" neuronal migration in FAS infants. Impairment of fine and gross motor function was also indicative of neural defects.

Clarren and Smith (1978) reviewed 245 cases of FAS and found several indicators of central nervous system dysfunction, with mental retardation being the most prominent. They stated that the sites of the observed brain malformations varied, with cerebellar dysplasias and heterotopic cell clusters being seen most often. Neurologic and behavioral abnormalities indicative of brain damage included neonatal tremulousness and irritability, hyperacusis, poor sucking ability, and childhood hyperactivity. In 1979 Clarren suggested an association between maternal alcoholism and neural tube defects after finding an increased incidence of meningomyeloceles and anencephaly in infants born to chronic alcoholic mothers.

Clinical evidence has indicated that the developing brain is one of the organs most sensitive to damage by alcohol. In those animal studies focusing on the association between central nervous system maldevelopment and maternal ethanol consumption, brain malformations in mice and rat offspring have included microcephaly, dilated or immature cerebral ventricles, absence of the corpus callosum, hydrocephalus, and exencephaly (Tze and Lee, 1975; Chernoff, 1977; Randall and Taylor, 1979; Diaz and Samson, 1980; Rasmussen and Christensen, 1980). Biochemical determinations of fetal brain DNA, RNA, and protein content have been used to demonstrate that prenatal consumption of ethanol by pregnant
rats or sheep can result in reductions in brain cell number but no decreases in brain cell size (Woodson and Ritchey, 1979; Potter et al., 1980).

Brain tissue from fetal and early postnatal rats and sheep which were exposed to ethanol either prenatally only, or both pre- and postnatally, has also been examined at the light microscopic level using both cell and myelin stains. Such studies have revealed abnormal or underdevelopment of neuronal connections, delayed cortical lamination, delayed myelination of a variety of fiber tracts, and decreased or retarded formation of dendritic spines (Rosman and Malone, 1976; Jacobson et al., 1979; Potter et al., 1980; Sherwin et al., 1980; West et al., 1981).

Developmental alteration of brain neurotransmitter levels following maternal ethanol consumption has just begun to be examined, yet initial results suggest that almost all neurotransmitter systems may be affected by perinatal ethanol exposure (Branchey and Friedhoff, 1976; Rawat, 1977, 1980; Detering et al., 1980a, 1980b, 1980c; Slotkin et al., 1980). Finally, there is some evidence that maternal ethanol consumption results in learning deficits in rodents (Martin et al., 1977; Streissguth et al., 1980).

Injection of near-term pregnant animals with \(^{14}\text{C}\) ethanol and subsequent examination of the distribution of that label in fetal central nervous system has demonstrated that the highest uptake of labeled ethanol occurs in the cerebellum (Idänpää-Heikkila et al., 1971; Ho et al.,
1972). Possibly related to this is the finding of Bauer-Moffett and Altman (1975) that cerebellar weight in the early postnatal rat following perinatal ethanol administration is reduced almost twofold compared with weight reduction in the rest of the brain.

In the rat, cerebellar development is known to occur largely in the first three weeks of postnatal life. Bauer-Moffett and Altman (1975, 1977) have administered alcohol to rat pups from postnatal days 3-20. Histological examination of the cerebellum at 21 days has shown a decrease in the width of all cortical layers and of the medullary layer as well. Autoradiography using [3H] thymidine has demonstrated that postnatal ethanol administration results in decreased numbers of both granule and Purkinje cells in the vermis, with cells in the early-maturing lobules more severely affected. These studies have shown that ethanol administration in the postnatal period affects both prenatally formed Purkinje cells and postnatally formed granule cells. The greater resistance of later maturing areas to the effects of alcohol suggests the possibility that the differential susceptibility of brain regions to ethanol may be associated with variations in their temporal pattern of development.

Anderson and Sides (1979) administered ethanol to rats postnatally on days 1-9 and subsequently prepared cerebellar tissue from 30-day-old animals for quantitative light microscopic analysis. In agreement with Bauer-Moffett and Altman, they found decreased numbers of granule cells, but the cell loss was scattered throughout all lobules. Moreover, they did not find any evidence of Purkinje cell loss. They concluded that alcohol only affects proliferating cells.
As for the effect of prenatally administered ethanol on cerebellar maturation, Volk (1977), Kornguth et al. (1979), and Volk et al. (1981) have reported delays in differentiation of the Purkinje cell cytoplasm and in disappearance of the external granular layer. It does appear, then, that ethanol adversely affects proliferating nerve cells and also may alter, whether directly or indirectly, the later maturational processes of cells which have already ceased to divide. The specific mechanisms behind the actions of ethanol on developing nerve cells, however, remain to be determined.

Effects of ethanol on neuronal RNA/protein synthesis during development

To date there has been a paucity of studies dealing with the effects of ethanol on RNA/protein synthesis in developing nerve cells. The research which has been carried out has involved purely biochemical methodology, and the results obtained have been mainly in agreement as to the inhibitory action of ethanol. Henderson and Schenker (1977) administered ethanol in a liquid diet to female rats several weeks before mating and throughout the gestation period and found the total brain RNA (whether expressed in terms of tissue wet weight or its protein concentration) in 3-day-old pups to be decreased by approximately 10-15%. DNA and protein concentrations and the rate of DNA synthesis were unchanged. Total RNA content of brain was similarly reduced in one-day-old rat pups exposed to ethanol prenatally (Ullrich and Dietzmann, 1980). These data, coupled with that of decreased activities of a variety of enzymes from the hippocampal gyrus of neonatal rats,
suggest that intrauterine ethanol exposure might result in a diminished synthetic capacity of neonatal brain.

Khawaja et al., in 1978b, isolated neuronal perikarya from the cerebral cortex of 8- and 18-day-old rat pups exposed to ethanol pre- and postnatally and measured their total RNA content as well as their capacity to incorporate a $^3$H-labeled mixture of 15 amino acids into protein. They found evidence of a slight increase in neuronal RNA concentration at 8 days and a greater increase at 18 days. Protein synthetic capacity was also found to be increased in 8-day-old pups, while there was a marked reduction in neuronal protein synthesis in the 18-day-old rats. They concluded that the stimulation of protein synthesis at 8 days postnatally may have been due to some sort of adaptive mechanism, while the depression at 18 days could possibly reflect the inability of even an adaptive phenomenon to handle a continuing ethanol insult.

Henderson et al. (1980) gave ethanol in a liquid diet to pregnant rats throughout gestation or administered ethanol acutely in the later stages of pregnancy. The brains of 20-day fetuses were obtained for determination of the incorporation of $[^{14}\text{C}]$ valine into protein. Results indicated that both ethanol-induced hypothermia and ethanol per se (with blood alcohol concentrations above 100mg%) significantly depressed net protein synthesis in vivo in fetal rats. It was concluded that the variable blood alcohol levels in an animal drinking ethanol over a 24-hour period may account for some cyclic depression-derepression of protein synthetic activity.
Intraperitoneal injections of ethanol to near-term pregnant rats demonstrated that maternal serum ethanol levels of 200mg% or more resulted in depressed fetal brain protein synthesis, whereas lower maternal ethanol levels enhanced fetal brain protein synthetic activity (Fisher et al., 1981).

The most definitive work in this field appears to be that done by Rawat (1975b). In this study, one group of pregnant rats was given a liquid diet containing 6% (w/v) ethanol throughout gestation, and the fetuses were taken from the mother just prior to parturition. A second group of pregnant rats was administered the same liquid diet containing ethanol throughout gestation and the first part of lactation, with pups zero to five days of age being sacrificed. Fetal and neonatal brains were either utilized for determination of total DNA and total RNA content or were processed for isolation of pH5 enzyme and ribosomal fractions, these fractions subsequently being used for examination of [U-14C] leucine incorporation into protein. Rawat observed a decrease in total DNA content and a decrease in total RNA content in the brains of fetuses and neonates exposed to ethanol. He also found that ethanol exposure resulted in a 30% reduction in the capacity of fetal brain ribosomes and a 60% depression in the capacity of neonatal brain ribosomes to synthesize protein. The ability of the pH5 enzyme fraction to synthesize tRNA was likewise decreased. The results of this study indicated that the processing of RNA in the fetal and neonatal rat brain was affected by chronic maternal ethanol ingestion. This alteration, in turn, apparently affected the capacity of cerebral ribosomes to synthesize proteins.
and may also have interfered with the synthetic capability of pH5 enzymes.

Determination of the effects of ethanol on brain RNA/protein synthesis is difficult even in the adult brain, since this is a most complex process dependent on a multiplicity of internal (biochemical and hormonal) and external (environmental) variables. In adult brain tissue, net protein levels are relatively constant, with protein synthesis and protein degradation occurring at approximately the same rates. In fetal and neonatal tissues, in marked contrast, net protein levels reflect the many dynamic processes that are occurring, with protein synthesis rates much increased over rates of protein degradation. The developing organism is "... a different metabolic species than the adult and must be regarded as such" (Miller, 1970, p. 1501). Consequently, interpretation of the data from fetal and neonatal studies is made more formidable, and speculation as to the functional significance of changes in RNA/protein synthesis in developing brain remains wide open.

Although it does appear that ethanol alters total brain RNA content during development, nevertheless the manner in which synthesis is modified and the class(es) of RNA which is (are) affected remain to be determined. Whether the apparent inhibition of protein synthesis affects all brain regions and brain cell types, or whether it is more restricted, is as yet unknown. It should be noted that none of these issues has been investigated on a cytological level. Such an approach
is almost essential in any explanation of the effects of ethanol on the subcellular machinery responsible for RNA and protein synthesis during brain maturation.

MALNUTRITION AS A FACTOR ACCOMPANYING ETHANOL CONSUMPTION:

EFFECTS ON DEVELOPMENT OF OFFSPRING

Importance of nutritional factors in the interpretation of the effects of ethanol on growth and developmental indices.

Since malnutrition often accompanies chronic alcohol ingestion, the effects of each upon cell metabolism need to be separated, if possible.

In 1963, Fuchs and Wagner showed that ethanol in high enough doses inhibited the release of oxytocin from the posterior pituitary in lactating rabbits. Later work (Fuchs, 1969) showed that the degree of inhibition bore a dose-dependent relationship to the amount of ethanol administered and that treatment with exogenous oxytocin reversed these effects and allowed resumption of lactation. Such studies indicate that the effects of postnatal malnutrition must be considered in any interpretation of the effects of ethanol on offspring growth and development.

Undernutrition of the pregnant mother and fetus must also be taken into consideration when ethanol is administered prenatally. Whatever the method of ethanol administration may be, care must be taken that the pregnant animal receives not only adequate calories but also suffi-
cient amounts of protein, vitamins, and other nutrients. There may also be additional factors such as the effects of ethanol on nutrient digestion, absorption, and transport, or a possible interference at the cellular or subcellular level with nutrient utilization (Lieber, 1979). Wunderlich et al. (1979) has shown that ethanol administration to the pregnant rat on gestation days 7-20 results in decreased placental RNA and protein content and decreased placental and serum rat chorionic mammotropin, the hormone responsible for accelerating transfer of nutrients from mother to fetus near the end of pregnancy.

Malnutrition and developmental impairment: Clinical (human) evidence

Intrauterine malnutrition has been shown to result in decreased birth weight, placental weight, and placental protein content as well as in the production of hypotonicity and hypoexcitability (or even apathy) in the newborn (Bhatia et al., 1979). At least a portion of this growth deficit can be compensated for very early in postnatal life if poor nutritional conditions are reversed (Davies et al., 1979). It is believed that the adult number of neurons in the human brain may be established as early as the 20th week of gestation (Evans et al., 1980). If intrauterine malnutrition occurs early enough and is sufficiently severe, at least some degree of mental retardation may develop; such a condition is irreversible.

The "growth spurt" of the brain in the human extends from about the end of the second trimester through the second postnatal year.
During this time period there is glial cell proliferation, myelination, and the establishment of neuronal connections. Early postnatal undernutrition, therefore, may restrict these maturational processes and retard intellectual development. Studies carried out on young children who have died of severe malnutrition have demonstrated decreased brain weight and reductions in DNA, RNA, and protein content; these latter reductions were equally severe in cerebrum and cerebellum and less severe in brainstem (Winick et al., 1970). The neuropathology of malnutrition does not appear to yield any focal lesions, just evidence of generalized brain impairment. Examination and testing of severely malnourished children who survive the first two years postnatally have revealed a decrease in head circumference and deficient mental development (Evans et al., 1980; Grantham-McGregor et al., 1980).

**Malnutrition and developmental impairment: Experimental (animal) evidence**

Since the vulnerable or critical period of brain development varies for different species and for different areas of the brain, the timing, duration, and extent of nutritional deprivation in any animal model will affect the final results obtained. Numerous studies, particularly on the rat, have provided evidence that undernutrition alters the composition of the brain during its development. Evidence is accumulating that the developing brain is increasingly vulnerable from conception to birth and that it becomes less vulnerable with increasing postnatal age (Pryor, 1975). This period of vulnerability appears to
be directly related to the rate at which each developmental process is occurring, e.g., processes such as neuroblast and glial mitosis, protein production, axodendritic growth, synaptogenesis, and myelination (Dobbing, 1970). Developmental malnutrition can be experimentally induced in a number of ways, yet the deficits that are seen are quite similar if consideration is given to the developmental timetable unique to each species.

Studies dealing with prenatal malnutrition have demonstrated decreased body and brain weight, decreased brain total DNA, RNA, and protein content, retarded postnatal weight gain, delayed development of certain physical features, altered myelination, changes in glutamate, norepinephrine, and dopamine metabolism, decreased open field activity, and modification of motivational factors (Peters, 1979; Charlebois and Fried, 1980; Detering et al., 1980a; Prasad and Agarwal, 1980; Bourre et al., 1981).

The few available studies on the effects of prenatal malnutrition at the cytological level report decreased numbers of large multipolar neurons in brain and spinal cord, fewer cells in the cerebral cortex, and reduction in cerebellar Purkinje cells (Shoemaker and Bloom, 1977; Wallingford et al., 1980).

Since the brain growth spurt in the rat and several other common laboratory animals occurs postnatally, the majority of research has dealt with the effects of postnatal (or pre- and postnatal) undernutrition on the developing nervous system. Because of his studies in the 1960's and 1970's, Winick is given credit for reviving the work done in
the early 1900's in this field, as well as for providing the impetus for the surge of publications in the late 1970's on perinatal malnutrition and brain development (see Shoemaker and Bloom, 1977). Effects reported include reductions (often drastic) in body weight and length with maintenance of normal body proportions, skeleto-muscular growth retardation, decreased brain weight and size (with cerebellum most affected), reduced total brain DNA, RNA, and protein content (indicating a decrease in cell number and/or size depending on the timing of the nutritional insult and brain region examined), decreased levels of certain free amino acids, decreased brain lipid concentration, lowered levels of at least certain neurotransmitters (norepinephrine and dopamine), alterations in growth of axons and dendrites, retarded myelination, and alteration of the biochemical processes of transcription and translation (Culley and Lineberger, 1968; Chase et al., 1969; Fish and Winick, 1969; Shoemaker and Wurtman, 1971; Toews and Lee, 1975; Shoemaker and Bloom, 1977; Yusuf et al., 1981).

Numerous postnatal studies have also been carried out at the light microscopic and, to a lesser degree, at the electron microscopic level. Light microscopic studies have provided evidence that malnutrition during lactation or throughout gestation and lactation results in a reduction in total area of the cerebellum and each of its layers, decreased cerebral cortical thickness and delayed lamination, decreased numbers of cells in the cerebellum (primarily granule cells) and the cerebral cortex (primarily glial cells), extension of the normal period
of cell proliferation in the cerebellum, delayed cytoplasmic maturation of cerebellar Purkinje cells and of neurons and glia in the cerebral cortex, evidence of decreased cell size, and reduction in length and extent of Purkinje cell and pyramidal cell dendritic networks (Bass, 1970; Bass et al., 1970; Altman and McCrady, 1972; Barnes and Altman, 1973; West and Kemper, 1976; Jordan and Howells, 1978; McConnell and Berry, 1978; Leuba and Rabinowicz, 1979a, 1979b; Pysh et al., 1979; Bernocchi and Scherini, 1980; Moraes e Santos, 1980). At the electron microscopic level, there is evidence of decreased numbers of synapses in the cerebellum, with scattered profiles of giant Purkinje cell spines capped by enlarged presynaptic boutons of parallel fibers. Also present in cerebellar tissue are membranous whorls in axons and axon terminals, along with increased glycogen in the terminals and in astrocytic cell bodies (neuronal cell bodies showing minimal changes) (Yu and Yu, 1977; Chen and Hillman, 1980). Recently Jones and Dyson (1981) reported altered development of cortical synapses in rats subjected to pre- and postnatal malnutrition.

There have been numerous investigations regarding the reversibility or irreversibility of malnutrition-induced alterations in the developing brain. In general it is believed that, in the rat, decreases in cell number due to undernutrition are irreversible, whereas decreases in cell size can be reversed with subsequent adequate nutrition (Fish and Winick, 1969). Examination of the functional significance of the changes permanently produced by pre- and/or postnatal malnutrition is
an area currently being investigated.

**Malnutrition and its effects on brain RNA/protein metabolism**

Overall RNA and protein synthesis are known to be much more active in the immature brain, as are the rates of protein turnover. In fact, neonatal development is characterized by an unusually high rate of anabolism never again seen in later life. In order for these high rates of synthesis to occur, there must be an adequate supply of nutrients. It is not surprising, therefore, that neonatal malnutrition has been shown to affect a multitude of synthetic mechanisms, RNA/protein synthesis being the most prominent.

Naismith (1973) examined the effects that restriction of maternal protein intake during pregnancy has on fetal growth in the rat. Under such conditions, he found evidence for the mother's ability to utilize protein more efficiently and to have protein reserves withdrawn from her own tissues and transferred to her fetuses. Zamenhof (1981) reported similar 'maternal efficiencies' in nine generations of rats subjected to a relatively mild but prolonged protein/energy malnutrition. Such adaptations in maternal protein metabolism are probably brought about through the action of hormones. In many cases, therefore, moderate restriction of maternal protein intake during pregnancy may have little effect on fetal growth and fetal metabolic processes. Severe forms of maternal undernutrition, however, may not allow the fetus to remain completely untouched. Determinations of the actual rates of fetal-
neonatal RNA and protein synthesis in the brain following prenatal undernutrition need to be performed.

In a study of the effects of postnatal protein-calorie malnutrition on cells in the rat forebrain, Moraes e Santos (1980) performed various biochemical analyses to demonstrate a reduction in cell size and a depression of protein synthesis. Bass and his co-workers (1970) specifically investigated rat somatosensory cortex and found a decreased RNA content per cell which correlated with a histological finding of delayed cytoplasmic maturation of ribosomal organelles following postnatal undernutrition. A histological study performed on Purkinje cell maturation following postnatal malnutrition found evidence of decreased cell size and decreased dendritic expansion, suggesting that neuronal metabolism (protein synthesis in particular being mentioned) had been altered (McConnell and Berry, 1978).

A few investigators have utilized direct methods to determine the effects of perinatal undernutrition on RNA and/or protein synthesis in brain tissue. Knoll-Köhler and Handke (1980) measured the ability of rat brain neuronal and glial nuclei to incorporate \[^{3}\text{H}]\text{UMP} \text{ (uridine monophosphate)} \text{ into RNA following pre- and postnatal malnutrition. They found that RNA synthesis was decreased (to a non-significant degree) in neuronal nuclei but was significantly increased in glial cell nuclei. They stated that the decrease in brain total RNA reported in other studies may have stemmed from less numbers of brain cells as well as from higher rates of RNA degradation in malnourished pups. A second study (Knoll-Köhler \text{ et al.}, 1980) provided evidence that long-term
protein malnutrition can significantly alter RNA/protein synthesis in neuronal nuclei as well.

Banay-Schwartz et al. (1979) looked exclusively at the effects of pre- and postnatal undernutrition on brain protein metabolism in 6-, 12-, 30-, and 42-day-old rat pups. They determined the rates of protein synthesis and protein breakdown in cerebrum, cerebellum, and brainstem following an injection of [U-\textsuperscript{14}C] tyrosine. Their findings indicated a decrease in both protein synthesis and protein breakdown, especially at six days postnatally, in the cerebellum and cerebrum of malnourished rats. Only small changes were noted in the brainstem. Since the inhibition of protein synthesis was always greater than the inhibition of protein breakdown, net protein deposition ("growth") was found to be decreased in the brains of underfed rats. Changes in the free amino acid pool of the brain were minimal and could not have accounted for the reported alterations in protein metabolism.

The most thorough work in this field has been carried out by deGuglielmone and his co-workers. In 1974, they reported their findings regarding the effects of malnutrition during lactation (postnatal days 0-20), and subsequent nutritional rehabilitation, on RNA synthesis in 10-, 20-, and 30-day-old rat brains (deGuglielmone et al., 1974). They examined the activity of DNA-dependent RNA polymerase in isolated brain cell nuclei and measured the \textit{in vivo} incorporation of [5-\textsuperscript{3}H] orotic acid into nuclear and cytoplasmic RNAs. Their results showed that DNA-dependent RNA polymerase activity was decreased in underfed rats at 10 and 20 days of age when compared to controls, yet
this difference disappeared at 30 days after a short period of food 
rehabilitation. Incorporation of labeled precursor into both micro-
smomal and soluble RNA was decreased at all ages, whereas incorporation 
into nuclear RNA was decreased only at 10 days postnatally. The 
relative specific radioactivity of nuclear RNA to microsomal RNA 
decreased with age in control rats but was increased in malnourished 
rats. From their findings they could not determine whether this 
alteration was due to impaired transport of RNA from the nucleus to 
the cytoplasm and/or whether it was due to increased degradation of 
cytoplasmic RNA. They concluded that early postnatal malnutrition 
in the rat does alter several aspects of brain RNA metabolism.

A continuation of this same work (Duvilanski et al., 1980) showed 
that neonatal malnutrition produced alterations of several facets of 
RNA/protein metabolism in 10-day-old rats, and that these alterations 
prevailed in 30-day-old animals even with nutritional rehabilitation 
having been instituted at 21 days. They obtained some evidence of 
increased RNase activity and increased degradation of mRNA (but not 
rRNA) in the cytoplasm of nerve cells. Their major finding, however, 
was one of impaired nucleocytoplasmic transport of mRNA and rRNA. Such 
alterations in RNA metabolism may have been at least partially re-
sponsible for the decreased brain protein synthesis they observed in 
rats at both postnatal ages. Their additional finding (with sucrose-
gradient centrifugation analysis) of a decrease in cerebral polysomes, 
together with an increase in monosomes, provides further evidence of a 
reduction in the protein synthetic capacity of the brain with neonatal 
underfeeding.
MAJOR BODY OF INVESTIGATIVE WORK

I. EFFECTS OF ETHANOL ON ADULT HAMSTER NEURONS:
LIGHT MICROSCOPIC STUDY

Introduction

In the golden hamster, the RNA- and protein-rich intranucleolar body (INB) is believed to serve as a reserve of nucleolar material destined to take part in the synthesis of cytoplasmic protein in nerve cells (LaVelle and LaVelle, 1975). The present light microscopic study was undertaken to determine whether INB structure (and therefore, presumably, RNA/protein synthetic activity) could be altered in vivo in the adult neuron with chronic ethanol ingestion. Facial motor neurons and cerebellar Purkinje cells were chosen for analysis because they constitute relatively homogeneous populations of cells cytoarchitecturally and because large proportions of both cell groups in the adult possess nucleoli with prominent intranucleolar bodies.

Materials and methods

Male and female golden hamsters (115-135g initial weight) were housed individually in plastic cages and placed on a 12 hour light/12 hour dark cycle. One group of hamsters received 15% (v/v) ethanol in water as their only drinking fluid, while a second group of animals (matched for sex and weight) received only water. Fluids were admin-
istered from inverted graduated glass bottles through stainless steel drinking tubes and were changed daily. All animals were provided with Purina rat chow and received two drops of Poly-Vi-Sol (Mead Johnson) multivitamin supplement once a week. Throughout the experimental time period, records were kept on the amount of laboratory chow and fluid consumed and on the weekly weight gain/loss for each animal. (See Supplemental Investigations, Section I, for an early attempt to administer a wholly liquid diet.)

At the end of a seven-week regimen, each hamster was anesthetized intraperitoneally with sodium pentobarbital (Nembutal) and perfusion-fixed through the heart with a 10% formalin-saline-gum acacia mixture (LaVelle, 1951). All animals were sacrificed between 9:00 and 11:00 in the morning. The brain of each animal was then exposed and immersed, still in situ, in the same fixative solution for a period of 24-72 hours. Subsequently each brain was completely dissected out, and the brainstem was double-embedded in Parlodion and Fisher TissuePrep and serially sectioned at 4μm on a sliding microtome. (See Appendix B for details of perfusion and tissue preparation.) Every tenth section was mounted and stained with buffered thionin at pH 4.46 for distinction of intracellular sites containing DNA and RNA (LaVelle, 1951; LaVelle and LaVelle, 1958a).

For the histological study, only those neurons which contained distinct, single nucleoli were used for quantitative analysis. The
counted cells included 1) those neurons which had no visible intra-nucleolar body (INB), 2) those neurons which had a very small, punctate, often tri-partite INB with particles less than 1 μm in diameter, and 3) those neurons which possessed a prominent INB 1 μm or larger in diameter. For each cell population, 300 cells from each animal were thus classified. This entailed observing the great majority of neurons in the sections of facial motor nucleus for any given animal. In the cerebellum, 300 Purkinje cells from lateral portions of each animal's hemispheres were chosen for analysis. Since the nucleolar architecture of Purkinje cells in all parts of the hemispheres is similar, 100 Purkinje cells at each of three different levels of the neuraxis (hypoglossal nuclear, mid-facial motor nuclear, and mesencephalic nuclear levels) were consistently utilized in cell counts. Control and experimental values were then averaged separately to obtain the percentages of cells with absent, punctate, and prominent intranucleolar bodies.

The diameters of prominent INBs from 100 facial and 100 Purkinje cells were also determined in each control and each experimental hamster and then averaged to obtain one control and one experimental value for each cell population.

The quantitative classification of nucleolar and intranucleolar structure and the measurement of INB diameter were carried out with the aid of a 100X oil immersion lens and a calibrated ocular micrometer.
disc. The results were submitted to statistical analysis using Student's t-test.

Cells were photographed according to the procedure outlined in Appendix D.

**Results**

During the seven-week experimental time period used in this study, neither illness nor death was observed among the animals. Although body weight fluctuated slightly from week to week for each animal, such changes were minimal and were found to have no significance when final control and experimental values were compared. Even though no differences were found between the two groups in body weight readings, it was determined that the amount of solid food consumed per day was less in the alcohol group (6.0g) than in the control group (7.5g). This decrease in food intake was determined to be statistically significant (p<0.01, see Table 1, Appendix E). Due to the relatively high caloric content of ethanol (7.1 Cals/g), however, the "alcoholic" hamsters actually ingested as many or more total calories per day than did the controls. There was considerable variability in the amount of fluid consumed per day, but overall there was little difference between the amounts of alcohol and water ingested by the two groups of animals (an average of 20-21ml of fluid per day
for both groups, see Table 1). In the experimental group, this value represents an average daily consumption of approximately 20g/kg of ethanol or almost 50% of the animal's total caloric intake. Other than an occasional exhibition of hyperexcitability and irritability or, even more rarely, the temporary occurrence of some loss of motor coordination, no marked behavioral alterations were seen in the alcohol-consuming animals.

In the hamster, the facial motor nucleus is located in a ventrolateral position in the medulla. The neurons form a distinct population of cells that is easily demarcated from the surrounding neuropil (Fig. 1A). Purkinje cells form an even more conspicuous neuronal population that is easy to delineate from the other cell layers which comprise the cerebellar cortex (Fig. 1B).

In sections of brainstem stained with thionin, the neuronal intranucleolar body (INB) was seen as a rounded, pink-purple mass located centrally in the nucleolus. The remainder of the nucleolus was relatively pale, except for a rim or cap of perinucleolar chromatin, which stained a deep blue color. The nucleus was also pale, while the Nissl substance within the cytoplasm took on a blue or purplish-blue hue. Photographs of typical nucleolar variations for facial motor neurons may be seen in Figure 2, those for cerebellar Purkinje cells in Figure 3. In addition, Figure 4 diagrammatically illustrates cell features and the classification system used in the counting of.
Counts confirmed that, in the adult animal, facial motor neurons normally have a larger percentage of cells which possess prominent intranucleolar bodies than do Purkinje cells (80% vs. 53%, Fig. 5). The latter population has a higher percentage of cells with punctate-sized INBs (33% vs. 11%) and a higher percentage of cells which lack any INB whatsoever (14% vs. 9%).

In both cell populations, alcohol ingestion resulted in a shift toward a greater percentage of cells with prominent INBs and correspondingly fewer cells with either punctate or no INBs. Although all but one sub-group of nucleolar changes were found to be highly significant (p<0.001), the differences between control and experimental values were greatest in the Purkinje cell population (Table 2, Appendix E). There the percentage of cells with prominent INBs increased from a control value of 53% to a value of 72% after chronic alcohol ingestion (Fig. 6). Concomitantly, the percentage of cells containing punctate INBs decreased from 33% to 19% of cells counted, and values for cells with no INBs decreased from 14% to 9%. In the facial motor nucleus, the shift to prominent INBs had already occurred during normal development. After alcohol administration the percentage of prominent INBs increased further from 80% to 87%, while only 6% and 7% of cells were characterized by punctate or absent INBs, respectively (Fig. 7). The shift in numbers of absent INBs in facial neurons was the only change
with alcohol that was borderline non-significant (p=0.0595).

As shown in Figure 8, diameters of prominent INBs in the neurons of alcohol-imbibing animals were significantly larger (p<0.001) when compared to controls. In facial motor neurons the mean diameter increased from 1.76 to 1.91 μm; in Purkinje cells it increased from 1.38 to 1.51 μm. Clearly, then, the prominent intranucleolar bodies in both populations of cells increased in size as well as in frequency following the chronic ingestion of ethanol by adult hamsters over a seven-week period.

**Discussion**

Previous studies have shown that golden hamsters exhibit a preference for alcohol over water and readily consume a 15% or 20% ethanol solution (Emerson et al., 1952; Arvola and Forsander, 1961, 1963). The value reported here of approximately 20 ml (20 g/kg) of fluid drunk per day conforms well to determinations made by other investigators for the hamster (Slighter, 1970; Sinclair and Sheaff, 1973; Ross et al., 1976; Geller and Hartmann, 1977; Thurman et al., 1978; Kulkosky and Cornell, 1979; Rudeen and Symmes, 1981). Reiter et al. (1974) and Geller and Hartmann (1977) proposed that the hamster's "highly active" pineal gland may play a partial role in regulating its ethanol preference. Yet Rudeen and Symmes (1981) have since provided evidence to suggest that the pineal only indirectly alters the hamster's
willingness to consume alcohol by influencing the animal's total daily fluid (water and/or ethanol) intake. With the exception of these few studies, no attempt has been made to determine the reason(s) behind this willingness to ingest alcohol solutions.

That the quantities of alcohol ingested in this study were rarely sufficient to produce an intoxicated state may be due to the reported ability of hamsters to rapidly metabolize even large quantities of alcohol (McMillan et al., 1977; Thurman et al., 1978; Harris et al., 1979; Kulkosky and Cornell, 1979). As shown in another section of this dissertation (Supplemental Investigations, Section II), blood ethanol levels in hamsters chronically consuming 15% alcohol were determined by gas chromatographic procedures. Although the results are preliminary, they show that hamsters do not maintain high blood ethanol levels for any extended period of time and that there is some tendency for hamsters to ingest larger quantities of alcohol at night.

Richter (1953) has stated that rats given ethanol decrease their food consumption in proportion to the additional calories they obtain through the alcohol. Such a phenomenon could explain the reduction in solid food consumed by the experimental animals in the present study, particularly since there was no loss of body weight in this group over the seven weeks. These findings are in accordance with those reported by Lamb et al. (1979) for the hamster. Since the animals appeared to remain in good health throughout the course of the investigation, it
was concluded that nutritional status was unlikely to have been a factor in any differences observed following consumption of ethanol.

Critical use has been made here of a labile intranucleolar body (INB), first reported by LaVelle and LaVelle in 1975 as a possible index of neuronal metabolic activity in the hamster. By cytochemical and extraction tests, the INB was demonstrated to be rich in RNA and protein and not to contain any appreciable amount of DNA. Ultrastructural evidence also showed that the body consists of an aggregation of fine granules localized centrally within the nucleolus (Kinderman and LaVelle, 1976). These studies, along with more recent work on developing neurons (McLoon and LaVelle, 1981b), demonstrate that the INB is absent during periods of cell growth and repair but appears when cells reach maturity or have completed their reconstitution after injury. Supportive evidence from autoradiographic (Kinderman and LaVelle, 1977; McLoon and LaVelle, 1981c) and scintillation (McLoon and LaVelle, 1981a) studies further demonstrates that, in hamster facial neurons, INB absence is correlated with times of intense cellular protein synthetic activity, while its presence coincides with a more stable metabolic state marked by less concentrated synthesis of protein.

The nucleolus is known to be the site for rRNA synthesis and RNP particle assemblage; these products are subsequently transported to the cytoplasm to be utilized in the synthesis of proteins (see Busch and Smetana, 1970; Dalgarno and Shine, 1973; DeRobertis and DeRobertis,
Precise correlations have been demonstrated between nucleolar morphological components and the sequence of rRNA synthesis within the nucleolus. (See, for example, Granboulan and Granboulan, 1965; Geuskens and Bernhard, 1966; Das et al., 1970; Royal and Simard, 1975; Hernandez-Verdun and Bouteille, 1979. See also review by Bouteille et al., 1974.) Furthermore, changes have been achieved in the cytological appearance of these components after treatment with chemical agents known to inhibit or otherwise alter the synthesis and/or use of rRNA by the cell. (For reviews see Busch and Smetana, 1970, Chapters 9 and 10; Simard et al., 1974.)

This knowledge concerning nucleoli in general, coupled with the information obtained in the initial INB studies in neurons, led to the conclusion that the INB is a site of RNA/RNP accumulation (LaVelle and LaVelle, 1975). More specifically, in light of the incorporation studies cited above, it is believed that the presence of the intranucleolar body in adult hamster neurons reflects a level of metabolic activity such that the rate of synthesis of nucleolar rRNA/RNP is greater than the rate of its removal from the nucleolus for cytoplasmic use, thus resulting in its stockpiling within the nucleolus. The INB per se is not viewed by this laboratory as a structural organelle with a unique function of its own, but rather as an aggregation of products belonging in a chain of synthetic events which are central to the maintenance of cell integrity. The signifi-
cance of the neuronal INB lies in the fact that, because of its histology visibility and its lability, it serves as a cytomorphological indicator of biochemical alterations in nucleolar, and therefore cellular, metabolism. The belief that morphological changes in this body reflect alterations in the balance between production and utilization of neuronal rRNA is in harmony with such diverse populations as HeLa cells (Studzinski and Gierthy, 1972), cultured human glial cells (Dupuy-Coin et al., 1978), and cultured chick fibroblasts (Goessens, 1978).

It should be noted that the absence of intranucleolar bodies in the neurons of many common laboratory animals (Jacob and LaVelle, 1977) implies a possible species difference in level of neuronal metabolic activity rather than necessarily a qualitative difference in the fundamental nature of the INB. The consistent presence of the INB in the hamster is viewed as fortuitous for purposes of experimentation.

In the last several years, a number of investigators have shown that the rate and extent of protein synthesis in the brains of adult animals can be altered through the ingestion or administration of ethanol. Virtually all of the studies reported to date have been biochemical in nature, and the results have sometimes been contradictory. However, the greater body of evidence points to an inhibition of protein synthesis in brain when ethanol is chronically administered to mice (Tewari and Noble, 1971; Noble and Tewari, 1973) or to rats
(Jarlstedt, 1972; Renis et al., 1975; Morland and Sjetnan, 1976; Tewari et al., 1977; Tewari et al., 1980). Studies dealing specifically with the chronic effects of ethanol on brain RNA metabolism suggest that this inhibition of protein synthesis may be interrelated with alterations in RNA transcription, transport, or overall metabolism (Noble and Tewari, 1973, 1975; Tewari et al., 1975; Tewari and Noble, 1977; Tewari et al., 1978).

In contrast to the largely biochemical studies performed by other investigators, the utilization here of the intranucleolar body provides an in situ, cytological index of changes in the activity of the neuronal machinery involved in RNA/protein synthesis. It is theorized that chronic ethanol use, resulting in central nervous system depression, inhibits the utilization of neuronal RNP and thus decreases the cell's protein synthetic capabilities. This appears evident in the buildup of "excess" nucleolar RNA/RNP as represented by the increase in size of the intranucleolar body. The ultrastructural study reported later in this dissertation (see Major Body of Investigative Work, Section III) was undertaken in an effort to localize more precisely the subcellular structures altered following chronic ethanol ingestion.
II. EFFECTS OF ETHANOL ON IMMATURE HAMSTER NEURONS: LIGHT MICROSCOPIC STUDY

Introduction

A survey of nucleolar development in several neuronal groups, including facial motor neurons and cerebellar Purkinje cells, was first reported for the guinea pig (LaVelle, 1956). Subsequently, LaVelle and LaVelle (1958a, 1958b, 1959) described a detailed developmental sequence for the structure of the nucleolus in maturing neurons of the facial motor nucleus of the golden hamster. A young nerve cell initially possesses multiple nucleoli but, as it matures, it acquires a single nucleolus. In the hamster, the RNA-rich intranucleolar body (INB) appears late in neuronal maturation (LaVelle and LaVelle, 1975). Alterations of the INB are believed to reflect changes in the RNA and protein metabolic activity of the cell.

Chronic alcohol exposure has been shown to increase the frequency and size of the INB in adult neurons of the golden hamster (see Major Body of Investigative Work, Section I). The purpose of the present investigation was to determine, at the light microscopic level, whether ethanol can also alter nucleolar development in young cells actively engaged in protein synthesis for growth. Postnatal day 20 was chosen as the end-point in this study because it marks a transitional period in facial motor neuron development when the final stage
of maturation is taking place and the INB is beginning to appear in substantial numbers. Any accelerated or inhibited development of the INB should therefore be demonstrable. Furthermore, a comparative element was added to the study since 20-day Purkinje cells are normally less developmentally advanced than those of the facial motor nucleus (LaVelle, 1956) and can thus provide evidence of the effects of alcohol on an earlier developmental stage.

**Materials and methods**

Adult golden hamsters were housed individually in plastic cages and placed on a 12 hour light/12 hour dark cycle. Prior to commencement of experimental procedures, all animals were provided with Purina rat chow and water *ad libitum*. In addition, each animal received two drops of Poly-Vi-Sol (Mead Johnson) multivitamin supplement once a week.

The female hamster has a very regular, 4-day estrous cycle. The day following estrus is marked by a thick, opaque discharge which can be expressed from the vagina. Before mating the animals, a minimum of three estrous cycles were followed in each female to ensure that she was cycling normally. She was then placed with a male on the night of estrus (ovulation) and left there until morning if it was observed that she was receptive and that coitus occurred. In the
morning she was returned to her own cage, and this date was recorded as gestation day one. Four days prior to parturition, which was expected on day 16, each hamster was provided with shredded paper for nesting purposes. Postnatal day one was considered to begin within 12 hours after birth of the last pup of the litter. Hamster pups remained with their natural mothers until time of sacrifice.

The day following mating, female hamsters were assigned to one of three treatment groups: Group C (controls) received water throughout the periods of gestation and lactation; Group G-L (gestation and lactation) received a 15% (v/v) ethanol solution as the sole drinking fluid throughout gestation and lactation; and Group G (gestation only) received 15% ethanol during the 16-day gestation period but subsequently received water in place of ethanol over the course of lactation. [In addition, a fourth but minor group (L: lactation only) was given water until birth of the litter, followed by a 15% ethanol solution as the sole fluid during the period of suckling. This group was included as a further check for the effects of malnutrition on body weight and general developmental progress (see Supplemental Investigations, Section III) but was not studied histologically.] In all groups of animals, the fluid provided to the mothers was the only fluid available to the pups after weaning. Beginning on the morning of the eleventh postnatal day, each glass
bottle was fitted with an elongated drinking tube in anticipation of fluid consumption by the pups. All groups continued to receive food pellets ad libitum, and the mothers continued to receive weekly doses of Poly-Vi-Sol.

Throughout pregnancy, the females were disturbed as little as possible. During the day following parturition, litter size was determined by inspection. However, in order to avoid excessive cannibalism, cross-fostering of litters was not performed, and pups were not handled until three days after birth. Daily observations of maternal behavior, together with daily monitoring of various developmental and behavioral indices for each litter, were conducted on postnatal days 3 through 19 (see Supplemental Investigations, Section III, for details). The pups were weighed on days 4, 9, 14, and 19.

On the morning of the 20th postnatal day, both male and female pups in each litter were perfusion-fixed for light microscopy. Double embedding, cutting (4μm), and staining (thionin) were the same as described for adult animals. (See details of method, Appendix B.)

Counts of nucleoli were performed on cerebellar Purkinje cells and facial motor neurons of pups from Groups C, G-L, and G, using the same criteria already outlined for adult hamsters (see pp. 54-55). In addition to the differential counting of single nucleoli containing prominent, punctate, or no intranucleolar bodies, a fourth category of cells containing multiple nucleoli was included because of their
presence in these more immature populations.

The quantitative classification of nucleolar and intranucleolar structure was carried out with the aid of a 100X oil immersion lens and a calibrated ocular micrometer disc. The results were submitted to statistical analysis using a one-way analysis of variance and Duncan's multiple range test in addition to supplementary procedures necessitated by differences in size of litter and body weights of pups (see Appendix C).

Results

Maternal behavior, development and survival of progeny

Of the 12 control females, all (100%) came to term successfully. Of the 19 females ingesting 15% alcohol (G-L and G groups inclusive), 16 (84%) delivered pups which survived the perinatal period. Of the three remaining females, two died during or after parturition; the third cannibalized her entire litter. Litter size at birth was not significantly different between control [mean = 9.2 pups (range 6-13), S.D. 2.5] and alcohol treatment [mean = 8.9 (range 4-14), S.D. 2.9] groups.

Both control (C) and alcohol-consuming (G and G-L) pregnant hamsters maintained levels of food and fluid intake during their gestation periods that were similar to those found prior to mating (approximately 7g solid food, 20-25ml fluid per day). During the
period immediately before and following parturition, fluid intake dropped similarly in all groups. On the other hand, mothers ingesting water and, to an even greater degree, those drinking alcohol increased their fluid intake during the lactation period. Neither apparent physical illness nor death occurred among nursing females from any treatment group.

Maternal behavior during pregnancy was little altered by ingestion of alcohol, except for somewhat heightened irritability and hyperactivity. When shredded paper was offered in the cages, all control females immediately made nests. Some "alcoholic" females likewise prepared nests, while others made partial or no attempts at nesting.

During the lactation period, maternal behavior differed considerably among the treatment groups. Control (C) hamsters and those withdrawn from alcohol 12-24 hours following parturition (G) took an interest in their young, and nursed and weaned them normally. Mothers introduced to alcohol 24 hours after giving birth (Group L) were irritable at times and overly protective of their young, but nursing appeared to proceed normally. Daily handling of C, G, and L pups by the investigator did not noticeably agitate any of the nursing females.

In contrast, females ingesting alcohol throughout both their gestation and lactation periods (Group G-L) often displayed abnormal, and at times bizarre, behavior. During the first several postnatal
days, they not only were irritable and overly protective of their young, but they became highly agitated when the pups were handled or wandered out of the nest. As the postnatal period progressed, these mothers became increasingly hyperactive, displaying marked irritability toward their pups during any extended periods of suckling, and usually making no attempt to retrieve them if they wandered out of the nest. They also experienced episodes of shaking and trembling, particularly in the morning, and often screamed and/or threw themselves against the sides of the cage upon sensing the approach of the investigator. Unlike non-alcohol-ingesting mothers, they rarely stockpiled small bits of pellets in anticipation of the pups' transition from a totally liquid to a solid and liquid diet. Cannibalism of pups that did not survive was much more frequent than in any other group of animals.

Because malnutrition must be considered as a possible factor in the interpretation of effects of ethanol, the body weights and general appearance of offspring in this study were observed carefully. Figure 9 is a graphic representation of the average weights for animals in each treatment group at postnatal days 4, 9, 14, and 19. In addition, Table 3 (see Appendix E) lists these weights, together with the p-values obtained from a statistical analysis of the data. At four days postnatally, there was no significant difference in the mean weight of the pups from any treatment group; all values lay between 4.3
and 4.5 grams. By nine postnatal days, pups in Groups L and G-L weighed significantly less (p<0.001) than those in Groups C or G. This weight difference was even greater at 14 days postpartum, and by 19 days the G-L animals weighed 34% less than controls, the L animals 37% less. Unexpectedly, pups subjected to alcohol solely during their fetal life (Group G) grew even faster and weighed more than control animals after birth. By day 19, this weight difference was statistically significant. Size of litter did not account for the weight differences among the four treatment groups.

For the first seven to ten days, while the ventral skin was still semi-transparent, it was possible to examine pups for the presence of milk in their stomachs. In all cases, milk was seen. Whether it was inadequate in amount during the period of maternal alcohol ingestion, or whether it lacked sufficient quantities of particular nutrients for growth, was not assayed. However, pups exposed to alcohol postnatally often acted as if they were not getting sufficient milk, and mothers of these litters (G-L in particular) were observed to have enlarged and inflamed nipples, as if the pups had suckled too forcefully.

Directly or indirectly, postnatal exposure to alcohol not only resulted in growth deficiency, as measured by decreased weight gain, but also appeared to affect the health and viability of these animals.
In addition to being "runty", many of the pups in Group G-L became increasingly weak from approximately 12 postnatal days onward and remained thin even after they began to eat solid food and to drink the alcohol solution on their own. Several of these pups resembled their mothers in undergoing periods of body quivering or trembling. Some were unsteady on their feet and often fell over when walking. A few had splayed hind legs as late as 20 days postnatally and partly dragged them as they moved about. Animals exposed to alcohol after birth only (Group L) were much less severely affected. Though often runty and thin and occasionally exhibiting body tremors, they nevertheless moved about quite well, and no splayed hind legs were ever observed. Animals in Groups C and G were generally found to be in good health, never exhibited body tremors, and roamed about in a normal fashion. Some observations on the developing motor skills and behavioral characteristics of pups in all four treatment groups are documented in Supplemental Investigations, Section III.

Among hamster young, a few are normally runts and usually die prematurely. Thus in this study, by 20 days postnatally, 4% of all control pups had died. Animals in Groups G and L exhibited mortality rates of 0% and 3%, respectively. The Group L young had a low death rate despite the fact that many were small in size and weighed much less than the control population. On the other hand, those pups in
Group G-L had a 51% mortality rate by 20 days postnatally, with the majority of deaths occurring in the third week of life. Cannibalism of the dead or dying pups by mother and littermates alike was much more frequently seen in this group of animals.

Differential cell counts

Figure 10 depicts the known sequence of nucleolar development in the hamster facial motor neuron as it is first seen with multiple nucleoli, then with a single nucleolus which lacks an intranucleolar body (INB), and finally with a single nucleolus containing either a punctate or prominent INB. The same pattern of nucleolar maturation also occurs in Purkinje cells.

In this study, thionin-stained facial motor neurons from 20-day hamsters (Fig. 11) closely resembled their adult counterparts, except for the markedly greater number of cells with multiple nucleoli in the younger animal group (Fig. 11A). An intranucleolar body was detected in many cells. Multiple nucleoli were even more characteristic of the Purkinje cell population (Fig. 12), while cells containing single nucleoli with or without an INB were relatively scarce (Fig. 13).

A few histological alterations were immediately observable in the facial motor neuron population from hamsters exposed to alcohol both pre- and postnatally (Group G-L). A small proportion of the
cells typically stained quite darkly, appeared shrunken, and displayed conspicuous, long, spindly processes (Figs. 14 and 15). No facial motor neurons from any other treatment group presented such an appearance. A large proportion of G-L cells also exhibited a central type of chromatolysis and/or extensive invaginations of the nuclear membrane (Fig. 16). In contrast to facial neurons, the 20-day ethanol-exposed Purkinje cells yielded no conspicuous differences from their controls.

Counts verified that Purkinje cells from 20-day-old animals normally have a less advanced type of nucleolar structure than do facial motor neurons (Fig. 17). (See also Table 4, Appendix E.) This is reflected in their having a much larger percentage of cells with multiple nucleoli (70% vs. 14% for facial neurons) and far fewer cells with single nucleoli containing either punctate-sized INBs (11% vs. 27%) or prominent INBs (3% vs. 46%). The percentage of cells displaying single nucleoli which lack INBs is approximately the same in both types of neurons (16% for Purkinje cells, 13% for facial motor neurons).

After exposure to ethanol (Group G-L), the facial motor nucleus had over twice as high a percentage of cells with multiple nucleoli (31%) as compared with controls (14%) (see graph, Fig. 18). The percentages of cells possessing single nucleoli and either punctate or prominent INBs were correspondingly decreased: from 27% to 20% for punctate and
46% to 35% for prominent INBs. These changes were highly significant (p < 0.001) and reflected a retardation in nucleolar development after ethanol. Interestingly, exposure to ethanol during the prenatal period alone (Group G) resulted in an even greater increase in the percentage of cells with multiple nucleoli (to 43%) and compensatory decreases in the percentages of cells with punctate (15%) or prominent (32%) intranucleolar bodies. These changes, too, were statistically significant (p < 0.001, see Table 4).

Figure 19 depicts the counts that were performed on Purkinje cells. Results were not as definitive as with the facial motor neurons and, in fact, showed a trend in the opposite direction, with a 4 to 5% decrease in cells containing multiple nucleoli after exposure to ethanol (p < 0.001 in both G and G-L groups). Comparison between the two experimental groups yielded no significant differences. Table 4 (Appendix E) provides a summary of all cell percentages and the p-values derived from a statistical analysis of the data for all three treatment groups.

An analysis of covariance was also performed on the same cell count data in order to make adjustments for weight and litter size. As Table 5 (Appendix E) shows, the results of this statistical test demonstrate that the nucleolar alterations produced after exposure to ethanol resulted from the timing of the alcohol treatment itself and not from changes in pup weight or size of litter.
Discussion

The results of this study indicate that ethanol alters the rate of neuronal nucleolar development and that it does this differentially depending upon the level of nucleolar (and cellular) maturation attained at the time of exposure. In particular, it appears that alcohol delays the conversion from multiple to single nucleoli. Normally, the great majority of facial motor neurons have acquired single nucleoli by 20 days postnatal age (LaVelle and LaVelle, 1958a). Yet after prenatal exposure to ethanol, this number is significantly decreased. Purkinje cells, on the other hand, are relatively unaffected since most of them normally still possess the multiple nucleoli of immaturity at 20 days.

The expectation that neurons of different developmental stages might react differentially to stress was previously established. Experimental studies involving immature and adult hamster facial motor neurons have indicated that successive stages in neuronal nucleolar and Nissl substance development can be correlated with varied types of cytological response to injury (LaVelle and LaVelle, 1958a, 1958b, 1959; LaVelle and Smoller, 1960; LaVelle, 1963). Although the histological appearance of developing Purkinje cells has been examined in a variety of animal species, including the hamster (Oster-Granite and Herndon, 1976), the connection between nucleolar development and responses to injury in the maturing Purkinje cell has not been studied.
It is well known that in the growth and differentiation of tissues and organs there are critical periods before and after which a given experimental insult may produce quite different results. This concept of critical periods may be extended to the development of individual cells and intracellular structures, in this case, particularly to the time of transition from multiple to single nucleoli. Although the inhibition of this transition by alcohol is clearly shown for facial neurons in this study, the exact time when alcohol begins to exert its effects on the cells is in question. The absence of gross abnormalities indicates that alcohol did not apparently affect the earliest stages of neurogenesis, migration, proliferation, and neuronal differentiation. It can be assumed, at least in broad terms, that afferents were reaching these cell groups and efferents were leaving them and that transmission of impulses was being effected, since the animals were able to suckle, walk, climb, and so forth.

Although all of these major developmental changes took place without overt histological evidence of abnormality, our results indicate that subtle, intracellular damage was nevertheless sustained and that it tended to preclude appropriate nucleolar development later on. In the hamster, most facial nucleoli undergo rapid conversion from multiples to singles in the first postnatal week (LaVelle and LaVelle, 1958a); most Purkinje cells still possess multiple nucleoli even at three weeks postnatal age. Continuous exposure to alcohol
throughout the prenatal and early postnatal periods adversely affects the normal postnatal conversion of facial motor neurons from multiple to single nucleoli. Yet alcohol must affect the cells prenatally, at even earlier stages of neuronal development, since exclusively prenatal exposure to alcohol (Group G in this study) also results in delayed nucleolar development of facial motor neurons. In fact, the retardation is even more pronounced. Neurons seem to be able to mature to at least the stage of multiple nucleoli under the influence of alcohol. Yet something happens to them so that they cannot readily go beyond this critical stage, even if alcohol is withdrawn. No long-term studies have yet been done to determine whether facial motor neurons can eventually recover and acquire their mature nucleolar configuration. Likewise, until longer survival times are used, the effect of alcohol on the nucleolar maturation of the later-developing Purkinje cell population remains unknown. Would they too, in due course, show an impaired transition from multiple to single nucleoli? Recent experimental evidence in rat Purkinje cells indicates that, at least in the case of cytoplasmic organelles, there is a delay in development after prenatal exposure to ethanol (Volk et al., 1981).

It should be noted that no cell counts were performed on 20-day animals exposed to alcohol during the postnatal period alone, since this group was included only for overt assessment of body size and general behavior. In what manner, if at all, the nucleolar morphology
of postnatally exposed neurons would be altered, without benefit of any prenatal "conditioning" with alcohol, awaits further investigation.

There are, of course, many aspects of neuronal development, other than the ones detailed here, which might be affected by exposure to alcohol. For example, the work of Bauer-Moffett and Altman (1975, 1977) and Anderson and Sides (1979) suggests that perinatal ethanol exposure results in the destruction of proliferating cell populations in rat cerebellar cortex. Evidence suggesting that ethanol may also delay or otherwise alter later maturational processes such as synaptogenesis, myelination, formation of dendritic spines, and neurotransmitter production has been provided by several investigators (Rosman and Malone, 1976; Rawat, 1977; Jacobson et al., 1979, Detering et al., 1980a, 1980b, 1980c; Sherwin et al., 1980; Slotkin et al., 1980; Borges and Lewis, 1981; West et al., 1981). To date, such studies have not utilized the golden hamster as an animal model.

Biochemical studies have shown that perinatal ethanol administration alters the RNA and/or protein synthetic activities of developing neurons and that the nature of this alteration (stimulation or depression) is apparently dependent not only upon the level and duration of ethanol exposure but also upon the type and developmental age of neurons being studied (Rawat, 1975b; Henderson and Schenker, 1977; Khawaja et al., 1978b; Henderson et al., 1980; Ullrich and Dietzmann, 1980; Fisher et al., 1981). Whether, and to what degree, developmental
irregularities can be linked to the action of ethanol on RNA/protein metabolism during neuronal maturation are significant questions that are only beginning to be answered. The expectation of maturational differences is high because of the results obtained after direct physical injury to neurons at different developmental ages and in the adult animal. In axotomized facial motor neurons, for example, there is a clear correlation between changing types of cytological response and cellular differences in the rate of incorporation of tritiated amino acids into protein (Griffith and LaVelle, 1971; Kinderman and LaVelle, 1977; McLoon and LaVelle, 1981a, 1981b, 1981c). Similarly, injury to maturing Purkinje cells has been related to subsequent defects in cellular RNA and protein synthetic capabilities (West and Kemper, 1976; McConnell and Berry, 1978; Pellegrino and Altman, 1979; Bernocchi and Scherini, 1980; Volk et al., 1981).

In addition to the differential nucleolar response to alcohol exposure reported here for 20-day facial motor neurons and Purkinje cells, there were other changes in individual facial nerve cells that did not appear in Purkinje cells, e.g., increased invaginations of the nucleus, central chromatolysis, and cell shrinkage. A highly invaginated nucleus may be indicative of a less "mature", more metabolically active cell, while the central chromatolysis is known to be a typical reaction of immature (15-day) facial motor neurons when axotomized (LaVelle and LaVelle, 1958b). Such nuclear/cytoplasmic
changes support the concept that alcohol delays the final stages of
cytopathological and functional maturation of at least some populations
of neurons and, furthermore, that it may cause a concurrent alteration
of the cellular machinery of RNA/protein metabolism.

Whereas mature neurons constitute a relatively stable metabolic
species as regards the types of proteins produced and the rates of
protein production and turnover, immature neurons represent a dynamic
metabolic species with a marked lability in the types of proteins
synthesized and degraded (Griffith and LaVelle, 1971; Lajtha et al.,
1979). Furthermore, Lajtha et al. (1979) have shown that the majority
of proteins in the immature brain are synthesized approximately four
times as fast and degraded at a rate three times as fast as most
proteins in the adult brain. Not surprisingly, the results of the
present study have demonstrated that the developmental response to
alcohol is different from that obtained in adult neurons (see Major
Body of Investigative Work, Section I). In adults, the nucleoli of
both facial motor neurons and Purkinje cells were advanced cytologically;
single nucleoli, many containing a punctate or prominent intranucleolar
body, were present. After chronic exposure to ethanol, the nucleoli
of these adult neurons did not revert back to the multiple form but
instead remained single and came to possess more frequent and larger
INBs. It was theorized that this reflected an upset in the balance of
RNA/RNP production and utilization such that rRNA and RNP were not
being used up as rapidly as usual in the synthesis of cytoplasmic proteins and therefore were being stockpiled in the nucleolus.

In young hamsters the delay in nucleolar maturation is presumably accompanied by a prolongation of an appropriate synthetic phase, including RNA and protein synthesis. Given that even normally the immature cell produces a different spectrum of proteins from the adult and not only synthesizes but also degrades these proteins at much higher rates, it can be speculated that immature neurons exposed to alcohol may be producing more "embryonic-type" proteins (Griffith and LaVelle, 1971) or even defective ones. This may involve the types of RNA being produced as well; it is not yet known whether multiple nucleoli as compared to single nucleoli synthesize the same RNA species and in the same amounts. There is a real need for both cytological and biochemical work to establish the differential susceptibility of specific cells or groups of cells to ethanol and, subsequently, to distinguish which portions of the RNA/protein synthetic chain of events are altered. With the exception of the recently published paper by Volk et al. (1981), which indicates a delay in ribosomal maturation, no light or electron microscopic studies have been reported on ethanol-induced alterations in nuclear, nucleolar, or ribosomal morphology of developing neurons following exposure to alcohol.
The significance of the effects of ethanol upon the developing brain is attested to by the growing volume of clinical evidence describing cases of the fetal alcohol syndrome in the human (see Review of Related Literature, pp. 27-30). The most severe effects observed on the offspring of ethanol-consuming mothers include microcephaly, incomplete brain development, severe mental retardation, and abnormal neuronal migration (Jones et al., 1973; Jones and Smith, 1973; Clarren and Smith, 1978). It now appears increasingly likely that even more "moderate" amounts of alcohol may produce behavioral abnormalities such as irritability and tremulousness in addition to deficits in motor development, poor sucking ability, and variable degrees of mental retardation (Little, 1977; Hanson et al., 1978; Martin et al., 1979; Ellis and Pick, 1980; Streissguth et al., 1980; Fish et al., 1981). The levels of ethanol to which developing hamster pups were subjected in this study were apparently not sufficiently high or prolonged to produce gross central nervous system defects. Yet more subtle evidence of neural damage was present, as individual pups showed signs of tremulousness and splaying of hind limbs.

An attempt was made in this study to simulate as closely as possible the route of ethanol intake and the quantities of ethanol consumed by many pregnant and lactating women. In order not to confound any effects presumed to be due directly to prenatal ethanol exposure with germ cell damage or reproductive organ malfunction
(Randall and Taylor, 1979), alcohol was not administered prior to mating. Blood ethanol levels were not measured for fear of adversely affecting the course of pregnancy. However, the levels of alcohol ingested were similar to those of virgin adult hamsters, and it has been shown in humans (Newman and Correy, 1980) and in rats (Abel and York, 1979) that pregnant as compared to non-pregnant subjects have slightly lower peak blood alcohol concentrations that are maintained for longer periods of time. That hamster fetuses and neonates were actually being exposed to ethanol was assumed since 1) the drug easily crosses the placenta and rapidly equilibrates between maternal and fetal blood (Baker, 1960; Mann et al., 1975; Ayromlooi et al., 1979), and 2) maternal milk contains virtually the same concentration of alcohol as is present in the circulating blood (Kesäniemi, 1974). Furthermore, since fetal and early neonatal animals are known to have lower ADH and ALDH activities than the adult (Rawat, 1976; Horton and Mills, 1979), it is highly probable that they are unable to metabolize ethanol sufficiently and that their blood ethanol levels, although comparable to those in the adult, are therefore potentially much more damaging.

Since malnutrition often accompanies chronic alcohol ingestion, the effects of each upon cell metabolism and bodily response need to be separated if possible. An organism possesses its maximal anabolic rates during development (Lajtha et al., 1979) and is therefore
especially sensitive to any lack of nutrients at this time. Using rats, Naismith (1973) and Zamenhof (1981) have shown that moderate restriction of maternal protein intake during pregnancy results in the mother's more efficient use of protein, along with an ability to transfer protein reserves from her own tissues to her fetuses. This fact, combined with the observations that pregnant, alcohol-consuming hamsters in this study ate and gained weight normally and gave birth to offspring of normal weight, suggest that fetuses of alcoholic mothers were not affected by malnutrition. Other investigators have drawn a similar conclusion for the rat (Lee and Leichter, 1980; Lindenschmidt and Persaud, 1980; Mendelson and Huber, 1980; Wiener et al., 1981).

The situation with progeny exposed to ethanol postnatally, however, is less clear. Ethanol, in sufficient quantities, is known to inhibit the release of oxytocin in animals (Fuchs and Wagner, 1963; Fuchs, 1969) and in man (Kesäniemi, 1974), thus inhibiting the secretion of milk. The occasional frenzied attempts by the hamster pups to suckle, together with the manifestation of inflamed nipples by nursing mothers, suggest that ethanol had at least partially inhibited maternal oxytocin release in this study. Body growth was certainly adversely affected by ethanol, particularly in those hamsters exposed to ethanol both pre- and postnatally (Group G-L). The small animal size, decreased body weight, unsteadiness of gait, and poor viability were most likely due to malnutrition. In contrast, hamster
pups exposed to ethanol only during their intrauterine life (Group G) were of good size, weight, health, and locomotion and, in fact, grew even larger postnatally than the controls. One can surmise that their general metabolic processes, free after birth from the alcohol depressant, now responded maximally to the demands of growth and concurrent repair.

It is significant, however, that nucleolar development in facial motor neurons did not advance normally after removal of alcohol, but retained a structural mode characteristic of younger, metabolically different cells. Evidently, nucleolar maturation was adversely (and possibly irreversibly) affected prior to birth, presumably as a direct effect of exposure to alcohol. It is possible that the retention of these cells in an even less mature state than those in Group G-L may be due to the greater demand for increased protein synthesis placed upon them after removal of alcohol. Perhaps, of course, they would ultimately recover if left free of alcohol long enough. The fact remains, however, that a marked and prolonged inhibition was effected by alcohol at an early, prenatal period when malnutrition was apparently not a determining factor.

Quite early in the course of this investigation, it was realized that the pups from any litter in any treatment group were not identical in body size or behavioral maturation. Individual weight differences were apparent even in control litters at four days postnatally and often
remained evident throughout much of the preweaning period. Correlated with this was a degree of differential susceptibility to the actions of ethanol, even within a single litter. The work of Abel and Greizerstein and Leichter and Lee (1979) tends to support this concept. In recognition of individual differences, and in order not to discard inadvertently those pups that might be most affected by ethanol pre- and/or postnatally, we did not cull litters to equalize their size. The results from the histological portion of this study suggest, however, that the effects of ethanol on nerve cells may be more consistent and specific than ethanol's actions on general body growth and development.

Some of the results of exposure to ethanol are probably unseen because of the high neonatal mortality rate. The maternal death and cannibalization of pups encountered in this study have also been reported for the rat (Tze and Lee, 1975), the rabbit (Fuchs, 1966), and the sheep (Potter et al., 1980). The fact that, among our hamsters, 51% of offspring exposed to alcohol both pre- and postnatally died prior to 20 days of age makes it quite likely that the cell count data from this (G-L) group may be somewhat skewed. Considering the amount of individual variation within a litter, it would appear that only those pups that were most hardy survived. The remainder, those presumably most affected by the ethanol, did not live to be included in the counts. Consequently, the difference in neuronal nucleolar
morphology between G and G-L treatment groups may not have been as pronounced as the results of this study tend to suggest. It would be important to next examine facial and Purkinje cell populations for their cytology before birth, at stages before 20 days postnatally, and at times long after, so as to trace the evolution of the differences between G and G-L neurons.

In studies on the effects of drugs on mammalian development, the role of the mother as an intermediary must be considered. Combined fetal and maternal mechanisms may be able to adapt to the detrimental effects of ethanol prior to birth, such that a degree of tolerance to the drug results. Furthermore, it seems quite likely that there may be different types of metabolic mechanisms operating at different stages of pre- and postnatal maturation to compensate for the effects of ethanol insult (Detering et al., 1979). Young animals exposed to alcohol on their own postnatally are clearly less competent to cope with the stress than when shielded by the mother prenatally. As already indicated, the factor of malnutrition became predominant in these pups. In line with the reality of individual differences, it is interesting to note that although many of these pups were unable to adapt metabolically and died in the second or third postnatal week, the remainder were able to mobilize intracellular metabolic mechanisms and at least partially compensate for the continuing
deleterious effects of ethanol. Consequently, they not only survived but physically grew and developed, although at a much slower pace than normal.

The effects of pre- and/or postnatal ethanol exposure on the growth and viability of offspring subsequent to the early neonatal period have been documented by a number of investigators, particularly in the last five to ten years. The decreased postnatal weight gain, increased variability in pup size, decreased viability, possible behavioral modifications, and delayed attainment of developmental parameters observed in this study are in agreement with the greater majority of papers published on this subject (see Review of Related Literature, pp. 32-34). The finding here that litters exposed to alcohol solely during the prenatal period were not adversely affected in their physical growth and development after birth likewise has some support in the literature (Øisund et al., 1978; Mendelson and Huber, 1980).

The timing and duration of ethanol exposure represent just two of many factors that play important roles in the survival and maturational outcome of ethanol-treated progeny. The alteration of maternal behavior by ethanol may affect the general care a litter receives, including the amount and quality of nursing that is provided. Since all pregnant and lactating hamsters ingested adequate amounts of food, were supplemented with additional quantities of vitamins,
gained weight, and always appeared in good health, it was assumed that their nutritional status had not been adversely affected. In contrast to the findings of Carver et al. (1953), ingestion of alcohol (or water) during the lactation period increased for almost all mothers. That ethanol was having some effect on these animals was evident in their behavior. The abnormal conduct of alcohol-ingesting mothers toward their offspring has been confirmed by many others. In particular, reports of maternal neglect or decreased maternal care have been published (Abel, 1978; Detering et al., 1979; Swanberg and Wilson, 1979; DaSilva et al., 1980). Regulation of maternal behavior in the hamster has been shown to be based upon endogenous maternal changes which occur prepartum and upon exogenous, litter-related factors which come into play in the postpartum period (Siegel and Rosenblatt, 1980; Swanson and Campbell, 1981). Whether the ethanol-induced alterations in maternal behavior originate with the mother (through hormonal changes as an example), or reflect a sensing by the mother that her pups are abnormal, is not known.

To our knowledge, the observation that "alcoholic" hamsters tend to engage in fewer nesting activities has not been previously reported. Likewise the occurrence of highly agitated and bizarre behavior, such as that exhibited by nursing, alcohol-imbibing hamsters, has heretofore not been noted in studies performed on other species. The type of behavior displayed (body tremors, squealing, exaggerated
Locomotor activity) is similar to that seen in laboratory animals undergoing withdrawal from alcohol. It is possible that rapid elimination of ethanol through maternal metabolic pathways and via breast milk results in periodic high and low levels of ethanol in the blood. Excessively abnormal behavior was almost always observed in the morning, a time when blood ethanol levels are normally low (see Supplemental Investigations, Section II) and when the pups are actively suckling.

Whether the effects seen in this study are due to some particular property of alcohol acting directly on neurons, or whether they reflect alterations in cellular metabolism due to alcohol-induced malnutrition, the fact remains that the cytological changes observed indicate an imbalance in metabolism (including RNA/protein metabolism) related to inherent cell maintenance. The results of the present investigation suggest that, in the hamster, there may be an increase in RNA/protein synthetic activity of the immature nerve cell following alcohol exposure. On the other hand, the few biochemical studies done to date in the rat generally point to a depression of brain RNA and/or protein synthesis in the developing brain after alcohol (Rawat, 1975; Henderson et al., 1980). Such apparent disagreement may reflect species differences, variations in the method and timing of ethanol administration, or differences in the neuronal populations.
chosen for study. There is an obvious need to determine the precise nature of ethanol's effects at the cellular or subcellular level. Some elucidation of the organellar morphology affected is discussed in the next section (Section III, Electron Microscopic Study).

III. EFFECTS OF ETHANOL IN THE ADULT AND IMMATURE HAMSTER NEURON: ELECTRON MICROSCOPIC STUDY

Introduction

Nucleolar changes have been seen with light microscopy in both adult and 20-day facial motor neurons and cerebellar Purkinje cells following chronic exposure to ethanol (see Major Body of Investigative Work, Sections I and II). The nature of the intracellular response depends upon the maturity of the cell during the period of exposure. Furthermore, the types of changes taking place have suggested an alteration in the protein synthetic machinery of neurons, thus providing cytological evidence for the changes in RNA/protein synthesis reported by biochemists. Light microscopy, however, cannot identify the portion of the synthetic chain most critically affected by ethanol, whether directly or indirectly. This may lie within the nucleolus itself, the nuclear pore structure, the ribosomal protein synthesizing machinery of the cytoplasm, or some other locus.

Consequently, a qualitative electron microscopic study was per-
formed in a preliminary attempt to reveal those subcellular elements altered as a result of chronic ethanol ingestion. Attention has been paid particularly to the appearance of the nucleolus, the nuclear envelope, the cytoplasmic ribosomal arrangement, and other organelles such as mitochondria and Golgi apparatus, which are involved in synthetic activity. As with the light microscopic portion of this dissertation, ultrastructural examination was carried out on facial motor neurons and cerebellar Purkinje cells of both adult and 20-day-old golden hamsters.

Materials and methods

The animals chosen for ultrastructural analysis included 1) adult hamsters which had ingested either tap water or a 15% (v/v) ethanol in water solution for seven weeks and 2) 20-day-old hamster pups which had been exposed to either water or to 15% ethanol continuously throughout their gestation and suckling periods. The care and feeding of the animals was as described elsewhere in this dissertation (see Major Body of Investigative Work, Sections I and II, Materials and methods).

During the mid-morning period of each appropriate day, adult and 20-day hamsters were anesthetized with sodium pentobarbital and fixed by intracardiac perfusion with a solution of 2% formaldehyde-2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). The brains
were retained in cold fixative overnight. Subsequently, pieces of tissue from the facial motor nucleus and from the lateral cerebellar hemispheres were trimmed into 1mm³ blocks, postfixed in 1.33% osmium tetroxide for 1.5 hours, dehydrated in graded ethanol, and embedded in an Epon-Araldite mixture. (See Appendix B for details of perfusion and tissue preparation.) Thin sections were cut with glass knives or with a diamond knife on a Porter-Blum MT-2 microtome, stained with aqueous uranyl acetate and lead citrate, and examined and photographed at 50kV on an RCA EMU3F electron microscope. In an effort to eliminate bias in the absence of quantitative analysis, a blind study of all tissue specimens was performed throughout.

Results

Control tissue: overview

In 1µm plastic sections stained with toluidine blue, adult facial motor neurons were easily distinguished from surrounding cell populations by their massive size and large dendritic bases (Fig. 20A). The Nissl substance within the cell cytoplasm stained a pale blue, while darker-staining particles represented lipofuscin. The nucleus was generally round to ovoid in shape and occasionally exhibited striations which, at the electron microscopic level, were seen to be invaginations of the nuclear envelope. Almost all of the cells possessed a single, prominent, very darkly staining nucleolus. The surrounding neuropil
consisted mainly of glial cells and myelinated axons.

In sections from 20-day-old animals, facial motor neurons appeared almost identical to those from the adult. However, the nuclei of the younger cells more frequently exhibited infoldings of their membranes and often contained multiple nucleoli (Fig. 20B).

In the adult brain, cerebellar Purkinje cells formed a single layer between the almost soma-free molecular layer and the cell-packed granule layer (Fig. 20C). Purkinje cells and their nuclei were usually oval in shape, and the apical dendrite was occasionally evident. A single, darkly staining nucleolus was commonly observed within the vesicular nucleus.

Purkinje cells from the 20-day-old hamster were similar to those in the adult with the exception that multiple nucleoli were much more prevalent (Fig. 20D).

With low-power electron microscopy, facial motor neurons from adult control hamsters (Ad-C-VII) displayed typical cellular organelles: mitochondria, Golgi, ribosomes (Figs. 21 and 22). Golgi apparatuses were particularly conspicuous in the cell soma and were predominantly located in a perinuclear position. Free ribosomes were dispersed throughout the cytoplasm, and "clumps" of rough endoplasmic reticulum (Nissl substance) were also observed (Fig. 21). Aggregates of lipofuscin were distributed in a random fashion. The euchromatic nucleus had a vesicular appearance, and occasional infoldings of its double
membrane were evident. Because of the large dimensions of the cell body and nucleus, thin-sectioning often failed to expose the presence of the single nucleolus. The general appearance of these cells resembled that type of large multipolar neuron commonly depicted in histology texts.

Low-power views of cerebellar Purkinje cells of adult control animals (Ad-C-Purk) revealed the same structures: prominent Golgi, free and membrane-bound ribosomes, large numbers of mitochondria, scattered lipofuscin (Fig. 23). However, the Purkinje cytoplasm was much more crowded with organelles than was the cytoplasm of adult facial motor neurons. Furthermore, the nuclear outline of these cells was usually more irregular, and invaginations of the nuclear envelope were more often evident. For the most part, the nuclei were devoid of heterochromatin, except as associated with the prominent, single nucleoli. The overall appearance of adult hamster Purkinje cells was quite similar to that reported for Purkinje cells of the rat and other species (Palay and Chan-Palay, 1974).

Purkinje neurons from control, 20-day-old hamsters (20D-C-Purk) often contained multiple nucleoli (Fig. 24). Otherwise, the nuclear/cytoplasmic morphology of this population of developing cells resembled that seen in the adult.

On the other hand, in facial motor neurons, there were ultrastructural differences between mature and immature cells. In some
ways, developing facial motor neurons (20D-C-VII, Fig. 25) were not unlike adult Purkinje cells in their subcellular appearance. The cytoplasmic organelles were closely packed, the round to oval nucleus was often uneven in contour, and deep invaginations of the nuclear envelope were evident. However, multiple nucleoli were rare, and there were more clumps of rough endoplasmic reticulum than were usually seen in Purkinje cells.

**Nucleolar fine structure**

After exposure to alcohol, no striking dissimilarities from the control appeared in nucleolar ultrastructure. In cells from any treatment group studied the nucleoli, whether single or multiple, bore certain characteristics in common. Portions of the pars granulosa were interspersed with portions of the pars fibrosa and with fibrillar centers and nucleolar vacuoles (Fig. 26). At least one clump of perinucleolar chromatin capped the nucleolar surface. In sections through the intranucleolar body (INB), a coalescence of the pars granulosa into a single, centrally-located aggregate was observed (Figs. 27 and 28). Depending upon the particular cut through the nucleolus, the amount and configuration of its components were found to vary (Figs. 29 and 30). Although single nucleoli were usually located in the center of the nucleus, away from cytoplasmic components, close association with free ribosomes in particular was often made
through invaginations of the nuclear envelope (Fig. 31). Most of these centrally-located nucleoli would be considered to be highly differentiated or developmentally advanced.

In contrast, among multiple nucleoli, many were typically located adjacent to the nuclear envelope (Fig. 24) and were seen in varying stages of development from purely DNA masses to DNA- and RNA-containing structures (Figs. 32-34). In all instances, the DNA-containing nucleolus-associated chromatin lay immediately next to the nuclear membrane.

Although exposure to alcohol did not produce any visible changes in the structural interrelationships of nucleolar components, it did, however, appear to modify other subcellular structures sufficiently so that alterations could be visually detected with electron microscopy. Validation of such changes awaits quantitative assessment. In the descriptions to follow, experimental groups are coded thus: adult facial motor neurons (Ad-Ale-VII), adult Purkinje cells (Ad-Alc-Purk), 20-day facial neurons (20D-Ale-VII), and 20-day Purkinje cells (20D-Alc-Purk).

Nuclear envelope

Modification of the nuclear envelope was often seen in alcohol-exposed cells. There appeared to be an increase in the numbers of nuclear pores, particularly in Ad-Alc-VII neurons (Fig. 35). Extensive
invagination of the nuclear envelope was noted in many 20D-Alc-VII cells (Fig. 36), providing ultrastructural confirmation of the light microscopic findings reported for this neuronal population (see Major Body of Investigative Work, Section II, Results).

Ribosomes, rough endoplasmic reticulum

Ethanol seemed to have its most consistent effect on the system of free and membrane-bound ribosomes in the cytoplasm. Neurons from alcohol-treated animals typically displayed a marked quantity of polyribosomes. A greater proportion of free as compared to membrane-bound ribosomes was particularly evident in Ad-Alc-VII cells, where, even at low magnifications, they seemed to "flood" the cytoplasm (Figs. 35A and 37). Ad-Alc-Purk and 20D-Alc-VII neurons also exhibited some tendency toward increased numbers of free ribosomes, these being densely distributed in various locations throughout the neuronal soma. In the latter cell population, large quantities of polysomes were concentrated on the cytoplasmic side of nuclear infoldings (Fig. 36). In spite of the impression of there being more free than membrane-bound ribosomes in alcohol-exposed cells, there could occasionally be found, in selected areas of cytoplasm, dispositions of these organelles which were virtually identical in both experimental and control neurons (Figs. 38 and 39).

In nerve cells from 20-day, alcohol-exposed animals only, there
was frequently observed a breakdown of the polysomal arrangement of free ribosomes into what might be termed "monoribosomes" or "monosomes" (Fig. 40). Unique to 20D-Alc-VII neurons was the shifting of membrane-bound ribosomes (RER) to the periphery of the cell soma and their almost total absence from the perinuclear area (Figs. 41 and 42). This provided electron microscopic evidence of the central chromatolysis that was seen in the same cells with light microscopy (see Major Body of Investigative Work, Section II, Results). Even with concentrated numbers of free ribosomes about the nucleus, most bound ribosomes were still found to accumulate more peripherally (Fig. 36). Unlike all other groups, the organization and distribution of free and membrane-bound ribosomes in 20-day Purkinje cells seemed unaltered by alcohol (Fig. 43).

An apparent difference was noted in the width of cisternae of RER in adult neurons after treatment with alcohol. Normally, the cisternal space in Ad-C-VII cells was very narrow (Fig. 44), and it seemed considerably widened with alcohol (Fig. 45). In Ad-C-Purk cells the cisternae were relatively wide to begin with (Fig. 46A) and were sometimes grossly enlarged after alcohol administration (Fig. 46B). Often, the rough endoplasmic reticulum located adjacent to the nucleus was most markedly altered. Figures 47A and 47B demonstrate at high magnification the extremes in cisternal width observed in this study.
Mitochondria

Another cellular constituent notably modified by ethanol was the mitochondrion. A comparison of typical control mitochondria with those from alcohol-treated animals often revealed the swelling of this organelle following ethanol exposure (Fig. 48). As a result of this swelling, the mitochondrial matrix became less electron-dense and there appeared to be an increase in intercristal distance. In rare cases, grossly enlarged mitochondria were seen with their internal and/or external configuration markedly disrupted (Figs. 49 and 50A). Such deformation was never observed in control neurons. Other mitochondrial alterations noted after alcohol exposure included peculiarities of shape (Fig. 50B) and marked elongation (Fig. 50C), particularly in Ad-Alc-Purk and Ad-Alc-VII cells. In addition, there was often "vacuolization" of mitochondria and striking irregularities of the external mitochondrial membrane in 20D-Alc-VII neurons (Fig. 51). Mitochondria from 20D-Alc-Purk cells exhibited the least alterations.

Golgi bodies

Golgi apparatuses in neurons from normal 20-day and adult hamsters were seen in their classical configuration, with well-organized stacks of cisternae and tubules (Figs. 48A and 52). In contrast the Golgi bodies from experimental cells, particularly 20D-Alc neurons, generally
seemed more prominent, more extensive, and more irregular in form. This difference was evident even at low magnification (Fig. 43). Details at higher magnification can be seen in Figures 40, 51, and 53.

**Lipofuscin**

Changes in one final organelle warrant mentioning here. Both the quantity and size of lipofuscin granules appeared greater following alcohol exposure in some Ad-VII and Ad-Purk neurons (compare Figs. 21 and 35A; 23 and 54). As with other observations in this ultrastructural study, confirmation awaits quantitative evaluation.

**Discussion**

The object of this portion of the investigation was to determine whether any visible ultrastructural changes could be elicited in neurons by exposure to ethanol, and, if so, whether they might further our interpretation of changes seen with light microscopy. To date, the observations have been confined to organelles which are in some way involved in RNA/protein synthesis, and they have been qualitative in nature. Any morphometric analysis for statistical purposes has been reserved for later in-depth pursuit.

When this work was begun, there was no published literature in the field of alcohol research that described intracellular alterations in neurons, in either adult or developing brains, at either the light
or electron microscopic level. Evidence of change had been provided almost entirely in biochemical, physiological, and behavioral terms (see Review of Related Literature, pp. 16-26, 35-43). In the past three years a few reports of subcellular changes in adult neurons have been published (Volk, 1978, 1980; Karwacka, 1980b). As yet, the recently published work by Volk et al. (1981) in the rat, together with the present study in the golden hamster, provide the only descriptions of intracellular alterations in immature neurons exposed to ethanol.

Several ultrastructural features related to the protein synthetic capability of the adult and developing neuron following exposure to alcohol can now be reported as preliminary, yet potentially significant, observations. A primary observation was the abnormality of ribosomal distribution in ethanol-exposed neurons. The proportionate increase of free over membrane-bound ribosomes in many experimental cells suggests a partial degranulation or disaggregation of the rough endoplasmic reticulum. Alcohol has been reported to cause such effects in the rat liver and heart (Dobbins et al., 1972; Rothschild et al., 1975; Baraona and Lieber, 1979). Similar morphological changes have not heretofore been described in the brain, although one group of investigators has published biochemical evidence for ethanol-induced conformational changes in rat brain microsomal membranes (Gruber et al., 1977), and others have reported decreased incorporation of labeled
amino acids into the cerebral membrane-bound ribosomes of alcohol-exposed animals (Noble and Tewari, 1975; Khawaja et al., 1978a; Lindholm and Khawaja, 1979). Therefore, the possibility exists that ethanol disturbs cytoplasmic protein synthesis in the nerve cell by altering the membrane structure of its endoplasmic reticulum.

In both populations of developing nerve cells there appeared to be increased numbers of "monoribosomes", which are usually thought of as representing the inactive ribosomal fraction (Zomzely-Neurath and Roberts, 1972). It is conceivable that the appearance of monosomes in growing neurons already intensely active metabolically may reflect the eventual exhaustion of the protein synthetic capacity of their active counterparts when they must also cope with the presence (and intracellular effects) of alcohol. It is also possible that ethanol exposure may lead to defects in mRNA molecules and/or in the ribosomes themselves and, consequently, to a decreased capacity of ribosomes to bind with mRNA. Noble and Tewari (1975) and Tewari et al. (1980), using the brains of chronic, alcohol-consuming adult rats and mice, have provided biochemical evidence for such an hypothesis. The increased prominence and irregularity of the Golgi bodies of 20-day, alcohol-exposed neurons likewise intimate that these cells may have reached the limits of their metabolic capabilities and may possibly be exhibiting signs of potential degeneration. (See Scherini et al., 1981, for a description of organellar changes attributed to either functional
variations or incipient degeneration in non-experimental Purkinje cells.

These ethanol-induced changes in the system of free and membrane-bound ribosomes were apparent in all cell populations studied with the exception of 20-day Purkinje cells. Volk et al. (1981) have reported that alcohol administered to female rats before and during pregnancy results in a delayed cytoplasmic maturation of Purkinje cells in their offspring. This developmental delay primarily involved the quantity and distribution of free and membrane-bound ribosomes. Interestingly, this delay was seen only during the very early postnatal period and by 17 days could no longer be observed. Since the present study involved examination of developing Purkinje cells from 20-day-old animals exclusively, the possibility that temporary alterations of ribosomal populations occurred in Purkinje cells of younger hamsters can not be ruled out.

In adult neurons exposed to ethanol, there was believed to be an increase in width of the cisternae of rough endoplasmic reticulum. This may indicate a partial inhibition of protein transport and/or secretion which would lead to intracellular accumulation of protein. Electron microscopic studies of adult rat cerebral cortex (Karwacka, 1980b) and biochemical studies using adult rat liver (Sorrell and Tuma, 1979; Tuma et al., 1980; Tuma and Sorrell, 1981) provide support for this hypothesis.
It was not surprising to find that disorganization of organelles responsible for protein synthesis was accompanied by alterations in the morphology of organelles responsible for the energetics of cell metabolism. The observation, in experimental cells only, of apparently normal mitochondria adjacent to those appearing grossly swollen suggested that poor fixation was not a factor. In immature and mature cell populations alike, the occurrence of distended, elongate, misshapen, and vacuolated mitochondria, coupled with disruption of their internal membrane structure, provided strong evidence that mitochondrial function was impaired as a result of exposure to ethanol. Almost identical structural changes have been reported for mitochondria in pancreas, skeletal muscle, and small intestine (Cederbaum and Rubin, 1975) as well as in the liver (French, 1971; Dobbins et al., 1972; Baraona and Lieber, 1979), the heart (Mall et al., 1980; Mattfeldt et al., 1980), and the brain (Karwacka, 1980b; Karwacka et al., 1980). There is some support in the biochemical literature for ethanol-induced impairment of enzymatic function (Kornguth et al., 1979) and respiratory activity (Karwacka et al., 1980) of neuronal mitochondria. Tewari et al. (1978) have shown that chronic ethanol consumption by rats alters RNA metabolism in brain mitochondria. From a somewhat different perspective, several investigators have provided evidence to suggest that the structural and functional changes in mitochondria after long-term alcohol exposure may be due to alteration in cytoplasmic and mito-
chondrial protein synthesis and consequent interference with mito-
chondrial membrane biogenesis (Burke and Rubin, 1979; Mall et al.,
1980; Mattfeldt et al., 1980).

In this study, ultrastructural changes in the nucleus were not
as numerous or varied as those in the cytoplasm. Among adult cells
there appeared to be an increased number of pores in the nuclear mem-
branes of facial motor neurons. Pore size may also have been enlarged.
Such observations may be indicative of an increase in activity on the
part of the cell; certainly the passage of substances across such
membranes would not appear to be impeded.

In 20-day animals exposed to ethanol, an increased irregularity
of the nuclear envelope was evident. In fact, the nuclear membranes
of facial motor neurons were often highly invaginated. The additional
presence of large numbers of free ribosomes on the cytoplasmic side
of the infoldings suggests that these are regions of very intense
metabolic activity.

The nucleoli of Purkinje and facial motor neurons from both
control and alcohol-consuming hamsters were ultrastructurally similar
in appearance. There was no evidence of the fragmentation of fibrillar
and granular components so often reported for other types of cells
after treatment with other agents which alter the synthesis and/or
utilization of rRNA by the cell (Busch and Smetana, 1970; Simard et al.,
1974). It has been shown with light microscopy (see Major Body of
Investigative Work, Section I) that exposure to ethanol results in statistically significant alterations in size of the intranucleolar body in adult neurons. The work of Kinderman and LaVelle (1976) has indicated that such variations are translated ultrastructurally as larger or smaller aggregates of ribonucleoprotein particles (pars granulosa) and that, functionally, they represent shifts in the balance of RNA/protein synthesis and utilization by the neuron.

The present observations with electron microscopy revealed nothing inherent to the nucleolus itself that can explain fluctuations in the amount of ribosomal precursor material either retained within (as the INB) or released from the nucleolus. Nor was there any evidence of blockage of this RNA/RNP at the nuclear envelope, since none of it was found to accumulate on the nuclear side of the envelope. In fact, the "channels" or pores that are presumed to be utilized for the passage of ribosome-like particles and other substances across this double membrane were either just as numerous in experimental neurons as in controls, or even more so. It seems likely, therefore, that the nuclear/nucleolar portion of the chain of synthetic events leading to protein production remains virtually intact, i.e., that rRNA transcription and RNA/RNP transport are capable of continuation during periods of alcohol exposure.

The use to which this material is put in the synthesis of cytoplasmic proteins, however, is in doubt. The apparent changes observed
in the amount and organization of free and membrane-bound ribosomes suggest that the protein synthetic process has in some way been altered. Biochemical evidence from other studies would indicate that the net effect is a decrease in the capacity to synthesize protein (Tewari and Noble, 1971; Jarlstedt, 1972; Noble and Tewari, 1973; Renis et al., 1975; Morland and Sjetnan, 1976; Tewari et al., 1977; Tewari et al., 1980). It is also possible that protein transport and/or secretion has been affected in these nerve cells.

The results of this preliminary electron microscopic study suggest that chronic ethanol exposure leads to alterations in a variety of cytoplasmic organelles involved in protein and/or energy metabolism and support the findings of earlier light microscopic investigations (see Major Body of Investigative Work, Sections I and II) as to the age-dependent nature of the interactions between nucleolus and cytoplasm following alcohol treatment. It is our belief that in adult hamster neurons, an apparent decrease in cytoplasmic demand for RNA/RNP after prolonged exposure to ethanol results in storage of the excess within the nucleolus, producing an enlarged INB. Other investigators have shown that the cytoplasm of a cell does contain a number of factors which can affect the metabolism of RNA as well as its release from the nucleus (Craig and Perry, 1970; Jacob et al., 1970; Planta et al., 1972; Weck and Johnson, 1978; Luetzeler et al., 1979). With regard to developing neurons, whether normal or exposed to ethanol,
an overall paucity of work (see Review of Related Literature, pp. 39-43) provides little supportive information on which to construct meaningful hypotheses about their differential reactions. It can not be emphasized too strongly that the developing organism is "... a different metabolic species than the adult and must be regarded as such" (Miller, 1970). Further discussion of comparative stages of maturation among the neurons described in this study, along with some speculation regarding their differential responses after exposure to ethanol, will be found later in the General Discussion.
SUPPLEMENTAL INVESTIGATIONS

I. ADMINISTRATION OF ETHANOL:

UTILIZATION OF A TOTALLY LIQUID DIET

Freund (1969) and Lieber et al. (1975) introduced the liquid diet method of ethanol administration in mice and rats respectively. The advantage of such a diet is that it permits these species to consume larger amounts of ethanol (up to 36% or more of their total caloric intake) than they would normally ingest by drinking an ethanol-water solution as part of a diet which includes standard laboratory chow or other solid food. A second advantage is that the liquid diet results in a relatively even distribution of fluid intake over a 24-hour period, with the maintenance of continually high blood ethanol levels. For these reasons, it was decided at the beginning of this dissertation project to administer an ethanol-containing liquid diet to adult hamsters.

Procedures and observations

The liquid diet chosen consisted of chocolate-flavored Nutrament (Drackett Products) containing either 35% of the total calories as sucrose (control) or as 6% (v/v) ethanol (experimental). All animals were initially introduced to the control regimen to allow them a period of adjustment to a totally liquid diet. However, within one week, all animals exhibited severe weight loss and loss of hair.
They were either sacrificed with an overdose of Nembutal and discarded or were placed back on a regular diet of water and laboratory chow, on which they eventually recovered and thrived.

**Comments**

Lamb *et al.* (1979) attempted to maintain hamsters on a liquid diet regimen but were unsuccessful since the animals "refused" to ingest the diet. The hamsters utilized in the present study showed no aversion to the chocolate-flavored Nutrament, but the results would suggest that either they did not consume sufficient quantities for their own well-being or that the diet's nutritional composition was totally inadequate for the hamster. It was decided to abandon the liquid diet as a means of administering ethanol in this investigation. In many ways, the regimen finally chosen was more comparable to the dietary practice of human alcoholics.
II. MEASUREMENT OF BLOOD ETHANOL LEVELS: GAS CHROMATOGRAPHIC STUDY

Despite its preference for ethanol solutions over water, the golden hamster has not been commonly used in alcohol research, and little is known about the titer (concentration) of alcohol in its bloodstream following ethanol ingestion. Consequently, 20 adult hamsters chronically consuming 15% alcohol for up to seven weeks were selected for determination of blood ethanol levels with gas chromatography.

Procedures

Blood samples were obtained under Nembutal anesthesia by cardiac puncture at 0800, 1400, 2000, and 0200 hours. Each sample was withdrawn into a heparinized syringe by means of a 5/8in, 25-gauge needle. Twenty μl of blood were mixed with 1-propanol (gas chromatograph internal standard) plus preservatives (sodium fluoride and sodium nitrite) in a 125 X 16mm Vacutainer and immediately chilled. Blood specimens from each hamster were prepared for triplicate analysis. All samples were frozen within 30 minutes of collection and were stored at -60°C until analyzed.

For gas chromatographic assay, each sample was warmed to room temperature. With a tuberculin syringe, 0.15ml of saturated oxalic acid was added to the sample by piercing the Vacutainer's rubber
stopper. The contents were then mixed on a Vortex mixer. To complete vaporization of the alcohol, each tube was wrapped in aluminum foil and placed in a water bath (55°C) for ten minutes. Two ml of the gas above the sample were subsequently withdrawn into a gas syringe and compressed so as to permit rapid injection of a bolus of gas ("head space gas") into a Perkin-Elmer Model 910 gas chromatograph equipped with dual hydrogen flame detectors. Stainless steel columns (6ft x 1/8in) were packed with Poropak Q/R. Column temperature was 170°C, injector temperature 190°C, and detector temperature 210°C. The carrier gas (helium) was delivered at a flow rate of 30ml/min. A signal fed from the flame detector to a calculating integrator (Perkin-Elmer M-2) and then to a recorder provided peak areas and heights in addition to gas elution times (Fig. 55).

A working reference solution containing a known concentration of ethanol (800μg or 0.8mg ethanol per ml of blood) was prepared by mixing the internal propanol standard, blood-bank blood, and ethanol reference standard together and pipetting 200μl of the mixture into Vacutainer tubes. At various times throughout the day, a working reference sample was mixed with oxalic acid, heated, and injected as a bolus of gas into the gas chromatograph.

Retention time for ethanol was 119-125 seconds and for propanol 238-246 seconds. The ratios of the peak area of the ethanol to the peak area of the propanol in the unknown sample (E/P_u) and in the
working reference solution \((E/Pr)\) were used to calculate the concentration of ethanol in the unknown sample \((Cu)\) as follows:

\[
Cu \text{ (mg/100ml)} = \left(\frac{(E/P_u)}{(E/Pr)} \times 0.8\right) \times 100.
\]

The above-outlined micromethod for determination of blood ethanol levels was devised by R.P. Sturtevant and has been shown to be a very sensitive, accurate, and reproducible assay procedure (Sturtevant, 1977 and personal communication).

**Observations**

Figure 56 depicts the mean 24-hour blood ethanol levels of adult hamsters chronically ingesting 15% alcohol. No statistical analysis was performed since different animals were utilized for determinations at each of the four time periods examined: 0800, 1400, 2000, and 0200 hours. Nevertheless, the results indicated that the animals had higher blood ethanol levels during the dark phase of their day-night cycle. Hamsters are known to be more active physically at night and would be expected to ingest more fluid at this time. The highest blood ethanol levels were achieved at 0200 hours (mean = 141mg%, highest individual value = 209mg%), while the lowest levels were recorded at 1400 hours (mean = 23mg%, lowest individual value = 2mg%).

The animals often slept after ingesting a moderate amount of alcohol and were sometimes difficult to arouse, but they rarely showed signs of any loss of motor coordination. They appeared hyper-
excitable and irritable on occasion, but such behavioral alterations were neither excessive nor prolonged.

Comments

This gas chromatographic evidence suggests that adult hamsters do not maintain high blood ethanol levels for any extended period of time. It can be reasonably assumed, therefore, that their brains do not suffer prolonged exposure to high concentrations of ethanol. The fact that the golden hamster rarely shows behavioral effects from short- or long-term alcohol ingestion has led other investigators to suggest that this animal is able to metabolize ethanol rapidly and that its central nervous system may be somewhat insensitive to the drug (Thurman et al., 1978; Harris et al., 1979). However, substantial evidence for or against this hypothesis is presently lacking.
III. EFFECTS OF ETHANOL EXPOSURE ON HAMSTER OFFSPRING: 
MATURATIONAL AND BEHAVIORAL OBSERVATIONS

Among the characteristic features of the fetal alcohol syndrome is a delay in the attainment of appropriate maturational levels of achievement. These are, presumably, the overt signs of impaired neuronal function. Although a controlled behavioral study was beyond the scope of this dissertation project, simple observations were nevertheless made of the external development and behavioral progress of offspring exposed to ethanol.

Procedures and observations

The few parameters selected for monitoring were rather simplistic and were related to normal activities of young hamsters. Furthermore, no attempt was made to identify each animal in a litter or to follow individual progress. Nevertheless, daily examination and recording of the indices attained by each litter revealed general trends which differed among the various treatment groups. The results are summarized in Table 6 (Appendix E).

For the most part, pups subjected to alcohol both pre- and post-natally (Group G-L) took longer to develop certain physical features (fur on their bodies, eye opening) and to accomplish particular physical tasks (adult-type ambulation, recognition of and withdrawal from a ledge, ability to climb up and over an obstacle, drinking from
a bottle) than did the pups in any other treatment group. They also were observed to suckle longer postnatally, while at the same time beginning to eat solid food earlier. Those hamsters exposed to alcohol only after birth (Group L) exhibited slight developmental delays in the indices examined, while offspring exposed to alcohol solely during the prenatal period (Group G) appeared to achieve these parameters just as fast as, and in some cases more quickly than, the control pups (Group C).

Comments

It is not surprising that the animals most affected by ethanol were those exposed both pre- and postnatally (Group G-L). The fact that many of these animals were unsteady on their feet and appeared uncoordinated (especially in the execution of movements involving the hind legs) could be due to their generally weakened condition and small size (see Fig. 9). Or it could be indicative of skeleto-muscular defects, cerebellar damage, and/or injury to a variety of brain areas involved in motor function. Others have reported similar findings with the rat (Martin et al., 1977; Rider, 1979; Diaz and Samson, 1980). The continuous body tremors exhibited by many G-L animals in this study suggested that exposure to alternating high and low levels of ethanol in the maternal milk (see Fig. 56) caused them to undergo periodic withdrawal-like reactions. Even those
animals that appeared to remain in relatively good health throughout the 20-day postnatal period were hyper-responsive in their behavior. A similar condition has been noted in rats (Diaz and Samson, 1980) and mice (Yanai and Ginsburg, 1979) exposed to alcohol.

Those young hamsters exposed to ethanol solely after birth (Group L) exhibited only slight delays in their attainment of the various developmental indices examined, despite the fact that most of them were as "runty" and small for their age as were those exposed to alcohol throughout gestation and suckling (see Fig. 9). However, the number of litters comprising the group was small (four), and not all developmental parameters were used in the evaluation of these pups. More rigorous testing of a larger number of offspring would be needed before any definitive comparisons could be made between pups subjected to postnatal ethanol exposure and those on the other three treatment regimens.

Hamster progeny which came in contact with ethanol in utero only (Group G) were surprising not only in their significantly greater growth rate when compared to controls (Fig. 9) but also in their more rapid achievement of the developmental and behavioral indices studied. It would appear that removal of ethanol at birth resulted in an "overcompensation phenomenon", with these animals growing, developing, and attaining physical attributes at a faster pace than would normally have been expected. Again, more refined testing procedures and more
precise criteria would have to be used before the true nature of this apparent growth "acceleration" could be determined.
GENERAL DISCUSSION

Ethanol may well be the most generally abused chemical agent used by man today. The magnitude of the problem in both adult and juvenile populations is reflected in the damage done to almost every organ of the body, including the brain. Following chronic ingestion of alcohol, the regions of the brain affected, as well as the degree of injury inflicted, are variable. Alcoholism is a disease which not only affects the body and brain of the drinking individual but may also cause severe problems for that individual's offspring. Since 1973 and the work of Jones et al. and Jones and Smith, the concept of a "fetal alcohol syndrome" has become firmly established, and the realization that maternal ethanol consumption during pregnancy and/or lactation may have devastating effects on the developing organism has increased, albeit slowly, among the general population.

The mechanisms by which ethanol exerts its damage, particularly at the cellular (metabolic) level, are only beginning to be understood. If a single locus for the action of ethanol on the individual cell is ever found, it will most likely involve changes in membrane production, structure, and function. Yet the means by which the action of the ethanol molecule on cell membranes could account for the diversity of pathology observed following ethanol exposure is a question for which an answer may be long in coming. An integration of the research efforts
of investigators from a variety of disciplines will be necessary. Considering both the enormous heterogeneity of the brain's neuronal constituents and the metabolic differences between its mature and immature neuronal species, inquiry into the effects of ethanol on the adult and developing brain is made that much more difficult.

The majority of studies concerned with the effects of alcohol on neuronal metabolism, specifically RNA/protein metabolism, have approached the problem on a purely biochemical level. Only recently have there been correlative cytological investigations to provide a morphological basis for the biochemical findings. Two basic pieces of information gave impetus to the present cytological study. First was the knowledge that nucleolar and Nissl substance development in the hamster neuron are closely related and reflect changes in intracellular protein metabolism during maturation (LaVelle, 1951, 1956; LaVelle and LaVelle, 1958a, 1958b; Griffith and LaVelle, 1971; McLoon and LaVelle, 1981a, 1981b, 1981c). Coupled with this was the discovery of an intranucleolar body which is able to serve as an in situ, cytological indicator of changes in the activity of the neuronal machinery involved in RNA/protein metabolism in the adult hamster (LaVelle and LaVelle, 1975; Kinderman and LaVelle, 1976, 1977).

The basic question, to which there was no guaranteed answer, was whether the nucleolus, which is known to respond to injury and to changes during maturation, could be structurally altered by exogenous
chemical means (e.g., by ethanol). Two additional questions were posed: 1) if ethanol affects the nucleolus, will it likewise affect other organelles involved in RNA/protein metabolism?, and 2) since mature and immature neurons are different metabolically, will their response to ethanol insult also differ? This study was necessarily exploratory in nature, asking a broad question and seeking general answers. Within the scope of the study, certain specific questions were asked and examined in statistical detail. Many more questions were raised than could be answered in the time permitted.

The major conclusion drawn from this investigation is that ethanol does structurally alter the RNA/protein synthetic machinery of the neuron. This was demonstrated in both facial motor neurons and cerebellar Purkinje cells. At the light microscopic level, there was evidence of an increase in size and frequency of the intranucleolar body (INB) in both populations of adult nerve cells following chronic ethanol consumption, and of a delay in the later stages of nucleolar development in young (20-day) facial motor neurons exposed to ethanol prenatally only or both pre- and postnatally. At the electron microscopic level, a variety of changes were seen in the organelles responsible for RNA/protein synthesis in both adult and 20-day nerve cells.

Young neurons are metabolically different entities from mature ones. It was to be expected (and was found) that the type of reaction
observed in this study appeared to depend upon the stage of maturation of the individual neuron at the time of exposure to ethanol. In fact, the exact fetal or postnatal age of the animal became less important than the morphology of the individual cell as a predictor of a cell's metabolic capabilities and, consequently, of the nature of its response to insult.

In many respects, the normal adult facial motor neuron is representative of the most "advanced" nucleolar structure and ribosomal arrangement seen in any mammalian neuron (LaVelle and LaVelle, 1975). Perhaps ultrastructurally this is expressed in its thin cisternae of rough endoplasmic reticulum, its relatively low density of free ribosomes, and the high proportion of RNA to DNA in its nucleolus. The adult Purkinje cell is somewhat less "advanced". Yet, as its well developed INB and response to ethanol indicate, its metabolic balance of RNA/protein synthetic processes is apparently such that its overall changes with ethanol parallel those of the adult facial motor neuron.

The normal 20-day facial nerve cell is known to be in its final stages of maturation cytologically and biochemically (LaVelle and LaVelle, 1975; Kinderman and LaVelle, 1976, 1977). However, in certain ultrastructural details, such as cytoplasmic density of organelles, preponderance of free ribosomes, and greater width of endoplasmic reticulum cisternae, it more closely resembles the adult Purkinje cell
than the adult facial motor neuron. In its response to ethanol, the degree of ribosomal disorganization and mitochondrial change parallel that of the adult Purkinje cell. Its marked nuclear infoldings and peripheralization of rough endoplasmic reticulum, however, are unique among the cell groups studied, indicating the existence of unique metabolic interrelationships. The displacement of rough endoplasmic reticulum to the periphery is the ultrastructural expression of the central chromatolysis observed in these neurons with light microscopy and is reminiscent of the type of response to axotomy differentially seen in 15-day hamster facial motor neurons (LaVelle and LaVelle, 1958b). This suggests that ethanol exposure throughout gestation causes some delay in neuronal maturation. The increased numbers of facial nerve cells containing multiple nucleoli at 20 days postnatal age, following treatment with alcohol, lend support to this suggestion and, furthermore, indicate that ethanol specifically inhibits the conversion from multiple to single nucleoli.

The typical 20-day Purkinje cell still possesses multiple nucleoli in varying stages of maturation and is the least "advanced" of all the cell groups investigated. The relative lack of changes in nucleolar structure and ribosomal disposition of this cell population following exposure to ethanol provides additional support for the notion that the earlier stages of neuronal development can proceed, at
least overtly, in the presence of ethanol.

There is a sort of paradox here. Ethanol seemed to have its major effect on a postnatal phenomenon, that is, the conversion from multiple to single nucleoli. Yet it clearly affected these neurons in some way before birth, since pups exposed to alcohol only prenatally (Group G) exhibited the same kind of profound nucleolar changes at 20 days as did those pups exposed to alcohol pre- and postnatally (Group G-L). Knowledge of the basis for the results obtained with the G and G-L treatment groups in this study would help determine the primary cause of the delay in nucleolar development in young cells with alcohol. This is of particular interest and awaits further investigation.

Interpretation of the effects presumed to result from ethanol exposure is made difficult by the seeming multifactorial origin of the alcohol-induced impairment. Differences in findings from one laboratory to the next are probably due to differences in methodology such as strain/species of animal used, timing and dosage of ethanol administered, route of ethanol administration, and overall diet. The complexity of the problem becomes that much greater when other factors such as the environment, hormonal changes, and maternal metabolism and behavior come into play.

Perhaps the greatest confounding factor in the interpretation of the effects of ethanol on the nervous system (especially the developing nervous system) is the question of proper nutrition.
However, in the present study, the realization that the neurons most severely affected cytologically were those 1) from adult hamsters which gained weight while consuming ethanol and which were in apparent good health, and 2) from 20-day animals which grew rapidly and had a high viability rate following prenatal ethanol exposure, led to the conclusion that ethanol is able to alter neuronal morphology without accompanying malnutrition. Any attempt to further separate direct, ethanol-induced effects from indirect actions of ethanol (undernutrition in particular) will necessitate utilization of experimental paradigms that are beyond the scope of this more exploratory type of investigation.

Perhaps the significance of this work lay in its demonstration of an in situ method for the observation of nucleolar and cytoplasmic changes by a chemical that has specific relevance to man and to society. The nature of this work has resulted in its intersection with a variety of research fields of interest. Due to the expanse of unexplored topics and the investigator's own interests, it was decided to pursue a number of different experimental projects with the hope that many would provide avenues for more exhaustive study in the future. The following represent a few of the most important of these prospective research approaches:

1) correlate neuronal nucleolar/INB changes resulting from ethanol exposure with autoradiographic uptake of amino acid into protein;
2) administer ethanol chronically to the adult rat or mouse in anticipation that neuronal populations from these species would develop an intranucleolar body;

3) at the ultrastructural level, perform a morphometric study in order to ascertain quantitatively the effects of ethanol on various components of the nucleolus and on different cell organelles and membranes involved in the synthesis of RNA/protein, e.g., disposition of ribosomes, width of rough endoplasmic reticulum cisternae, size and length of mitochondria, infoldings of the nuclear envelope, number and size of nuclear pores;

4) sacrifice hamster offspring at a more extensive series of prenatal and postnatal ages in order to determine the progressive effect of ethanol on neuronal development.

Although the results presented here have dealt only with nerve cells, which offer the advantage of cytologically large nucleoli, the implications extend to the broader question of the molecular biology of nucleoli in general. The metabolic activities of the nucleolus are generally so sensitive to the demands of the cell that even normal nucleolar morphology varies from cell type to cell type (Busch and Smetana, 1970). The histological observation that neuronal nucleoli undergo definite structural changes related to the increase (or decrease) of cytoplasmic RNA/RNP is of fairly long standing (LaVelle, 1951, 1956; LaVelle and LaVelle, 1958a, 1958b), as is evidence
correlating nucleolar morphology to specific aspects of the synthetic process.

There is a wealth of literature concerning nucleolar changes induced by a variety of drugs. For instance, actinomycin D is known to cause segregation of nucleolar components (separation of pars granulosa from pars fibrosa) in numerous types of cells, from Rous sarcoma virus-transformed chicken embryo fibroblasts (Connan et al., 1980) to cells of the rat preoptic area (Quadagno et al., 1980). Segregation is considered to be a "marker" for inhibition of nucleolar RNA synthesis caused by an agent's binding with rDNA. On the other hand, α-amanitin produces fragmentation of the nucleolus into its granular and fibrillar elements (Barsotti et al., 1980; Connan et al., 1980). Fragmentation is believed to reflect disturbance in the processing of nucleolar RNA. Cycloheximide is known to alter several steps in the intranucleolar maturation of rRNA (Craig and Perry, 1970). Like α-amanitin, its effects occur at the post-transcriptional level. Ultrastructurally, the nucleolus shows an increased compactness characterized by a uniform distribution of RNP components. Additional drugs which functionally inhibit the nucleolus include 2-mercapto-1(8-4-pyridethyl) benzimidazole, ethionine, benzo (a) pyrene and other carcinogens, and anthramycin (one of a variety of antibiotics) (Simard et al., 1974; Goessens, 1978; Calin and Dragomir, 1980).
In the presence of some stimulatory agents, the nucleolus (particularly its granular portion) hypertrophies. Presumably these substances somehow block the release of nucleolar RNA. Perhaps the most commonly used stimulatory drug has been thioacetamide (Busch et al., 1963).

A number of individuals have utilized agents other than drugs in an attempt to relate changes in nucleolar appearance with modification at specific sites in the chain of events involving the production, transfer, and utilization of RNA/RNP. Dietary deficiencies (Luetzeler et al., 1979), electrical stimulation (Busch and Smetana, 1970), supranormal temperatures (Simard et al., 1974), viral infection (Dupuy-Coin et al., 1978), tumor transplantation (Potmesil and Goldfeder, 1971), and dark-induced blockage of mitosis (Kwiatkowska and Maszewski, 1980) represent just a few of the methods that have been employed.

The findings from this dissertation project show that alcohol may retard the eventual maturation of the nucleolus in young nerve cells and appears to alter the size and frequency of the intranucleolar body in adult nerve cells. Yet the nucleoli in themselves are not unduly modified by the drug, as no fragmentation or major segregation of nucleolar constituents was observed. Ultrastructurally, the obvious distortions are seen in cytoplasmic organelles, such as the system of ribosomes and endoplasmic reticulum in particular, in the more mature
cells (20-day facial motor neurons and both facial and Purkinje adult cells).

Synthesis of protein represents the culmination of a chain of events which "begins" in the nucleus with production of the three species of RNA and "ends" in the cytoplasm with ribosomes serving as the site for assemblage of polypeptide chains. The primary role of the nucleolus appears to be in the formation of ribosomal subunits destined for the cytoplasm. However, many of the proteins which become incorporated into ribosomes are not synthesized in the nucleolus but are transported there after being manufactured in the cytoplasm (Royal and Simard, 1975; Miller and Gonzales, 1976). Similarly, the nucleolus itself appears to play an additional role in the transfer of extranucleolar RNA (mRNA, tRNA) as well as rRNA across the nuclear envelope (Sidebottom and Harris, 1969; Jordan and Loening, 1977). Nuclear-cytoplasmic interactions are believed to play an important role in adult cells, and an even greater role in immature cells, in the regulation of transcriptional and translational processes (Weck and Johnson, 1978). There are multiple potential sites for modification of events at the nuclear, nucleolar, and/or cytoplasmic levels. The intricacies that are woven into this chain of synthetic events make it quite difficult to interpret alterations in nucleolar structure following drug (ethanol) exposure.
The results observed here attest to the close association between the nerve cell's RNA and protein synthetic machinery; morphological alteration of the one is accompanied by modification of the other. An attempt has been made to explain in a general way the mechanisms behind the changes observed. One of the most remarkable findings has been the ability of these young neurons to adapt to pre- and/or post-natal ethanol insult. Purkinje and facial motor cells alike showed marked "plasticity" structurally and (presumably) functionally.

It is of some import that so many different laboratories, including our own, have tested in such diverse ways their theories of nucleolar structure as related to function and have arrived quite independently at a conclusion linking morphological changes to an altered balance in the production and use of rRNA (Studzinski and Gierthy, 1972; Dupuy-Coin et al., 1978; Goessens, 1978). It should be remembered, however, that due to variation in the morphological composition and appearance of the nucleolus in different cell types, there exists a broad spectrum of changes in the cellular (including nucleolar) reactions to drugs and other agents. Attempts are being made to resolve the very complex nature of these responses. Even now, the implications of the significance of these findings to an eventual understanding of at least some disease processes can begin to be surmised.
SUMMARY

This investigation sought to determine whether the nucleolus of the adult and/or developing nerve cell could be morphologically altered by exogenous chemical administration. The chemical agent selected was ethanol, the experimental animal of choice was the golden hamster, and the cell populations studied were adult and 20-day facial motor neurons and cerebellar Purkinje cells.

The results of this combined light and electron microscopic study have led to the following conclusions:

1) Chronic ethanol ingestion by adult hamsters elicits an increase in size and frequency of the intranucleolar body in both facial motor neurons and Purkinje cells.

2) Exposure to ethanol prenatally or both pre- and postnatally causes a retardation in nucleolar development, such that there is a delayed transition from multiple to single nucleoli in facial motor neurons.

3) There is a differential nucleolar response to alcohol exposure depending upon the level of nucleolar (and cellular) maturation attained during the period of ethanol insult. Purkinje cells, being normally less advanced than facial motor neurons, show minimal alteration at 20 days of age.

4) Ethanol-induced alteration of nerve cell morphology appears
to occur independently of the effects of malnutrition. The latter appears to be an inhibitory factor in general body growth and development during the early postnatal period.

5) Following ethanol administration, the nuclear/nucleolar portion of the chain of synthetic events leading to protein production appears to remain virtually intact. Preliminary findings with electron microscopy suggest, however, that a variety of cytoplasmic organelles involved in the energetics of RNA and protein metabolism are adversely affected by chronic ethanol exposure.


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FIGURE 1

Low-power views of normal adult hamster brain tissue. Thionin stain.

A. Facial motor nucleus: the group of large neurons lying at lower center of photograph, near ventral surface of medulla. Midline of medulla lies at left edge of photograph. (63X)

B. Cerebellar cortex with its molecular (Mol), Purkinje (P), and granule (Gr) cell layers. (190X)
FIGURE 2

Representative neurons of the facial motor nucleus of the adult hamster showing typical nucleolar structure. Thionin stain.

A. Open arrowhead indicates perinucleolar chromatin; solid arrow points to a prominent intranucleolar body. Control animal. (1900X)

B. Cell (a) contains a large, single nucleolus capped below with perinucleolar chromatin and containing a prominent intranucleolar body. Cell (b) contains one small nucleolus with no intranucleolar body. Alcohol-exposed animal. (1900X)

C. Arrow indicates a large, prominent intranucleolar body. Control animal. (1900X)

D. A prominent intranucleolar body is visible in the cell on the left, even at this lower magnification. Control animal. (1250X)
FIGURE 3

Representative Purkinje cells of the cerebellar cortex of the normal adult hamster showing typical nucleolar structure. Thionin stain. (1900X)

A. Large, single nucleolus with a dark cap of perinucleolar chromatin (to the left) and a prominent intranucleolar body (arrow).

B. The large nucleolus (arrow) contains a punctate-sized intranucleolar body (see dot in middle).

C. The nucleolus (arrow) contains two punctate-sized intranucleolar granules.

D. Arrow points to nucleolus containing no intranucleolar body. The large, dark cap is nucleolus-associated chromatin.
Diagrammatic representation of the nucleolar classifications used for both facial motor neurons (top row) and cerebellar Purkinje cells (bottom row). For each nerve cell containing a distinct, single nucleolus, the intranucleolar body was judged to be absent (Abs.), punctate (Punc., with one or more small particles), or prominent (Prom., measuring $\mu$m or more in diameter). Indicated in the diagrams are: nucleolus (NCL), intranucleolar body (INB), nucleolus-associated chromatin (NAC), nuclear membrane (NM), Nissl substance (NS). The two cell types are drawn to appropriate comparable dimensions.
INB CHARACTERIZATION, FACIAL MOTOR NEURONS

Abs.  Punc.  Prom.

INB CHARACTERIZATION, PURKINJE CELLS

Abs.  Punc.  Prom.
FIGURE 5

Percentages of Purkinje cells and facial motor neurons with absent, punctate, or prominent intranucleolar bodies in normal (control) adult hamsters. Prominent bodies predominate, particularly among facial neurons.
CONTROL
ADULT

P = Purkinje Cells
F = Facial Motor Neurons

% Cells Counted

INB Absent       Punctate INB       Prominent INB

INB Absent

P
F

P
F

P
Percentages of Purkinje cells with absent (Abs.), punctate (Punc.), or prominent (Prom.) intranucleolar bodies in normal (white bars) and alcohol-consuming (hatched bars) adult hamsters. A shift to the right, toward greater frequency of prominent bodies, is evident. Differences in nucleolar characterization after administration of alcohol are highly significant (p<0.001). S.D.<3.4. N=13.
Percentages of facial motor neurons with absent (Abs.), punctate (Punc.), or prominent (Prom.) intranucleolar bodies in normal (white bars) and alcohol-consuming (hatched bars) adult hamsters. The shift in percentages of absent intranucleolar bodies after alcohol exposure is (marginally) non-significant (p=0.0595). All other differences in nucleolar characterization are highly significant (p<0.001). S.D.≤2.6. N=13.
FIGURE 8

Diameters of prominent intranucleolar bodies of Purkinje cells and facial motor neurons in normal (white bars) and alcohol-consuming (black bars) adult hamsters. The increase in diameters with alcohol is significant ($p \leq 0.001$). S.D. $\leq 0.11$. N=13.
Purkinje Cells  Facial Motor Neurons

Diameter in μm

2.00  1.75  1.50  1.25  1.00  0.75  0.50  0.25  0.00

Control  Alcohol
Mean weight in grams of hamsters at postnatal days 4, 9, 14, and 19. Treatment groups include: hamster pups exposed to water (Control) or to alcohol throughout the gestation and suckling (lactation) periods (Alcohol, G-L); hamster pups exposed to alcohol during the gestation period only (Alcohol, G); and hamster pups exposed to alcohol during the suckling period only (Alcohol, L). Weights among all treatment groups are not significantly different at 4 days postnatal age. At later ages, the following weight differences are significant: 9 days -- Control and G vs. G-L and L groups; 14 days -- Control and G vs. L vs. G-L groups; 19 days -- G vs. Control vs. G-L and L groups. N=189. For details, see Table 3, Appendix E.
Diagrammatic representation of nucleolar development in a nerve cell. The facial motor neuron of the hamster is used as an example. Maturation proceeds from multiple chromatin masses (upper left), to multiple nucleoli showing increasing amounts of RNA (shown as empty rings here), to a single nucleolus showing progressively less nucleolus-associated chromatin and a progressively larger intranucleolar body (lower right). Indicated in the diagrams are: nuclear membrane (NM), nucleolus (NCL), nucleolus-associated chromatin (NAC), intranucleolar body (INB).
Multiple Nucleoli

Single Nucleolus

NUCLEOLAR DEV. - HAMSTER FACIAL MOTOR NEURONS
FIGURE 11

Representative neurons of the facial motor nucleus of the 20-day-old hamster showing typical nucleolar structure. Thionin stain.

A. Within the nucleus (arrow) are depicted one prominent central nucleolus and three peripheral nucleoli. Control animal. (1190X)

B. The central nucleolus is capped by perinucleolar chromatin (arrows) and lacks an intranucleolar body. Control animal. (1900X)

C. Several punctate-sized intranucleolar granules are evident within the nucleolus. Control animal. (1900X)

D. Arrow points to a single, prominent intranucleolar body. Dark masses of nucleolus-associated chromatin cap opposite surfaces of the nucleolus. Alcohol-exposed animal. (1900X)
FIGURE 12

Representative Purkinje cells of the cerebellar cortex of the 20-day-old hamster. Thionin stain.

A. Purkinje cell layer lies between molecular (Mol) and granule (Gr) layers of cerebellar cortex. Note frequency of Purkinje cells containing multiple nucleoli; two such cells are indicated by arrows. Alcohol-exposed animal. (760X)

B. Higher power views of multi-nucleolated Purkinje cells. Control animal. (1190X)

C. Purkinje cell (P) possessing four nucleoli. Alcohol-exposed animal. (1900X)
FIGURE 13

Purkinje cells of 20-day-old hamsters. Cells with single nucleoli are indicated by arrows. Thionin stain.

A. Alcohol-exposed animal. (1900X)
B. Alcohol-exposed animal. (1900X)
C. Control animal. (1250X)
Figure 14

Comparison of control and alcohol-exposed facial motor neurons in 20-day-old hamsters. Thionin stain. (475X)

A. Control. Thionin stains cell bodies and only proximal portions of dendritic processes.

B. Alcohol-exposed. Cell bodies are shrunken and stain darkly with thionin. Dendritic processes now take up the stain and are spindly in appearance.
FIGURE 15

Facial motor neurons of alcohol-exposed, 20-day-old hamsters. Thionin stain.

A. Dark, shrunken, spindly cells are evident. Two cells indicated by arrows are seen at higher magnification in B. (475X)

B. Peripheral cytoplasm stains intensely. Central chromatolysis is evident in the less intense staining of cytoplasm which immediately surrounds the nucleus. (1190X)
FIGURE 16

Facial motor neurons of alcohol-exposed, 20-day-old hamsters. Thionin stain. (1190X)

A. Nuclear invaginations (arrows) are evident. Cell on left also displays a mild, central chromatolysis.

B. Two neurons displaying central chromatolysis and nuclear invaginations (arrows).

C. Neurons exhibiting varying degrees of central chromatolysis.
Percentages of Purkinje cells and facial motor neurons with either multiple nucleoli or single nucleoli containing no (absent), punctate, or prominent intranucleolar bodies (INB). Counts were performed on cells from normal, 20-day-old hamsters. Multiple nucleoli are highly characteristic of Purkinje cells; facial motor neurons more typically contain single nucleoli, often displaying a prominent INB.

FIGURE 17
Multiple Nucleoli

INB Absent

Punctate INB

Prominent INB

P = Purkinje Cells

F = Facial Motor Neurons

CONTROL

20 Days of Age

% Cells Counted

Less Developed

More Developed
Percentages of 20-day-old hamster facial motor neurons with multiple nucleoli, single nucleoli lacking intranucleolar bodies (INB Abs.), single nucleoli with punctate INBs (Punc.), or single nucleoli with prominent INBs (Prom.). Cells counted were from control pups (white bars), pups exposed to ethanol during the gestation and nursing (lactation) periods (G-L, hatched bars), and pups exposed to ethanol during the gestation period only (G, cross-hatched bars). Differences in nucleolar characterization after alcohol are significantly different ($p<0.001$) in all cases with the exception of INB-Absent nucleoli. The shift is toward multiple nucleoli after exposure to alcohol. N=80. (See Table 5, Appendix E, for details of statistical analysis.)
FACIAL MOTOR NEURONS

- Control
- Alcohol, G-L
- Alcohol, G

% Cells Counted

Multiple Nucleoli
INB Abs.
Punc. INB
Prom. INB

Less Developed  More Developed
FIGURE 19

Percentages of 20-day-old hamster Purkinje cells with multiple nucleoli, single nucleoli lacking intranucleolar bodies (INB Abs.), single nucleoli with punctate INBs (Punc.), or single nucleoli with prominent INBs (Prom.). Cells counted were from control pups (white bars), pups exposed to ethanol during the gestation and nursing (lactation) periods (G-L, hatched bars), and pups exposed to ethanol during the gestation period only (G, cross-hatched bars). In almost all cases, differences in nucleolar characterization after alcohol are not significantly different. N=80. (See Table 5, Appendix E, for details of statistical analysis.)
PURKINJE CELLS

- Control
- Alcohol, G - L
- Alcohol, G

% Cells Counted

Less Developed → More Developed
FIGURE 20

Plastic thick sections of normal brain tissue from adult and 20-day-old hamsters. Toluidine blue stain. (475X)

A. Adult facial motor neurons; three are labeled (F).

B. Twenty-day-old facial motor neurons (F). Arrow indicates cell containing multiple nucleoli.

C. Adult cerebellar cortex. Two of the Purkinje cells are labeled (P).

D. Twenty-day-old cerebellar cortex. Arrows indicate two Purkinje cells containing multiple nucleoli.
FIGURE 21

Facial motor neuron from normal adult hamster. Arrow = nuclear invagination; N = nucleus; RER = rough endoplasmic reticulum; G = Golgi; L = lipofuscin. (8600X)
FIGURE 22

Facial motor neuron from normal adult hamster. Perinuclear Golgi bodies (G) are conspicuous. M = mitochondria. (10,800X)
FIGURE 23

Purkinje cell from normal adult hamster. Compared with the normal adult facial motor neuron (Fig. 22), the Purkinje cell cytoplasm is "packed" with organelles. Nucleus (N) is irregular in outline. NCL = nucleolus; L = lipofuscin; G = Golgi bodies; R = ribosomes. (10,800X)
FIGURE 24

Purkinje cell from normal, 20-day-old hamster. Multiple nucleoli (Mu) are evident. (8600X)
Facial motor neuron from normal, 20-day-old hamster. Deep nuclear invagination (NI) is evident. Uneven nuclear outline and organelle-packed cytoplasm are characteristic. (Compare with normal adult Purkinje cells, Fig. 23.) NCL = nucleolus; G = Golgi bodies; RER = rough endoplasmic reticulum. (10,800X)
FIGURE 26

Purkinje cell nucleolus from an alcohol-ingesting adult hamster. Nucleolar structure is characteristic of that found in most cell types. (56,700X)

Key to Abbreviations Used Throughout Figs. 26-34.

NAC = Nucleolus-associated chromatin
PF = Pars fibrosa
PG = Pars granulosa
FC = Fibrillar center
V = Nucleolar vacuole
FIGURE 27

Nucleolus from an alcohol-exposed adult facial motor neuron. Note aggregation of the pars granulosa into a prominent intranucleolar body (INB). (41,600X)
Nucleolus from a normal adult Purkinje cell. Contrast the sharpness of the granularity of the intranucleolar body (INB) with the softer-grained nature of the nucleolus-associated chromatin (NAC). (56,700X)
FIGURE 29

Nucleolus from a normal adult Purkinje cell. This cut shows a large cap of nucleolus-associated chromatin and a paucity of pars granulosa material. Nucleolar vacuoles and fibrillar material are also observed. (56,700X)
FIGURE 30

Nucleolus from a normal, 20-day-old hamster. All characteristic components of the nucleolus are present. Arrows indicate probable fibrillar centers. (41,600X)
FIGURE 31

Portion of a nucleolus in close proximity to cytoplasmic ribosomes (R) lying in an invagination of the nuclear envelope (arrow). Facial motor neuron, 20-day alcohol-exposed hamster. (41,600X)
Developing nucleolus from a normal, 20-day-old Purkinje cell. DNA-containing material (NAC) is located adjacent to the nuclear envelope (NM), while RNA-containing material (mainly PF at this early stage) projects further into the nucleus. Arrows indicate two nuclear pores. M = mitochondrion; RER = rough endoplasmic reticulum; R = free polyribosomes. (56,700X)
FIGURE 33

One nucleolus of a multi-nucleolated Purkinje cell from a 20-day-old, alcohol-exposed hamster. A relatively small amount of chromatin is located next to the nuclear envelope (NM), while the remainder of the nucleolar material is developing into distinctive pars granulosa and pars fibrosa regions. G = Golgi apparatus; L = lipofuscin. (56,700X)
Nucleolus from a normal, 20-day-old Purkinje cell. Although still located along the nuclear envelope (NM), this nucleolus has almost reached its mature appearance cytologically. Compare with Figs. 32 and 33 for prior developmental stages. (56,700X)
FIGURE 35

Portions of facial motor neurons from adult hamsters chronically ingesting ethanol. (8600X)

A. In portion of nucleus shown, the nuclear envelope pores (arrows) are conspicuously numerous. Within the cytoplasm, the quantity of free ribosomes and the number and size of lipofuscin granules are increased over the normal (compare with Figs. 21 and 22).

B. Pores can be seen along the entirety of the nuclear envelope (NM).

Key to Abbreviations Used Throughout Figs. 35-54.

G = Golgi apparatus
L = Lipofuscin
M = Mitochondrion
N = Nucleus
R = Free ribosomes
RER = Rough endoplasmic reticulum
FIGURE 36

Portion of a facial motor neuron from a 20-day-old, alcohol-exposed animal. The extensive invaginations (NI) of the nuclear envelope hold great quantities of cytoplasmic polysomes (R). Membrane-bound ribosomes (RER), on the other hand, are usually found in a more peripheral position. A part of the nucleolus (NCL) can also be observed. (8600X)
Facial motor neuron from an alcohol-consuming adult hamster. A characteristic feature of these cells is the large number of free ribosomes. (Compare with control, Fig. 22.) Nuclear envelope pores (arrows) are relatively conspicuous. (8600X)
FIGURE 38

Cytoplasm of a normal, 20-day-old facial motor neuron. Distribution of organelles is characteristic, including position of rough endoplasmic reticulum adjacent to the nucleus. (26,500X)
FIGURE 39

Cytoplasm of a facial motor neuron from a 20-day-old, alcohol-exposed hamster. Distribution of organelles is similar to that seen in the control (Fig. 38) with the exception that the rough endoplasmic reticulum is located toward the periphery of the cell cytoplasm. (26,500X)
FIGURE 40

Cytoplasmic detail of facial motor neurons from 20-day-old, alcohol-exposed hamsters.

A. Arrows indicate sites where polysomal arrangement of free ribosomes appears to be breaking down into monosomes. Such a phenomenon was only observed in cells from alcohol-treated animals. (56,700X)

B. Regions containing monoribosomes (arrows) are dispersed among population of polysomes. Note extent and irregularity of Golgi apparatus. (41,600X)
Facial motor neuron from a 20-day-old, alcohol-exposed hamster. Rough endoplasmic reticulum is located in periphery of cell cytoplasm and is absent from perinuclear area. (Compare with control, Fig. 25.) (10,800X)
Facial motor neuron from a 20-day-old, alcohol-exposed hamster. As seen also in Fig. 41, rough endoplasmic reticulum is displaced to the cell periphery. Such a central chromatolytic response is characteristic of this particular cell type and treatment group. (8600X)
FIGURE 43

Purkinje cell from a 20-day-old, alcohol-exposed hamster. Distribution of ribosomes is similar to that seen in control (Fig. 24). Note prominent Golgi bodies. Mu = multiple nucleoli. (8600X)
FIGURE 44

Characteristic appearance of the cytoplasm of a normal adult facial motor neuron. Narrow width of cisternae of rough endoplasmic reticulum is typical of this cell type. (41,600X)
Cytoplasmic detail of an adult facial motor neuron from an alcohol-consuming hamster. Compare cisternal width of rough endoplasmic reticulum with that found in control (Fig. 44). (41,600X)
Comparison of cisternal width of rough endoplasmic reticulum in control and alcohol-treated adult Purkinje cells. (41,600X)

A. Control cell. Normal width of RER cisternae is greater than that seen in adult, control facial motor neurons (Fig. 44).

B. Alcohol-treated cell. Note swollen cisternal space of RER. Large numbers of free ribosomes are also evident.
Comparison of extremes of cisternal width of rough endoplasmic reticulum in this study. (56,700X)

A. Normal adult facial motor neuron. Cisternal width is narrow.

B. Alcohol-exposed adult Purkinje cell. Cisternal width is markedly swollen.
Comparison of mitochondria from control and alcohol-exposed neurons. (56,700X)

A. Control neuron (adult Purkinje cell). Mitochondria are characteristic of those observed in Purkinje and facial motor neurons of both adult and 20-day-old control hamsters. On the left is a Golgi apparatus in its classical configuration.

B. Alcohol-exposed cell (20-day facial motor neuron). Mitochondria are markedly swollen, and their matrices are less electron-dense than in the control.
Mitochondrial configurations frequently observed in alcohol-exposed nerve cells only.

A. Grossly enlarged mitochondrion with its internal (cristal) structure markedly disrupted. Compare with adjacent mitochondria (arrows) and with those in Fig. 46A. (Twenty-day facial motor neuron, 41,600X)

B. Mitochondrion ballooned out to exaggerated dimensions. There is almost complete obliteration of its internal structure. Arrows point to mitochondria of normal size (see also Fig. 47A). Note, in addition, the swollen cisternae of rough endoplasmic reticulum. (Adult Purkinje cell, 56,700X)
FIGURE 50

Examples of mitochondrial configurations observed in alcohol-exposed nerve cells.

A. Two mitochondria from a 20-day facial motor neuron. The one on the left is swollen and exhibits disruptions of both its internal and external membranes. (56,700X)

B. Mitochondrion from an adult Purkinje cell. Note its enlarged dimensions and peculiarity of shape. (56,700X)

C. Markedly elongate mitochondrion. (Adult facial motor neuron, 41,500X)
FIGURE 51

Portion of a facial motor neuron from a 20-day-old, alcohol-exposed hamster. The labeled mitochondrion exhibits irregularities of its external membrane and contains, within, a single vacuole. An extensive Golgi apparatus, somewhat irregular in form, is also evident (compare with typical Golgi bodies in other neurons at the same magnification, Figs. 48A and 52). (56,700X)
FIGURE 52

Golgi bodies in characteristic configurations of well-organized stacks of cisternae and tubules. (Normal 20-day Purkinje cells, 56,700X)

A. Golgi apparatus adjacent to nuclear envelope (NM).

B. Golgi apparatus located in periphery of cytoplasm.
Types of Golgi bodies particularly evident in neurons from 20-day, alcohol-exposed hamsters. Such Golgi contrast sharply with those from normal 20-day and adult neurons (Figs. 48A and 52). (56,700X)

A. Golgi apparatus appears disorganized and irregular in form (20-day facial motor neuron). See similar configuration in Fig. 48B.

B. Compare size and organization of this Golgi apparatus (from a 20-day Purkinje cell) with those from control cells (Fig. 52).
Purkinje cell from an adult hamster chronically ingesting alcohol. Compare size and quantity of lipofuscin granules (arrows) with that typically found in control cells of the same type (Fig. 23). NCL = nucleolus; NI = nuclear invagination. (10,800X)
Characteristic readings obtained after injection of a (gaseous) mixture into the gas chromatograph.

On the left is graphically displayed the elution times and peak heights of gases within the injected sample as obtained from a recorder connected to the chromatograph. "Ethanol peak" represents amount of ethanol (here converted to a gas) present in original blood sample obtained from hamster. "Propanol peak" represents known amount of propanol added to test sample and serves as gas chromatograph internal standard.

On the right is numerically displayed the elution times and peak areas of the same gases as they are determined by a calculating integrator.
**Ethanol Peak**

---

**Sample Injection**

(0 sec.)

**Propanol Peak**

(238 sec.)

---

**CALCULATING INTEGRATOR**

<table>
<thead>
<tr>
<th>TIME</th>
<th>AREA</th>
</tr>
</thead>
<tbody>
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<td>33254</td>
</tr>
<tr>
<td>238</td>
<td>176784</td>
</tr>
</tbody>
</table>

---

**RECORDER**
FIGURE 56

Mean blood ethanol levels, over a 24-hour period, in adult hamsters chronically ingesting a 15% alcohol solution. Blood was obtained by cardiac puncture under light anesthesia and subsequently prepared for gas chromatographic analysis. Values on ordinate represent mean blood ethanol levels in mg%. Values on abscissa represent time periods at which blood samples were taken.
The diagram shows the mean blood ethanol concentration in mg% at different times of the day, categorized by the phase of the day:

- **Light Phase** (represented by white bars)
- **Dark Phase** (represented by black bars)

The x-axis represents the times of the day:
- 1400 hrs.
- 2000 hrs.
- 0200 hrs.
- 0800 hrs.

The y-axis represents the mean blood ethanol concentration in mg%.

At 0200 hrs., there is a significantly higher mean blood ethanol concentration, indicating a peak in the dark phase compared to the light phase at other times.
APPENDIX A: NUMBERS OF HAMSTERS UTILIZED

The approximate total number of animals used for this dissertation project was 425. Data from 404 of these hamsters were incorporated into the body of this dissertation; the remainder were either discarded for reasons of poor health or because of experimental procedural errors.

The number of adult hamsters utilized was 109. Forty (males and females) were monitored for daily levels of food and fluid consumption; twenty-six of these were subsequently chosen for cell counts (facial motor neurons, cerebellar Purkinje cells) at the light microscopic level. In addition ten adults were selected for the liquid diet study, twenty for the gas chromatographic study, and eight for electron microscopic investigation. Finally, thirty-one females were mated and monitored for fluid consumption throughout their gestation and/or lactation periods. Twenty-five of these hamsters gave birth to offspring which were used in the developmental portion of this study.

A total of 270 newborns were assessed for attainment of behavioral and maturational indices throughout their postnatal period of development. Two-hundred-and-three of these 270 were weighed at 4 days postnatal age and, depending on their survival, were subsequently weighed at 9, 14, and 19 days of age. Cell counts were made on 81 of the hamsters that survived until 20 days postnatal age. Lastly, fifteen 20-day-old hamsters (exposed to water or ethanol throughout their periods of gestation and suckling) were used for electron microscopic investigation.
APPENDIX B: DETAILS OF TISSUE PREPARATION

Light Microscopy

At the end of each series of experiments and prior to perfusion-fixation, adult or juvenile hamsters were weighed and anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, 64.8mg/cc). The dosage administered was dependent on the age and weight of each animal, adults receiving a dose of 0.12cc/100g, 20-day-old hamsters receiving 0.025cc/25g. A gravity-directed perfusion set-up, consisting of two suspended intravenous drip bottles connected to a common Y-type tubing, allowed for the sequential flow of saline and formalin solutions. A 20-gauge, 5/8in hypodermic needle, blunted by filing off its bevel, was attached to the lower end of the Y-tube apparatus and used as an intracardiac conduit for the proper perfusate.

The chest cavity of each anesthetized animal was rapidly opened, and a cut was made in the left ventricle of the heart to permit advancement of the blunted needle into the aorta. As the flow of perfusate through the animal's circulatory system was initiated, the right atrium was punctured in order to allow escape of the perfusion solutions.

The animals were initially perfused with a saline wash solution until the liver and skin were cleared and no blood was seen flowing out of the right atrium. A fixative solution (10% formalin in saline)
immediately followed, perfusion continuing until internal organs were firm and the animal became rigid. Both wash and fixative solutions contained gum acacia for maintenance of the proper osmolarity (LaVelle, 1951) and were perfused at room temperature. The brains were quickly exposed and immersed \textit{in situ} in the same fixative solution for at least 72 hours to ensure complete fixation.

Subsequently, each brain was completely dissected out, and the brainstem-cerebellum was prepared for light microscopic study. Tissue was dehydrated in a graded series of alcohols, infiltrated with several changes of Parlodion mixtures (a 6\% mixture as the highest concentration), and embedded in 6\% Parlodion. Hardening of the tissue blocks occurred with overnight exposure to chloroform vapors in a desiccator jar. Blocks were then trimmed, cleared in several changes of benzene, and infiltrated with paraffin (Fisher TissuePrep) \textit{in vacuo} for one hour at 15 pounds negative pressure. The double-embedding techniques outlined above were devised by LaVelle (1951) and LaVelle and LaVelle (1958a, 1958b) for the hamster brain and were found to yield good cellular and subcellular detail with no tissue shrinkage.

Brainstem-cerebellar tissue was serially sectioned at 4\textmu m on a Model 860 American Optical sliding microtome using the 'wet blade' technique. Every tenth section was mounted and stained with buffered thionin at pH 4.46 for distinction of intracellular sites containing DNA and RNA (LaVelle, 1951; LaVelle and LaVelle, 1958a).
Electron Microscopy

Intracardiac perfusion-fixation was performed on anesthetized animals (see p. 221). The initial perfusate of physiological saline (room temperature) was rapidly followed by a 2% formaldehyde-2.5% glutaraldehyde solution in 0.1M phosphate buffer (Sorenson's) containing 4% sucrose. The pH of the latter solution was adjusted to 7.4. In preparation of the final fixative solution, a 70% concentration of glutaraldehyde (Polysciences, EM grade) was utilized, while the formaldehyde was prepared from paraformaldehyde and sodium hydroxide (Peters, 1970).

Following perfusion the brain was exposed, fresh fixative was poured over its surface, and the entire animal was sealed in a plastic bag and placed overnight in the refrigerator. This procedure ensured maximal hardening and protected the brain from distortion during handling and cutting. The next morning, a section of pons-medulla containing the facial motor nucleus was located under a binocular dissecting microscope and removed from each side of the brainstem. Each facial motor nucleus was cut into two or three portions. Pieces of tissue from the lateral portions of the cerebellar hemispheres were also obtained.

Tissue specimens were left in cold fixative (0°C) for 30-60 minutes, rinsed with cold sucrose-phosphate buffer, and postfixed for 1.5 hours in osmium tetroxide (1.33% in phosphate buffer, 0°C). Subse-
sequently the tissues were again rinsed with cold phosphate buffer, dehydrated in a graded ethanol series (reaching room temperature with 100% ethanol), and infiltrated with varying concentrations of propylene oxide and plastic (1:1, 1:3, and 100% plastic). The specimens were flat-embedded in the same plastic mixture (Epon 812 and Araldite 502) and left overnight in a 37°C oven and one week in a 60°C oven for complete polymerization.

Tissue blocks were sectioned at 0.5-1.0 μm on a Porter-Blum MT-2 microtome and stained with toluidine blue for orientation purposes. Upon location of facial motor neurons or cerebellar Purkinje cells, blocks were retrimmed and cut at 600-900 Å (showing silver to gray interference colors). Sections were mounted on uncoated, 200-mesh copper grids and were stained with saturated aqueous (5%) uranyl acetate and Reynold's lead citrate. Sections were examined and photographed at 50kV on an RCA EMU3F electron microscope.
APPENDIX C
APPENDIX C: STATISTICAL ANALYSES

Results were submitted to statistical analysis using Student's t-test whenever a comparison was being made between two treatment groups only. When the data from three treatment groups were compared, a one-way analysis of variance was initially performed to determine if there were any significant differences among the three group means. In order to identify where those differences lay, an additional statistical procedure (Duncan's multiple range test) was employed. In the cell counts performed on 20-day-old hamsters, the analysis of covariance procedure was used in order to adjust the treatment means of the dependent variables for differences in the values of corresponding independent variables (pup weight and size of litter).

Various texts were consulted in order to determine the most applicable statistical procedures (Steel and Torrie, 1960; Morrison, 1967; Remington and Schork, 1970; Armitage, 1971). The advice of Dr. William Henderson, statistician at Hines VA Hospital, and the use of his computer facilities in the statistical analysis of these data are gratefully acknowledged.
APPENDIX D: PHOTOGRAPHIC PROCEDURES

Photography of light microscopic sections was performed on a Zeiss Ultraphot II microscope with Kodak Pan-X (ASA 32) film. An additional contrast filter (golden amber) was utilized for enhanced subcellular detail.

Electron microscopic sections were photographed on Kodalith Estar base 70mm film (Kodak LR film 2572).
Average daily consumption of food (Purina rat chow) and fluid (water or 15% ethanol in water) by adult hamsters. Statistical test used: Student's t-test.

<table>
<thead>
<tr>
<th>Food, Fluid Consumed</th>
<th>Control Group</th>
<th>Experimental Group</th>
<th>Statistical Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food</td>
<td>Mean grams per day (and S.D.)</td>
<td>t-value</td>
<td>df</td>
</tr>
<tr>
<td>Food</td>
<td>7.5 (0.5)</td>
<td>6.0 (0.3)</td>
<td>2.59</td>
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<tr>
<td>Fluid</td>
<td>Mean milliliters per day (and S.D.)</td>
<td>t-value</td>
<td>df</td>
</tr>
<tr>
<td>Fluid</td>
<td>20.9 (2.5)</td>
<td>20.2 (1.8)</td>
<td>0.23</td>
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TABLE 2

Characteristics of nucleolar morphology in adult hamster neurons, with and without chronic exposure to ethanol. For each cell population: Subgroup A shows mean percentages of cells exhibiting different formations of the intranucleolar body (INB); subgroup B shows mean diameters of the prominent intranucleolar bodies. Statistical test used: Student's t-test.

<table>
<thead>
<tr>
<th>Nucleolar Morphology</th>
<th>Non-exposed Controls</th>
<th>Exposed to Ethanol</th>
<th>Statistical Evaluation</th>
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<tr>
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<td></td>
<td>t-value</td>
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**FACIAL MOTOR NEURONS**

<table>
<thead>
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<th>Mean Percentages (and S.D.)</th>
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<tbody>
<tr>
<td>A. INB prominent</td>
<td>80.2 (2.6)</td>
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<tr>
<td>INB punctate</td>
<td>11.0 (1.0)</td>
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<tr>
<td>INB absent</td>
<td>8.8 (2.4)</td>
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<table>
<thead>
<tr>
<th>Mean Diameters in Microns</th>
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<tr>
<td>B. Prominent INB</td>
<td>1.76 (0.08)</td>
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**PURKINJE CELLS**

<table>
<thead>
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<th>Mean Percentages (and S.D.)</th>
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<td>A. INB prominent</td>
<td>52.8 (2.7)</td>
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<tr>
<td>INB punctate</td>
<td>33.5 (3.4)</td>
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<tr>
<td>INB absent</td>
<td>13.7 (2.6)</td>
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</table>

<table>
<thead>
<tr>
<th>Mean Diameter in Microns</th>
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<tbody>
<tr>
<td>B. Prominent INB</td>
<td>1.38 (0.11)</td>
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TABLE 3

Postnatal weights of control hamster pups and of those chronically exposed to ethanol pre- and/or postnatally. Statistical tests used: one-way analysis of variance, Duncan's multiple range test.

<table>
<thead>
<tr>
<th>Postnatal Age in Days</th>
<th>Non-exposed Controls (Group C: 7 litters)</th>
<th>Prenatal Exposure (Group G: 5 litters)</th>
<th>Postnatal Exposure (Group L: 4 litters)</th>
<th>Postnatal Exposure (Group G-L: 9 litters)</th>
<th>Statistical Evaluation</th>
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<tr>
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<td>4.5 range 2.8-7.2</td>
<td>4.3 range 2.5-4.9</td>
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<td>F-value 0.59 df 199 p &gt;&gt; 0.05</td>
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<td>9</td>
<td>8.4 range 4.4-11.5</td>
<td>8.8 range 6.1-13.4</td>
<td>7.4 range 4.5-8.8</td>
<td>7.4 range 5.1-11.5</td>
<td>F-value 14.09 df 199 p &lt;&lt; 0.001</td>
</tr>
<tr>
<td>14</td>
<td>13.7 range 10.2-17.6</td>
<td>14.3 range 8.4-23.4</td>
<td>10.6 range 5.3-13.1</td>
<td>9.4 range 5.1-17.3</td>
<td>F-value 36.20 df 185 *p &lt;&lt; 0.001</td>
</tr>
<tr>
<td>19</td>
<td>24.4 range 10.0-36.0</td>
<td>29.1 range 18.3-40.3</td>
<td>15.4 range 10.0-19.0</td>
<td>16.2 range 7.7-30.0</td>
<td>F-value 69.04 df 186 **p &lt;&lt; 0.001</td>
</tr>
</tbody>
</table>

There are no significant differences among any of the groups at 4 days of age. Thereafter, Groups L and G-L are consistently and significantly different from groups C and G. At these later ages, additional significant differences are as follows:

* Group G-L is significantly different from Group L.

** Group G is significantly different from Group C.
TABLE 4

Mean percentages of neurons exhibiting different patterns of nucleolar morphology in 20-day-old hamsters chronically exposed to ethanol during development.
Statistical tests used: One-way analysis of variance, Duncan's multiple range test.

<table>
<thead>
<tr>
<th>Nucleolar Patterns</th>
<th>Facial Motor Neurons</th>
<th>Purkinje Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Percentages</td>
<td>Statistical Evaluation</td>
</tr>
<tr>
<td></td>
<td>Non-exposed Controls</td>
<td>Pre-natal Exposure</td>
</tr>
<tr>
<td></td>
<td>(Group C: 7 litters)</td>
<td>(Group G: 5 litters)</td>
</tr>
<tr>
<td>Non-exposed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-natal Exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre- and Postnatal Exposure</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Facial Motor Neurons**

<table>
<thead>
<tr>
<th>Nucleolar Patterns</th>
<th>Multiple nucleoli</th>
<th>Single nucleoli</th>
</tr>
</thead>
<tbody>
<tr>
<td>INB* prominent</td>
<td>45.6</td>
<td>31.7</td>
</tr>
<tr>
<td>INB punctate</td>
<td>27.2</td>
<td>15.5</td>
</tr>
<tr>
<td>INB absent</td>
<td>13.0</td>
<td>10.1</td>
</tr>
</tbody>
</table>

**Purkinje Cells**

<table>
<thead>
<tr>
<th>Nucleolar Patterns</th>
<th>Multiple nucleoli</th>
<th>Single nucleoli</th>
</tr>
</thead>
<tbody>
<tr>
<td>INB prominent</td>
<td>3.3</td>
<td>3.7</td>
</tr>
<tr>
<td>INB punctate</td>
<td>11.2</td>
<td>14.5</td>
</tr>
<tr>
<td>INB absent</td>
<td>15.6</td>
<td>16.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Statistical Evaluation</th>
<th>F-value</th>
<th>df</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Facial Motor Neurons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple nucleoli</td>
<td>249.30</td>
<td>78</td>
<td>** p&lt;&lt;0.001</td>
</tr>
<tr>
<td>Single nucleoli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INB* prominent</td>
<td>60.56</td>
<td>78</td>
<td>** p&lt;&lt;0.001</td>
</tr>
<tr>
<td>INB punctate</td>
<td>47.07</td>
<td>78</td>
<td>** p&lt;&lt;0.001</td>
</tr>
<tr>
<td>INB absent</td>
<td>17.26</td>
<td>78</td>
<td>** p&lt;&lt;0.001</td>
</tr>
<tr>
<td>Purkinje Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple nucleoli</td>
<td>13.19</td>
<td>78</td>
<td>† p&lt;&lt;0.001</td>
</tr>
<tr>
<td>Single nucleoli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INB prominent</td>
<td>1.59</td>
<td>78</td>
<td>†† p&gt;0.05</td>
</tr>
<tr>
<td>INB punctate</td>
<td>18.99</td>
<td>78</td>
<td>† p&lt;&lt;0.001</td>
</tr>
<tr>
<td>INB absent</td>
<td>1.61</td>
<td>78</td>
<td>†† p&gt;0.05</td>
</tr>
</tbody>
</table>

*INB = intranucleolar body.
**Groups G and G-L are significantly different from group C and from each other.
***Group G is significantly different from groups C and G-L.
†Groups G and G-L are significantly different from group C, but not from each other.
††There are no significant differences between groups.
Mean percentages of neurons exhibiting different patterns of nucleolar morphology in 20-day-old hamsters chronically exposed to ethanol during development. Treatment means of the dependent variables (multiple nucleoli, INB absent, INB punctate, INB prominent) were adjusted for differences in values of the corresponding independent variables (pup weight and litter size). Statistical test used: Analysis of covariance.

<table>
<thead>
<tr>
<th>Nucleolar Patterns</th>
<th>Mean Percentages</th>
<th>Statistical Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-exposed Controls</td>
<td>Pre-natal Exposure</td>
</tr>
<tr>
<td>Facial Motor Neurons</td>
<td>(Group C: 7 litters)</td>
<td>(Group G: 5 litters)</td>
</tr>
<tr>
<td>Multiple nucleoli</td>
<td>14.2</td>
<td>42.7</td>
</tr>
<tr>
<td>Single nucleoli</td>
<td>45.6</td>
<td>31.7</td>
</tr>
<tr>
<td>INB* prominent</td>
<td>27.2</td>
<td>15.5</td>
</tr>
<tr>
<td>INB punctate</td>
<td>13.0</td>
<td>10.1</td>
</tr>
<tr>
<td>INB absent</td>
<td>69.9</td>
<td>65.0</td>
</tr>
<tr>
<td>Purkinje Cells</td>
<td>3.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Multiple nucleoli</td>
<td>11.2</td>
<td>14.5</td>
</tr>
<tr>
<td>Single nucleoli</td>
<td>15.6</td>
<td>16.9</td>
</tr>
<tr>
<td>INB prominent</td>
<td>13.0</td>
<td>10.1</td>
</tr>
<tr>
<td>INB punctate</td>
<td>69.9</td>
<td>65.0</td>
</tr>
<tr>
<td>INB absent</td>
<td>3.3</td>
<td>3.7</td>
</tr>
<tr>
<td>*INB = intranucleolar body.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>**Groups G and G-L are significantly different from group C and from each other.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>***Group G is significantly different from groups C and G-L.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Groups G and G-L are significantly different from group C, but not from each other.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| ++There are no significant differences between groups.
TABLE 6

Attainment of developmental and behavioral characteristics in young hamsters following exposure to ethanol.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Animal Age in Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-exposed Controls (Group C: 7 litters)</td>
</tr>
<tr>
<td>Appearance of pigmented hair</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>7.0-9.0</td>
</tr>
<tr>
<td>Average</td>
<td>8.2</td>
</tr>
<tr>
<td>Beginning to eat solid food</td>
<td>9.0-11.0</td>
</tr>
<tr>
<td>Range</td>
<td>10.3</td>
</tr>
<tr>
<td>Ambulation with hind legs under body</td>
<td>8.0-16.0</td>
</tr>
<tr>
<td>Range</td>
<td>11.9</td>
</tr>
<tr>
<td>Withdrawal from a ledge</td>
<td>7.5-17.0</td>
</tr>
<tr>
<td>Range</td>
<td>12.4</td>
</tr>
<tr>
<td>Beginning to drink from bottle</td>
<td>13.5-15.5</td>
</tr>
<tr>
<td>Range</td>
<td>14.5</td>
</tr>
<tr>
<td>Climbing over wire-mesh cylinder (4 inches)</td>
<td>8.5-18.0</td>
</tr>
<tr>
<td>Range</td>
<td>15.0</td>
</tr>
<tr>
<td>Opening of eyes</td>
<td>14.5-18.5</td>
</tr>
<tr>
<td>Range</td>
<td>15.5</td>
</tr>
<tr>
<td>Average</td>
<td></td>
</tr>
</tbody>
</table>
The dissertation submitted by Carole R. Dunmire-Graff has been read and approved by the following committee:

Dr. Faith W. LaVelle, Director
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Associate Professor, Anatomy, Loyola Medical Center

Dr. James Hazlett
Associate Professor, Anatomy, Wayne State University

Dr. Mary Manteuffel
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Dr. Philip Ulinski
Associate Professor, Anatomy, University of Chicago

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 8, 1981

Date

[Signature]

Director's Signature