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STUDIES ON THE ORNITHINE DECARBOXYLASE OF RAT LIVER

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Alan Joseph Bitonti, B.A.

* * * * *

The Ohio State University
1978

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I would like to express my sincere thanks to my adviser, Dr. Daniel Couri, for his guidance towards the completion of this work. He has always allowed the intellectual freedom necessary for my development as an independent scientist.

Special thanks to Barbara Bayer and Russell Savage for their help in the conception of this project and their guidance and friendship in the first years of my graduate training.

Thanks to the other students in our group -- Bob, Pam, Steve, Mohamed, Karl and Joe -- for their discussions, advice, and friendship and especially for their expert assistance in the laboratory.

My appreciation is also extended to Mrs. Nancy Coldren for her aid in the preparation of this manuscript.

To my future wife, Joan, thank you for making the last two years the best in my life. You have contributed more to my success than you will ever know.

Finally, to my Mother and Father, thanks for 26 years of love and understanding that have brought me this far. Your support and encouragement always allowed me to continue, even when I thought I couldn't. I hope I can always make you as proud of me as I am of you.
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Physiological Chemistry. Drs. R. Nuenke, A. Merola, H. Sprecher

Biochemical Methods. Drs. R. Nuenke and F. Och

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INTRODUCTION

CYTOPLASMIC CONTROL OF NUCLEAR FUNCTION

Well before the turn of the century, the obvious morphological division of the cell into nucleus and cytoplasm gave rise to speculations concerning the functional significance of such a division. In the writings of August Weismann (1893), it can be seen that the idea of the cell being totally dependent on the nucleus for viability was well founded on basic biological observations. The nucleus was considered the "master mind" of the cell, directing all cellular function including the determination of the heritable characteristics of the cell. Weismann stated that "although much remains to be decided by future investigation, that the nature of the cell is really decided by the elements of the nucleus, is definitely established".

Though Weismann was correct in concluding that the nucleus directed cytoplasmic function, he did not foresee the possibility that the cytoplasmic environment could in turn influence nuclear function. He did guess that "the life of the cell is a continual interchange of substance between the cell (cytoplasm) and the nucleus", but failed to grasp the full importance of his statement, believing nucleocytoplasmic
exchange to be a one way phenomenon in which invisible "biophors"
from the nucleus escaped into the cytoplasm. Years later, observations
in the field of embryology were to change this thinking.

In 1925, E.B. Wilson wrote that "no investigator would today
maintain that the nucleus or the chromosomes are the sole agents of
heredity. On the contrary, cytological research has clearly
demonstrated that the protoplasm plays an important part in development".

The first line of evidence supporting this conclusion was an
observation on changes in the biochemical composition of the nucleus
during different stages of the cell cycle. The nucleus avidly bound
certain chemical dyes and presented a multicolored appearance upon
microscopic examination. Chemical dyes of basic nature were thought to
be specific for the nucleus and were referred to as "nuclear" dyes as
opposed to the "cytoplasmic" or acidic dyes. During certain states in
development there would be "enormous growth of the chromosomes,
accompanied in some cases by a complete reversal of their staining-
reactions (which) seems to indicate a progressive accumulation of
protein-components". The nucleus bound acidic dyes more avidly than
basic dyes and stained as if it contained cytoplasmic components,
probably cytoplasmic proteins.

Experiments conducted by Boveri (1909-1910) on Ascaris megalcephala
eggs documented that nuclear function was dependent on the cytoplasmic
environment (cited in Wilson, 1925). During cleavage of fertilized eggs,
some cells remained "totipotent" and retained their entire complement
of chromosomes intact. Other cells underwent "diminution", losing
portions of their chromosomes. The former were designated "germ" or "stem" cells while the latter were designated "somatic" cells. Each embryo contained only one stem cell if the cleavage was normal (Figure 1, A-C). However, if the plane of cleavage, and therefore the distribution of cytoplasmic components to the daughter cells was shifted by centrifugation of the embryo, often more than one "stem" cell would arise (Figure 1, D-F). From these observations it was concluded that "the behavior of the nuclei in these eggs is determined by the cytoplasm in which they lie; the whole nuclear content being retained only in the cells which receive the particular cytoplasmic region predestined to form the "stem" cells. This is the first solid evidence that cytoplasmic factors controlled chromosomal activity and determined the history of the cell in development. Excellent reviews and detailed descriptions of these and other experiments can be found in Gurdon, 1968 and Wilson, 1925.

The preceding experiments are classics in the area of nucleocytoplasmic interaction but the methodologies of the time were too crude to precisely define which functions of the nuclei were under cytoplasmic control. Presently it is accepted that the major function of the nucleus is the production of all types of ribonucleic acid (RNA) which in turn act in the cytoplasm to synthesize proteins. Thus, if the cytoplasm is to influence cellular development by controlling nuclear activity, this influence would probably be manifested at the level of RNA synthesis. It is conceivable that factors from the cytoplasm could
NORMAL CLEAVAGE

CLEAVAGE OF CENTRIFUGED EGGS

In each case the future stem-cells are indicated by parallel lines.

after Wilson, 1925

FIGURE 1
DEMONSTRATION THAT NUCLEAR FUNCTION IS UNDER
THE INFLUENCE OF THE CYTOPLASMIC ENVIRONMENT
enter the nucleus and selectively activate or repress chromosomal genes. The techniques necessary to demonstrate this type of control, however, were only recently developed.

Working with *Xenopus laevis* embryos and ova, J.B. Gurdon (1965) proved that the synthesis of RNA was controlled by the cytoplasm. The experiments involved the transplantation of endoderm cell nuclei (from neurula stage embryos) into enucleated, unfertilized eggs. Normally neurula endoderm cell nuclei actively synthesize ribosomal RNA (rRNA). Upon transplantation to the unfertilized egg, no rRNA synthesis could be demonstrated. Instead, the transplanted nuclei reverted to the synthesis of heterogeneous RNA typically synthesized during cleavage stages. The synthesis of rRNA then recommenced after gastrulation occurred and continued undisturbed during further development. After transplantation there was also a great increase (30-fold) in nuclear volume. It is now thought that the nuclear swelling was a consequence of influx of proteins into the nucleus. It has been suggested that the migrating proteins are responsible for the changes in genetic activity.

Puff formation in the polytene chromosomes of *Drosophila hydei* salivary gland nuclei can be induced by temperature shock or treatment with the insect hormone ecdysone. Puffing is thought to be due to gene activation, the puffs being areas of the genome associated with high rates of RNA synthesis. Berendes (1968) found that one of the first events in puff formation was the accumulation of acidic proteins in the puffing zones. This accumulation was demonstrated by an increased fast green dye binding capacity in the puff regions and occurred prior to
increased synthesis of RNA. It was concluded that the acidic proteins are responsible for the gene activation (increased RNA synthesis).

Holt (1970) confirmed Berendes' work in Drosophila hydei and demonstrated further that the proteins which accumulated in the puffed regions of the chromosomes were not newly synthesized after puff induction but existed in the cytoplasm prior to the application of ecdysone or temperature shock. Radioactive amino acids were injected into the cytoplasm of salivary gland cells at the same time that the puff-inducing stimulus was applied. No incorporation of radioactive label into the puff regions occurred. It was concluded that preformed proteins existed in the cytoplasm which, upon application of a proper stimulus, were able to move into the nucleus and modulate chromosomal activity.

In similar experiments, Helmsing and Berendes (1971) extracted all the proteins from induced salivary gland nuclei. The proteins were then subjected to gel electrophoresis. It was demonstrated that during induction, a new species of protein appeared in the nucleus. Again, as in Holt's work, the protein appeared to be a preexistent molecule since no incorporation of labelled amino acids could be shown in the accumulated protein. The protein was found to be a nonhistone, that is, probably not a normal constituent of chromatin.

The phenomenon of protein accumulation during periods of intense gene activation appears to be a generalized phenomenon occurring in many different species in response to widely varying stimuli. Merriam (1969) prelabelled Xenopus laevis oocytes with radioactive amino
acids and irradiated their nuclei. He then transplanted nuclei from blastula cells (of the same species) into the labelled cytoplasms. Puromycin was added to the cells to prevent continued protein synthesis and the fate of radioactively labelled cytoplasmic proteins was followed. The transplanted nuclei immediately swelled to enormous volumes as large amounts of radioactivity (much of it in proteins with molecular weights greater than 5000) migrated into the nucleus. At this same time the nuclei changed from basophilic staining character to acidophilic staining character, reminiscent of experiments performed many years before (Wilson, 1925). The nuclei from the blastula cells, which normally synthesized DNA, ceased this activity and began synthesizing RNA. Again it appeared that application of a proper stimulus caused the migration of preformed proteins from cytoplasm to nucleus, proteins which may be modulators of gene activity.

Studies by various investigators demonstrated that inhibitors of protein synthesis, in particular cycloheximide, could inhibit RNA polymerase activity and RNA synthesis in vivo. Because cycloheximide had no effect on RNA polymerase activity when added directly to nuclei in vitro (Higashinakagawa and Muramatsu, 1972), another mechanism for the drug effect on RNA synthesis had to be found.

Administration of cycloheximide inhibited the rapid increase in the labelling of hepatic rRNA (with $^{14}\text{C}$-orotic acid) normally seen after partial hepatectomy (Higashi et al., 1968) or treatment with phenobarbital (McCauley and Couri, 1971). These studies suggested that the synthesis of rRNA is subject to some translational influence. The
latter study also showed that the drug-induced rRNA synthesis was more sensitive to cycloheximide inhibition than rRNA synthesis in untreated livers.

Willems et al. (1969) also found a decrease in the synthesis of rRNA after cycloheximide addition to cultured HeLa cells. They demonstrated that the only species of RNA that was not labelled by $^{3}$H-UTP in the presence of cycloheximide was 45S or ribosomal precursor RNA. Their experiments brought out some very important points. First of all they concluded that the decrease in RNA synthesis was not due to a rapid decline in the number of RNA molecules as has been suggested by others (Yu and Feigelson, 1972). After removal of the cycloheximide from the culture media, rRNA synthesis rapidly recovered. Willems et al. therefore proposed that a rapidly turning over protein existed whose continued synthesis was necessary for the maintenance of rRNA synthesis. They proposed further that this short-lived protein acted at the point of initiation of RNA synthesis on the DNA template. If the protein factor acted primarily on the chain elongation step of RNA synthesis, its disappearance after cycloheximide administration would presumably cause more incomplete rRNA molecules to be formed. This was not the case, however, and it was concluded that the factor was an initiation promoting factor.

The cycloheximide effect on RNA metabolism has also been shown in normal animals. Muramatsu et al. (1970) and Gross and Pogo (1974) showed that cycloheximide rapidly inhibited the synthesis of rRNA and
the activity of RNA polymerase I in normal rats and concluded that there must be a short-lived protein factor necessary for sustained RNA polymerase I activity.

Indirect evidence for the existence of cytoplasmic proteins which function as regulators of nuclear RNA polymerase I activity has also been obtained in studies utilizing the mushroom toxin α-amanitin. Alpha-amanitin inhibited the synthesis of both mRNA (RNA polymerase II activity) and rRNA (RNA polymerase I activity) in the rat liver when administered in vivo (Jacob et al., 1970; Tata et al., 1972). This contrasts sharply to the toxin's in vitro effects. Alpha-amanitin is a specific inhibitor of RNA polymerase II when added to isolated nuclei (Kedinger, 1970). Both Jacob and Tata concluded that α-amanitin remains a specific inhibitor of RNA polymerase II in vivo and as the mRNA content of the cell drops, the synthesis of a protein activator of RNA polymerase I ceases. Thus α-amanitin indirectly decreases RNA polymerase I activity. It should be noted that α-amanitin had no effect on general protein synthesis (Jacob et al., 1970).

Further support for the proposed protein initiation factor came from the work of Franze-Fernandez and Fontanive-Senguesa (1973) and Cereghini and Franze-Fernandez (1974). These investigators found that RNA polymerase I activity and rRNA synthesis was enhanced by the addition of amino acids to Ehrlich ascites cells in culture. These workers concluded that the added amino acids stimulated the synthesis of the RNA polymerase I initiation factor.
Finally, studies have demonstrated that the addition of cell-free cytoplasm to homologous isolated nuclei stimulated RNA polymerase activity (Thompson and McCarthy, 1968; Young, 1976). This finding directly showed that RNA polymerase activity was regulated by cytoplasmic factors, presumably proteins. The goal of further experiments would logically be the identification and purification of specific cytoplasmic proteins that have as one of their properties the ability to stimulate the synthesis of rRNA.

Nearly ten years ago Stein and Hausen (1970) and Seifart (1970) identified cytoplasmic protein factors that stimulated RNA polymerase II activity in nuclei of calf thymus and rat liver, respectively. Very recently a factor which stimulated RNA polymerase I activity was identified in rat liver (Goldberg et al., 1977). The latter factor stimulated rRNA synthesis at the initiation step rather than the elongation step.

Very recently another protein, ornithine decarboxylase, has been proposed as an initiation factor for the RNA polymerase I reaction (Manen and Russell, 1975). The evidence for this hypothesis will be reviewed in detail in the next section.

**ORNITHINE DECARBOXYLASE AS AN INITIATION FACTOR FOR RNA POLYMERASE I**

Ornithine decarboxylase (ODC) was first described in mammalian tissues in 1968 by three laboratories (Russell and Snyder, 1968; Pegg and Williams-Ashman, 1968; Janne et al., 1968). ODC is the rate limiting enzyme of polyamine biosynthesis (Pegg and
Williams-Ashman, 1968). The enzyme is responsible for the decarboxylation of L-ornithine to form putrescine (1,4-diaminobutane) (Figure 2).

The demonstration that an increase in ODC was one of the earliest biochemical changes in regenerating rat liver (Russell and Snyder, 1968; Fausto, 1969) and the livers of rats treated with growth hormone (Russell et al., 1970; Jänne and Raina, 1969) suggested that ODC might have some role in rapid growth processes. ODC also was found to be increased in rapidly growing tumors (Russell and Snyder, 1968). In recent years, in fact, it has been shown that increased ODC activity is an early response to just about any stimulus which increases the metabolic activity of the liver cell (see Morris and Fillingame, 1974 for references). Another interesting feature of ODC is its extremely short half-life of 10-20 minutes (Russell and Snyder, 1969). This half-life is the shortest of any known mammalian enzyme (Snyder and Russell, 1970) and further suggested ODC as an important regulatory molecule.

The link between ODC and growth processes was strengthened when simultaneous changes in ODC activity and RNA synthesis or nuclear RNA polymerase activity were described. After growth hormone treatment, both ODC activity and RNA polymerase activity reached maximal approximately 4 hours after administration of the hormone (Jänne and Raina, 1969). Fausto (1969) demonstrated that in regenerating rat liver, livers of rats treated with casein hydrolysate (amino acid mixtures) and in livers of thioacetamide treated rats, that the
FIGURE 2

THE POLYAMINE BIOSYNTHETIC PATHWAY
incorporation of $^{14}$C-orotic acid into RNA increased in parallel to the increases in ODC activity. Höltta and Raina (1973) found that glucagon, dibutyryl cAMP, and theophylline also caused paralleled changes in the two enzyme activities. Finally, it was shown by Russell (1971) that in rats treated with phenobarbital, 3-methylcholanthrene, 3,4-benzpyrene, heparin, and clofibrate, increases in ODC activity were always accompanied by increased incorporation of $^{3}$H-UTP into RNA.

Both Jänne and Raina (1969) and Fausto (1969) found that the stimulation of RNA synthesis was primarily due to stimulation of RNA polymerase I activity and the stimulation of ribosomal RNA synthesis. This specificity for stimulation of RNA polymerase I was also found by Young (1976) after phenobarbital stimulation of ODC activity.

Another line of evidence which suggested a link between ODC and RNA polymerase I was work done with normal and anucleolate mutants of Xenopus laevis (Russell, 1971b). Russell described a specific link between the presence of nucleoli in developing embryos and the course of ODC synthesis in these organisms. Prior to gastrulation in normal Xenopus embryos, most enzymes were at a rather low level and remained so until gastrulation. However, in the early blastula stages before gastrulation, both ODC and RNA polymerase I activity increased dramatically. Throughout the further development of the embryo, there were always parallel changes in the two enzyme activities. There was an especially large increase in both enzyme activities shortly after hatching. In anucleolate mutants there was an absolute lack of rRNA
synthesis (no RNA polymerase I activity)\(^1\) and depressed ODC activity. It is especially significant that the depressed ODC activity was a specific effect, other enzymes (lactate dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase) being the same in normal and anucleolate mutants.

In 1975, evidence for a more direct link between ODC and RNA polymerase I was obtained. In studies on the effects of 3-isobutyl-1-methylxanthine (MIX) on ODC and RNA polymerase activity, it was discovered that the enzyme activities increased in parallel after drug treatment (Manen and Russell, 1975). After 4 hours of treatment with MIX, ODC activity was 50-75 times control and RNA polymerase activity (both I and II are stimulated) increased approximately 2-fold, the increase in the RNA polymerases occurring shortly after the ODC increase. Treatment with cycloheximide revealed that both enzymes turn over with the same apparent half-lives, approximately 15 minutes. It was further demonstrated that the addition of a partially purified preparation of ODC to isolated nuclei increased RNA polymerase I activity (RNA polymerase II was unaffected). RNA polymerase I activity increased in a linear fashion with the addition of increasing units of ODC activity. It was proposed that ODC was a stimulatory factor for the RNA polymerase I reaction.

\(^1\)This finding is expected since the nucleolus appears to be the sole site in the nucleus for ribosomal RNA synthesis (Perry, 1969).
Two years later these same investigators purified ODC greater than 1000-fold on an affinity chromatography column which utilized RNA polymerase I as an affinity ligand (Manen and Russell, 1977a). This purification procedure supposedly demonstrated a direct physical interaction between the two enzyme molecules. Further evidence for ODC being an initiation factor was also obtained. Purified ODC (1000-fold) was added to an RNA polymerase I reaction in which both the incorporation of $\gamma^{32}$P-ATP and $^{14}$C-ATP into RNA were measured. The addition of ODC increased the incorporation of $^{32}$P-ATP greater than $^{14}$C-ATP incorporation. This is evidence that initiation of RNA synthesis was affected more than the elongation of RNA chains.\(^2\)

One important point that has not been considered completely in past studies is the availability of the ODC molecule to the intranuclear compartment and thus to RNA polymerase I in rat liver nuclei. Reports have been contradictory as to nuclear ODC content. In 1970, Russell and Snyder found that chick embryos had as much as 50% of their total cellular ODC activity in the nucleus. This finding could not be confirmed by Eloranta et.al., (1976), though some ODC activity (2-10% of total cellular ODC activity) was found in the nuclear fraction. It is one of the goals of this work to more clearly define the presence of ODC in the nucleus with particular attention being paid to the

\(^2\)Synthesis of the RNA molecule is initiated with the incorporation of a triphosphate nucleoside whereas internal bases are incorporated as monophosphate nucleosides. Thus, if the incorporation of $\gamma^{32}$P-ATP increases more than $^{14}$C-ATP, it indicates the incorporation of an initial base is increased more than the incorporation of internal bases.
possibility that drugs may alter nuclear ODC content and thus affect RNA polymerase I activity.

MECHANISMS OF NUCLEOCYTOPLASMIC EXCHANGE

The proposal that ornithine decarboxylase (ODC) acts as an initiation factor for RNA polymerase I presupposes accessibility of the enzyme (ODC) to the intranuclear compartment and that this accessibility is subject to change during different physiologic or drug-induced states. How the accessibility of materials to the intranuclear compartment can be controlled will be discussed here.

It is important to understand the general morphology of the nuclear envelope before considering the mechanism of nucleocytoplasmic exchange (for a complete review of the nuclear envelope morphology and function see Busch, 1974a). The nuclear envelope consists of a pair of membranes running parallel to one another, forming a narrow space between them called the perinuclear cisterna. Functions for the two different membranes are not yet strictly defined. One of the most interesting features of the nuclear envelope is the nuclear pore complexes that interrupt both nuclear membranes at regular intervals, forming what appears to be a site of communication between the cytoplasm and the nucleoplasm (Figure 3).

The nucleopores are not simply holes in the membrane but appear to be highly complex, highly organized structures that have several distinct biochemical constituents. The pore complexes consist of an annulus, pore diaphragm, intrapore material, and the pore opening
FIGURE 3

THE MORPHOLOGY OF THE NUCLEAR MEMBRANE AND ITS PORE COMPLEXES
itself (Figure 3). All these structures may play some role in nucleo-cytoplasmic interaction though it isn't yet clear exactly what the role for each component is. One function of the nuclear pore complex, as a whole, that appears to be well established is that of mediating the movement of materials, particularly macromolecules, between the cytoplasm and the nucleus.

The first evidence for movement of macromolecules through the nuclear pores is the work of Feldherr (1962). He injected polyvinylpyrrolidone-coated gold particles into the cytoplasm of amebae. The gold particles are electron opaque and their locations are easily seen on electron micrographs. After injection of the gold particles, cells are examined at various times. At 1-2 minutes post injection, most gold particles were cytoplasmic. At 10 minutes the concentration of gold particles in the cytoplasm and nucleoplasm were roughly equal and at 24 hours the gold was highly concentrated in the nucleus. The experiment showed a progressive movement of gold particles into the nucleus but more important was the finding of particles within the nuclear pore complex, thus implicating the pore as a route of entry. Also, the particles were always in the exact center of the pore which suggested some sort of central channel through which passage occurred.

Transport through the nuclear pores has also been shown for proteins and ribonucleic acids, the two types of macromolecules that would most likely be transported in the cell. Stevens and Swift (1966) demonstrated the appearance of newly synthesized RNA granules in the nuclear pores. Nuclei from Chironomus tummi (midge) salivary gland
cells, contained polytene chromosomes with Balbiani rings which were actively synthesizing RNA. The RNA granules synthesized in the Balbiani ring were initially released into the nucleoplasm. Later these granules were found in transit through the pore complex and on either side of the nuclear envelope. The RNA granules, as they passed through the pores, took on an elongated, cylindrical appearance, as if they were squeezing through a central channel in the pore.

Paine and Feldherr (1972) injected fluorescent-labelled proteins into the cytoplasm of living cockroach (Periplaneta americana) oocytes and followed the proteins' movements by fluorescence electron microscopy. The protein tracers were of varying molecular weights and it was shown that transport into the nucleus through the pores was a function of molecular weight. Small proteins (myoglobin, MW = 17,500; lysozyme, MW = 14,500; cytochrome c, MW = 13,000) entered the nucleus very rapidly while larger proteins (bovine serum albumin, MW = 67,500; ovalbumin, MW = 45,000; ferritin, MW = 465,000) moved more slowly. The observation that even a protein molecule the size of ferritin could enter the nucleus, albeit very slowly, is significant.

From these experiments it is clear that large molecules probably transverse the nuclear membrane through the nuclear pores. It should also be established that the transport of molecules through the pore can be regulated during different physiologic or drug-induced states for the pore complex to assume some importance in cell regulatory processes.

There is evidence in the literature that the nuclear pore complex is not a static structure but is subject to change during changes in
the physiologic state of the organism or the cell (Blackburn, 1971). It appears, also, that these changes in the nuclear pores correlate well with changes in nuclear metabolism.

Experiments have shown that increased DNA and RNA synthesis correlates with increases in the nucleopore frequency. Tokuyasu et al., (1968) showed that in cultured human lymphocytes, stimulated to growth with phytohemagglutinin, there were parallel changes in the rate of RNA synthesis and the number of nucleopores in the nuclear membrane. Maul et al., (1972), using the same model system, demonstrated a biphasic change in the number of nucleopores. The first increase in number of pores was correlated with an increased RNA synthesis while the second increase in nucleopore number was correlated with an increase in DNA synthesis.

The frequency of nucleopores has also been shown to shift at different stages in development. Merriam (1962) conducted a comparative study on the dynamic aspects of the nuclear envelope. He found that immature frog oocytes which were much more active in RNA synthesis than mature oocytes, had 40% more nucleopores. It was also demonstrated that after fertilization there was a progressive loss of nucleopores with increasing differentiation of the cell and decreasing metabolic activity (King, 1955).

However, not all studies support the hypothesis that an increased number of pores is the most important factor regulating nucleocytoplasmic exchange. Wunderlich (1969) showed that there was an inverse relationship between pore frequency and pore diameter in different physiologic
states of *Tetrahymena pyriformis*. In all cases, as the pore frequency increased, pore diameter decreased so that total pore area per nuclear surface area remained a constant ratio of 0.32. This finding suggested a complex control of nucleocytoplasmic exchange rather than regulation by a simple increase in pore frequency. It is also possible that exchange is regulated by the intrapore material.

Feldherr (1969) noted that in the nuclei of cells of different species (ameba, frog oocytes, roach oocytes) there was a wide variation in total pore area. There was also a wide variation in the rates of nucleocytoplasmic exchange, but variations in these two parameters were not correlated. The rate of nucleocytoplasmic exchange did correlate with the amount of opaque material located in the nuclear pore complex, however, suggesting that changes in the pore diaphragm or in material lining the pore walls (Figure 3) may regulate nucleocytoplasmic exchange. It was also interesting in this study that the cells which had the highest metabolic activity had the highest nucleocytoplasmic exchange, suggesting a regulatory function for the exchange processes.

There was also an obvious difference between immature and mature frog oocytes in the amount of opaque material located in the pore complex (Merriam, 1962). Again there was a link between the structural components of the pore and regulation of nucleocytoplasmic exchange. Merriam suggested that there might be a difference in the thickness of the pore diaphragm which stretches across the pore opening.

The nuclear pore must not be considered as a structural entity only. It has a complex biochemical character which includes at least
one enzyme thought to be involved in nucleocytoplasmic exchange. In mouse choroid plexus epithelial cells, Yasuzumi and Tsubo (1966) showed that the nuclear membrane contained a Mg\(^{2+}\)-dependent ATPase activity that was most highly concentrated in the pore complex.

Another study on young and aging human testis (Yasuzumi et al., 1967) showed that the ATPase activity of the nuclear pore might be related to nucleocytoplasmic exchange. Yasuzumi observed that young testis had more frequent nucleopores and more intense ATPase activity in these structures. Aging human testis had fewer nucleopores and little or no ATPase activity in these pores. Since the younger cells are more metabolically active and more active presumably in nucleocytoplasmic exchange, Yasuzumi speculated that "regulation of nucleocytoplasmic exchange of macromolecules (is) dependent on the activity of the ATPase located within the pores of the nuclear envelope".

Two other studies are also suggestive of ATPase regulation of nucleocytoplasmic exchange. Kawashima et al. (1971) showed that the transport of nucleolar proteins into the nucleus is an energy (ATP) dependent process and can be inhibited by dinitrophenol. Schumm and Webb (1972) showed that RNA transport out of the nucleus is dependent on ATP and can be inhibited by beryllium nitrate, a specific inhibitor of the nuclear pore ATPase (Cutler, 1974).

POLYAMINES AND NUCLEIC ACIDS

The physiologically important polyamines are putrescine, spermidine, and spermine. Their synthesis is shown in Figure 2. Putrescine
(diaminobutane) is formed by the decarboxylation of L-ornithine. Ornithine decarboxylase appears to be the rate-limiting step of the pathway (Pegg and Williams-Ashman, 1968). Spermidine is formed by the addition of a propylamine moiety to one of the terminal amine groups of putrescine. The propylamine group is donated by decarboxylated S-adenosylmethionine (formed by S-adenosylmethionine decarboxylase) and is added to putrescine through the action of a propylamine transferase, spermidine synthase. Spermine is produced by the addition of a second propylamine group to the opposite end of the spermidine molecule. The propylamine group is added through the action of spermine synthase (see Jänne, 1967 for details of the enzymatic steps).

Polyamine biosynthesis can be inhibited by various drugs. ODC is inhibited by derivatives of its substrate, ornithine. These derivatives include hydrazino-ornithine (Harik et al., 1974) and α-methylornithine (Mamont et al., 1976). S-adenosylmethionine decarboxylase can be inhibited by methylglyoxal bis(guanylhydrazone) (Pegg, 1973). Effects of these compounds on cellular events other than polyamine biosynthesis are either contradictory or entirely unknown.

The polyamines have a wide range of physiologic effects in many species (Tabor and Tabor, 1976; Bachrach, 1973). They are necessary growth factors in at least some mammalian cells (Ham, 1964). Some effects of the polyamines are associated with nucleic acids and protein synthesis. The polycationic polyamines bind to negatively charged DNA and mRNA (Bachrach, 1973) as well as to ribosomes (Raina and Telaranta, 1967) and transfer RNA (Cohen, 1970). The binding suggests that the
polyamines are involved in the synthesis and turnover of the important intracellular macromolecules.

Changes in polyamine levels are correlated with rapid growth and increased RNA synthesis in the liver after partial hepatectomy (Heby and Lewan, 1971). Increased polyamines are also related to drug-induced changes in RNA synthesis (Manen and Russell, 1977a; Young, 1976).

RNA polymerase activity in isolated nuclei is stimulated strongly by spermidine and spermine and weakly by putrescine (Moruzzi et al., 1975; Jänne et al., 1975). Protein synthesis is also stimulated (Hershko et al., 1961). The polyamines obviously lend themselves well to the role of intracellular regulatory molecules.
STATEMENT OF THE PROBLEM

It is presently accepted that cytoplasmic protein molecules migrate into the nucleus prior to and during periods of intense gene activity and that the migrating pool of protein molecules contains specific factors which initiate new RNA synthesis (Gurdon, 1968). Protein factors have been isolated which are able to stimulate RNA polymerase activity (Stein and Hausen, 1970; Seifart, 1970; Goldberg et al., 1977). A factor very recently proposed as an initiation factor for rat liver RNA polymerase I is the polyamine biosynthetic enzyme ornithine decarboxylase (Manen and Russell, 1975). If ornithine decarboxylase (ODC) is to be considered a possible mediator of drug effects on nuclear RNA polymerase activity, then nuclear ODC content should increase after treatment of animals with agents which cause simultaneous increases in the activities of both enzymes. In this work this possibility will be examined.

The chemical agent that will be studied is 3-isobutyl-1-methyl-xanthine (MIX). MIX increases both cytoplasmic ODC and nuclear RNA polymerase activity (Manen and Russell, 1975) and thus appeared to be a good agent with which to work.

Initially it was desirable to measure ODC activity in rat liver cytoplasm and nuclei to determine whether or not there were changes in
the subcellular distribution of ODC activity after drug treatment.

Three possible mechanisms for increased ODC activity within the nucleus may be involved. These are:

1. Migration of the enzyme (ODC) from cytoplasm to nucleus
2. Activation of ODC molecules already present in the nucleus
3. De novo synthesis of the ODC enzyme in the nucleus upon drug stimulation

Using various treatments with MIX and an inhibitor of protein synthesis, cycloheximide, administered in vivo, either in combination or separately, possibility 1 could be examined. To test the third possibility it would also be necessary to show inhibition of nuclear labelling of ³H-leucine incorporation into protein, whereas the first mechanism is tested by the measurement of ODC activity after the various drug treatments. The second mechanism could be examined in vitro.

Because MIX is a potent inhibitor of cAMP phosphodiesterase (Beavo et al., 1970), it was also desirable to discover whether or not effects measured after MIX administration were due to a direct effect of MIX or due to an indirect action of the drug mediated by cAMP. In vivo and in vitro work utilizing dibutyryl cAMP would determine whether or not an elevation of intracellular cAMP might be responsible for any or all of the effects seen with MIX.

If the subcellular distribution changes (i.e. if nuclear ODC content is increased) after treatment with 3-isobutyl-1-methylxanthine, then ODC might indeed mediate the effect of the drug on RNA polymerase in the nucleus. To test the hypothesis, RNA polymerase will be measured under the same treatment conditions as used in ODC activity studies. If increased ODC activity in the nucleus could be dissociated from increased
RNA polymerase activity in certain situations, previous proposals involving a direct link between the two enzymes would be brought into question. If, on the other hand, there was a direct correlation between increased nuclear ODC activity and RNA polymerase activity, then the previous proposal that ODC is a possible initiation factor would be supported.

The polyamines (putrescine, spermidine, and spermine) stimulate RNA polymerase (Bachrach, 1973). Intracellular putrescine levels are increased in rat liver after treatment with MIX (Manen and Russell, 1977a). It is therefore necessary to consider an alternate route by which RNA polymerase I may be stimulated. Rather than stimulation by ODC directly, RNA polymerase might be stimulated indirectly by the biosynthetic products of ODC, namely putrescine, spermidine, and spermine. Although Manen and Russell (1977a) showed no effect of putrescine on RNA polymerase (using both intact and sonicated nuclei as the source of the enzyme) it is possible that spermidine and spermine may increase RNA polymerase activity, as reported in other systems (see Bachrach, 1973).

An approach to study the contribution of polyamines to polymerase activity involves the use of an inhibitor of the polyamine biosynthetic enzyme, S-adenosylmethionine decarboxylase, to block spermidine and spermine production after treatment of an animal with MIX. The inhibitor chosen for this study was methylglyoxal bis(guanylhydrazone) (MGBG) (Pegg, 1973). RNA polymerase activity was to be measured after these drug treatments.
ODC has been implicated as an important factor in all rapid cellular growth processes (Snyder and Russell, 1970), especially in tumor growth (Russell and Snyder, 1968). It is thought by some workers that increases in ODC activity may be an obligatory event in the carcinogenic process (O'Brien, 1976). If increased cytoplasmic ODC is a factor in carcinogenesis, then the migration of ODC into the nucleus may also play a fundamental role in the initiation or promotion of the tumor process. It is thus proposed to study the effects of a liver carcinogen (thioacetamide) on the subcellular distribution of ODC. The effects of thioacetamide on RNA polymerase I and II will be investigated to discover whether or not there was a relationship between nuclear ODC activity and RNA polymerase activity.

ODC may play a role in the increase in the hepatic drug metabolizing enzymes after certain drug treatments (Byus et al., 1976). Thus, the effects of 3-methylcholanthrene, a known stimulator of the liver drug metabolizing enzymes, on the subcellular distribution of ODC will be investigated.

Nuclear membrane adenosine triphosphatase (ATPase) activity will be studied as a possible biochemical or molecular mode of action by which MIX increases the transport of ODC from the cytoplasm to the nucleus. It was suggested by Yasuzumi (1966) that the ATPase of the nuclear pore complex in the nuclear membrane might have some function in the transport of macromolecules through the nuclear membrane.

Finally, it is of interest to study the specificity of MIX on the subcellular distribution of ODC. To test the structure-activity
relationship, aminophylline, a drug very similar to MIX will be used. Aminophylline (1,3-dimethylxanthine) differs from 3-isobutyl-1-methylxanthine (MIX) in having a methyl group at its 3 position rather than the isobutyl moiety found in MIX. ODC activity in both cytoplasmic and nuclear compartments will be measured to determine whether or not any changes after MIX could be achieved by its close structural analog, aminophylline.
METHODS

A. Animals

Male albino Wistar rats weighing between 125 and 175 grams were used in all experiments. The animals were housed in a controlled environment (temperature, 22°C; humidity, 50%) with a 12 hour light/dark cycle beginning at 6:00 AM. Animals were given food and water ad libitum for all experiments.

B. Preparation of Tissues

A single method of preparation of subcellular fractions (with slight modifications for certain experiments) was used for all experiments to avoid variations caused by the tissue preparation alone. The method selected is modified from Sadowski and Steiner (1968) and Eloranta et al (1976).

Rats were killed by decapitation. The liver was exposed and perfused in situ with 10 ml of ice cold 0.25 M sucrose-Tris-Mg\textsuperscript{2+} buffer (STM buffer) through the hepatic vein using a 10 ml syringe equipped with a disposable 18G needle. The 10 ml volume was sufficient to rid the liver of most red blood cells. A noticeable blanching of the liver
occurred, the color changing from a dark reddish-brown to a light tan. The STM buffer consisted of 0.25 M sucrose, 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2.5 mM dithiothreitol (Cleland's reagent), and 0.1 mM Na₂H₂EDTA. The STM buffer also contained 0.3 mM pyridoxal phosphate when ornithine decarboxylase was to be assayed.

After perfusion, the entire liver was excised and placed in ice cold 0.25 M STM buffer for rinsing. The liver was then blotted on an absorbent pad, weighed on an analytical balance, and homogenized in 5 volumes (5 ml/1 g) of 0.25 M STM buffer with 5 strokes in a Potter-Elvehjem type glass tissue grinder equipped with a teflon pestle.

Homogenates were subsequently centrifuged for 10 minutes at 750 x g in a Sorvall Model RC-2B ultracentrifuge equipped with an SS34 rotor. The centrifuge was temperature controlled at 0°C. The supernatant was centrifuged at 20,000 x g for an additional 20 minutes (or 100,000 x g for 60 minutes where indicated). The supernatant of 20,000 x g was designated "cytoplasm".

The 750 x g pellet was resuspended in 2.3 M STM buffer, i.e. 2.3 M sucrose replacing 0.25 M sucrose in the STM buffer, (10 ml/g tissue) using 2 strokes of a teflon-glass tissue grinder described above. This suspension was then centrifuged at 40,000 x g for 60 minutes in the Sorvall ultracentrifuge. The supernatant was discarded and the pellet taken up in 0.25 M STM buffer (0.5 ml/g tissue) using a hand held, ground glass tissue grinder. This fraction was designated "nuclei".
C. Assay of Ornithine Decarboxylase Activity

To examine the ornithine decarboxylase activity in rat liver, the procedure of Russell and Snyder (1968) was modified and used as follows.

Subcellular fractions (cytoplasm and nuclei) were prepared as described above. The nuclear fractions were sonicated prior to measurement of ODC activity. The enzyme reaction was then carried out in 10 ml Erlenmeyer reaction flasks equipped with rubber stoppers and polyethylene center wells (Kontes Glass Co.). The center wells contained 200 μl of a 2:1 solution of ethanolamine and 2-methoxyethanol (Eastman Organic Chemicals) to trap $^{14}$CO$_2$ liberated in the ODC reaction. The reaction mixture contained 0.5 ml of the enzyme preparation, 0.5 μCi of D,L-ornithine-1-$^{14}$C (specific radioactivity = 45 mCi/m mole, New England Nuclear), and a volume of sodium-potassium phosphate buffer (pH 7.2) sufficient to make a final volume of 2 ml. Final concentrations of pyridoxal phosphate and dithiothreitol were 0.75 μM and 0.5 μM, respectively.

The reaction was initiated by the addition of radioactive substrate after 15 minutes preincubation of the reaction mixture at 37°C in a Dubnoff metabolic shaker. The reaction mix was then incubated for 45 minutes at 37°C to determine release of $^{14}$CO$_2$. At 45 minutes 0.5 ml of 5 N H$_2$SO$_4$ was injected into the reaction mix through the rubber stopper, both to terminate the reaction and to drive the $^{14}$CO$_2$ from the solution. The flasks were further incubated for 30 minutes to allow complete reaction of the $^{14}$CO$_2$ with the contents of the center well.
Center wells were removed from the flasks and placed in liquid scintillation counting vials containing 10 ml of toluene-PPO-POPOP cocktail and 2 ml of ethanol. The toluene-PPO-POPOP cocktail was made by dissolving 100 g of PPO (Packard) and 1.25 g POPOP (Packard) in 1000 ml toluene to make a concentrated solution. 40 ml of this concentrate was then dissolved in a liter of toluene prior to use. Radioactivity was determined in a Packard Tri-Carb Model #3255 liquid scintillation spectrometer. Counting efficiency was approximately 90% as determined by automatic external standardization. Final results were corrected for efficiency and expressed as pmoles $^{14}CO_2$ evolved/hr/mg protein.

To correct for nonenzymatic release of $^{14}CO_2$ during the incubation, blanks consisting of enzyme preparations precipitated with 0.5 ml of 5 N H$_2$SO$_4$ prior to addition of $^{14}$C-ornithine, were run. Blanks usually were between 50 and 200 DPM.

D. Assay of RNA Polymerase Activity

Nuclei were prepared essentially as described above except that buffers lacked both pyridoxal phosphate, which inhibits RNA polymerase activity (Bayer, 1977), and Na$_2$H$_2$EDTA. Also, the final suspension of nuclei was in 25% glycerol solution containing 10mM Tris-HCl (pH 7.9) and 1 mM MgCl$_2$ (instead of the 0.25 M STM buffer). RNA polymerase activities are very stable with time in the glycerol solution (Spelsberg et al., 1974).
The method of assay for RNA polymerase (both types I and II are essentially those of Glasser and Spelsberg (1972). The only modification was the addition of both Mg\(^{2+}\) and Mn\(^{2+}\) to all reactions (Spelsberg and Wilson, 1976).

All reactions contained:

- 10 μmoles Tris-HCl (pH 7.9)
- 2.0 μmoles KCl
- 0.4 μmoles MnCl\(_2\)
- 0.5 μmoles MgCl\(_2\)
- 0.1 μmoles KH\(_2\)PO\(_4\)
- 0.1 μmoles each of CTP, GTP, and ATP
- 0.4 μmoles mercaptoethanol
- 2.5 μCi \(^3\)H-UTP (41.6 Ci/mmole, New England Nuclear)
- 50 μl nuclear preparation (added last to initiate reaction)

To the reactions designed to measure RNA polymerase I was added 0.2 μg α-amanitin (Calbiochem), a specific inhibitor of RNA polymerase II activity (Kedinger, 1970). To the reactions designed to measure RNA polymerase II was added (NH\(_4\))\(_2\)SO\(_4\) to a final concentration of 0.25 M. Distilled water was added to all reactions to a final volume of 250 μl.

The reactions were carried out in 12 x 75 mm disposable glass tubes at 15°C, 10 min, in a Dubnoff metabolic shaker. The temperature was maintained by the addition of ice to the water bath. At 15°C ribonuclease activity is nil and thus doesn't interfere with the measurement of polymerase activity.

The reactions were terminated by the addition of 1 ml of ice cold 10% trichloroacetic acid (w/v). Samples were then centrifuged at 1000 x g for 10 minutes in a Sorvall GLC-1 tabletop centrifuge equipped with a type HL-4 rotor. The supernatant was decanted and discarded. The pellet was washed twice by resuspending in 2 ml of ice cold
5% TCA (w/v) - 1% tetrasodium pyrophosphate (Na₄P₂O₇; w/v) with a vortex mixer. After the final centrifugation, pellets were suspended in 2 ml of 5% TCA-1% Na₄P₂O₇ by sonication and were then filtered on a Millipore filter apparatus equipped with a glass fiber filter (pore size = 0.45 μm). Suspensions were drawn through the filter by a vacuum attachment, trapping RNA and DNA on the filter. Filters were washed twice with 2 ml aliquots of toluene to remove TCA. Filters were removed from the filtration apparatus and air dried for 30 minutes on an absorbent pad. When completely dry, the filters were added to 10 ml of the toluene-PPO-POPOP cocktail described above. Radioactivity was measured as above and efficiency was approximately 30%.

To improve the reproducibility of duplicate assays, DNA coprecipitated with the RNA was measured. This was done as follows.

Filters were removed from the liquid scintillation counting vials and again allowed to air dry for at least 30 minutes on absorbent pads. Filters were then placed in 12 x 75 mm test tubes and 2 ml of 10% perchloric acid (v/v) added to the tubes. The samples were then placed in a hot water bath at 90°C for 30 minutes to solubilize the DNA from the filters. The hydrolysates were decanted and DNA content was measured.

DNA was quantitated by the diphenylamine method of Burton (1956) as modified by Giles and Myers (1965). This entailed adding 2 ml of a 4% diphenylamine solution (in glacial acetic acid) to the 2 ml of the DNA sample solution. A 0.1 ml aliquot of a 16 mg/100 ml acetaldehyde solution was added to the mixture and the samples were allowed to stand overnight at 30°C. A blue color developed and absorbance at 595 nm was measured.
in a spectrophotometer. Final data for RNA polymerase activity was then expressed as DPM $^3$H-UTP incorporated/µg DNA, then converted to percentage of control values.

E. ATPase Activity in the Nuclear Membrane

The activity of Mg$^{2+}$-dependent adenosine triphosphatase (ATPase) in the nuclear membrane was measured by the determination of inorganic phosphate formed in the reaction. Nuclei were prepared as above with no modifications of the STM buffers. Cytosol referred to in these experiments is the 100,000 x g supernatant, 60 minutes, in a Beckman L265B ultracentrifuge equipped with a SW27 rotor.

The incubation mixture for activity determination was that described by Franke et al., (1970). The mixture contains:

- 20 mM Tris-HCl pH 7.5
- 100 mM NaCl
- 10 mM KCl
- 5 mM MgCl$_2$
- 5 mM ATP (buffered with Tris to pH 7.0)
- 2.5 mM dithiothreitol

Various drugs and cytosol were added to the incubations. In each case, distilled water was added to bring the final volume to 1.5 ml.

The reaction was started by the addition of ATP and incubated for 30 minutes at 37°C in a Dubnoff metabolic shaker. The reaction was terminated by pipetting 100 µl of the reaction mixture into a test tube containing silicocotungstic acid- H$_2$SO$_4$-Isobutanol:Benzene (prepared as described below). The latter step also prepared the samples for the determination of inorganic phosphate.
The ATPase reaction was shown to be linear with both time of incubation and concentration of the enzyme preparation (Figure 4).

Inorganic phosphate was determined by the method of Berenblum and Chain (1938) as modified by Martin and Doty (1949) and described by Lindberg and Ernster (1956). The method was chosen because it can be used in the presence of a small amount (less than 0.2 mg) of protein.

As already stated above, 100 µl of the ATPase reaction mixture was pipetted into a 12 ml conical centrifuge tube containing the following:

- 0.4 ml 5 M H₂SO₄
- 1.0 ml Silicotungstic acid
- 3.0 ml distilled water
- 5.0 ml Isobutanol:Benzene (1:1)

To this mix was added 0.5 ml 10% ammonium molybdate (w/v). The tubes were capped and shaken vigorously by hand for 15 seconds. A faint yellow color could often be seen in the organic phase. This indicated the extraction of the phospho-molybdate complex. Next, 2.5 ml of the organic phase was removed from the centrifuge tube and mixed with 2.5 ml of 3.2% H₂SO₄ (in absolute ethanol) in a small test tube. 0.5 ml of 10% stannous chloride (w/v in concentrated HCl) was added to give a blue color. The absorbance of the samples was determined at 730 nm. The assay was linear for concentrations of inorganic phosphate between 0.2 and 10 mM (Figure 5).

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3 This reagent was made by dissolving 26.7 g Na₂SiO₃·9H₂O (sodium metasilicate) and 397 g Na₂WO₄·2H₂O (sodium tungstate in 2 liters of distilled water. 75 ml of concentrated H₂SO₄ was added and the solution was refluxed for 5 hours. After cooling the solution was filtered through Whatman #40 filter paper and diluted two-fold prior to use.
ATPase: DEPENDENCE ON TIME OF INCUBATION AND ENZYME CONCENTRATION

ATPase activity in isolated nuclei was measured as described in Methods. The dependence of the reaction on time of incubation (A) and protein concentration (B) was determined. Results are expressed as μmoles inorganic phosphate (P$_i$) formed per hour per mg protein. Each point represents the mean of triplicate determinations. Vertical bars represent the standard error of the mean.
FIGURE 4

ATPase: DEPENDENCE ON TIME OF INCUBATION AND ENZYME CONCENTRATION
FIGURE 5

INORGANIC PHOSPHATE STANDARD CURVE

Inorganic phosphate standards (0-10 mM) were made up by dissolving Na$_3$PO$_4$ in distilled water. 100 µl aliquots of each standard were assayed as described in Methods. Each point represents the mean of triplicate determinations.
FIGURE 5

INORGANIC PHOSPHATE STANDARD CURVE
F. Incorporation of $^3$H-Leucine into Protein

Animals were administered 20 µCi of $^3$H-leucine (110 Ci/mmol, New England Nuclear) intraperitoneally. In all cases a 30 minute pulse labelling period was used. At the end of this time, rats were sacrificed by decapitation and subcellular fractions were prepared as described above. Cytoplasm in this case represents a 100,000 x g, 60 min, supernatant. Nuclei were sonicated prior to further treatment.

Aliquots of 3 ml of cytoplasm or nuclei were mixed with 3 ml of 10% TCA (w/v) to precipitate proteins. The samples were then centrifuged at 1,000 x g for 5 minutes in a Sorvall GLC-1 tabletop centrifuge equipped with an HL-4 rotor. The supernatant was decanted and the pellet was dissolved in 1 N NaOH. Samples were dissolved more quickly if they were sonicated. 1.0 ml aliquots of the protein solutions in NaOH were added to Combusto-cones (Packard) and allowed to air dry overnight. On the following day, the samples were oxidized in a Packard Tri-Carb Model #306 Sample Oxidizer. This instrument traps all the tritium label as $^3$H₂O and automatically adds it to a liquid scintillation cocktail, in this case, Monophase 40 (Packard). Samples were then counted in a Packard Tri-Carb Model #3255 Liquid Scintillation Spectrometer at 30-35% counting efficiency as determined by external standardization.

Since it was desired to express final results as DPM $^3$H-leucine incorporated per mg protein, protein concentrations of the NaOH-protein solutions were estimated by the method of Bradford (1976).
G. *Cell-free System for Studying the In Vitro Transport of Ornithine Decarboxylase*

The method for measurement of in vitro transport of macromolecules was that of Schumm and Webb (1972). Though the method was originally designed to measure the active transport of RNA out of the nucleus, the mechanisms for nuclear membrane transport of any type of macromolecule might be similar enough to apply similar methods of measurement. The method was modified slightly and used as follows.

Incubation mixtures contained:

- 50 mM Tris-HCl pH 7.5
- 25 mM KCl
- 2.5 mM MgCl₂
- 0.5 mM CaCl₂
- 0.3 mM MgCl₂
- 5.0 mM NaCl
- 2.5 mM Na₂PO₄
- 5.0 mM Spermidine (3HCl, Sigma)
- 2.0 mM Dithiothreitol
- 2.0 mM ATP
- 0.3 mM Pyridoxal phosphate
- 2.5 mM Phosphoenolpyruvate (KCl, Sigma)
- 35 Units Pyruvate Kinase (456 units/mg protein, Sigma)

These reagents were prepared prior to the day of the experiments and kept frozen at -20°C.

The incubations also contained 3 ml of cytoplasm prepared as described above (100,000 x g supernatant) and 1 ml of nuclei in suspension, also prepared as above. These amounts were equivalent to 0.5 g and 2.0 g liver wet weight for cytoplasm and nuclei, respectively. It is important to note here that the cytoplasm and nuclei were both obtained from untreated animals. Incubations were in a total volume of 5.0 ml.
Amounts of each drug to be added \textit{in vitro} were calculated on the assumption that the entire intraperitoneal dose would be distributed to the liver. Approximately one fifth of this maximum dose was then added to the incubations. The determination of the proper dose of MIX is used as an example of these calculations.

The \textit{in vivo} dose of MIX was 10 mg/kg. Therefore, 1 mg of MIX was given to a 100 g rat which had a liver weighing approximately 4 g. If all the drug was distributed to the liver, the final maximum concentration of MIX was 0.25 mg MIX/g liver. Nuclei equivalent to 2 g of liver was added to each incubation so that 0.5 mg of MIX had to be added to each incubation. This 0.5 mg would be in a total volume of 5.0 ml so that the final concentration \textit{in vitro} would be 450 \mu M. A final concentration of 100 \mu M (10^{-4} M) was arbitrarily chosen for addition to the incubations from these calculations. Similar calculations and reasoning also determined the dose of cAMP which was added to incubations in other experiments.

The incubations were carried out in 50 ml Erlenmeyer flasks open to the air. Temperature was maintained at 37°C and flasks were shaken in a Dubnoff metabolic shaker. Nuclear suspensions were the last addition to the flasks to initiate transport determinations.

At selected times after nuclear suspensions were added, the reactions were terminated by pouring the contents of the flasks into 20 ml of ice cold 2.3 M STM buffer in 50 ml tissue grinders. Presumably if the transport was energy and temperature dependent, it would be nil at 0°C (Schumm and Webb, 1972). Incubation mixtures were then suspended
in the 2.3 M STM buffer by gentle homogenization (2 strokes at low speed) with a teflon pestle. These suspensions were then centrifuged at 40,000 x g for 60 minutes in a Sorvall RC-2B centrifuge to reisolate nuclei. The nuclear pellet was resuspended in 2 ml of 0.25 M STM buffer as described above. Ornithine decarboxylase activity and protein concentrations were also determined as described above.

H. Protein Determination

The method of Bradford (1976) was used for the quantitative estimation of protein concentrations in all experiments. This method was chosen because it could be used in the presence of chemical agents which interfered with the method of Lowry et al., (1952). These agents included Tris buffer, dithiothreitol, and EDTA, all of which were components of the homogenizing and suspending buffers.

The method of Bradford utilizes binding of Coomasie Brilliant Blue G-250 (Sigma) to primary amino groups in proteins. Upon dye-binding there is a change in absorbance at 595 nm which is quantitatively measured in a Beckman Model #26 dual beam spectrophotometer. The assay is linear up to 50 µg/ml, using bovine serum albumin as a standard. All samples were diluted (usually 200-400 fold) to bring them within the linear range of the assay.

I. Time Course of Investigations

Ornithine decarboxylase is a very labile enzyme. After drug stimulation its activity rises to maximum very rapidly (4 hours) and
declines to control levels by 6 hours, in most cases. Therefore, the time course used in this study is usually 4-6 hours. Because both ODC (Hayashi, 1972) and the RNA polymerases (Glass and Spelsberg, 1972) have diurnal variations all experiments were begun at the same time daily. For convenience, initial injections were given between 9:00 and 10:00 AM and rats sacrificed at hourly intervals afterwards. The time course for single drug experiments was relatively straight forward. In experiments in which 3-isobutyl-1-methylxanthine (MIX) and cycloheximide (CH) were given in combination, the schedule was as follows:

(A)

\[
\begin{array}{cccccccc}
CH & MIX & X & X & X & X & X & X \\
-0.5 & 0 & 1 & 2 & 3 & 4 & 5 & 6 \\
\end{array}
\]

TREATMENT TIME (HR)

\(X = \text{SACRIFICE TIME}\)

Schedule A indicates that cycloheximide was given 30 minutes (0.5 HR) prior to MIX administration. Preliminary experiments (Figure 6) showed that 30 minutes was the time at which maximal inhibition of protein synthesis was reached. It was desired that MIX be given at a time following cycloheximide so that the induction of enzyme activity could not be due to de novo protein synthesis.
In experiments in which injection of $^3$H or $^{14}$C-leucine was used, the time course was as follows:

B)

\[
\begin{array}{cccccccc}
\text{CH} & \text{MIX} & \text{LEU} & \text{X} & \text{LEU} & \text{X} & \text{LEU} & \text{X} & \text{LEU} & \text{X} \\
-0.5 & 0 & 1 & 2 & 3 & 4 & 5 & 6 \\
\end{array}
\]

TREATMENT TIME (HR)

LEU = $^3$H- or $^{14}$C-LEUCINE

X = SACRIFICE TIMES

Schedule B was planned so that the 30 minute pulse label of $^3$H- or $^{14}$C-leucine encompassed the time point of interest. Control animals in all experiments were normally sacrificed at zero time or along with the 1 hour animals.

J. Drugs

The 3-Isobutyl-1-methylxanthine (MIX) was obtained from Aldrich Chemical Co. It was necessary to dissolve this drug in 20% ethanol (v/v). This ethanol concentration has been shown to have no effect on ODC activity (Manen and Russell, 1975). The dose of MIX was 10 mg/kg.

The N$^6$O$^2$'-Dibutyryl adenosine-3':5'-cyclic monophosphoric acid (dibutyryl cAMP) was obtained from Sigma Chemical Co. A dose of 3 mg/100 g body weight was given. This dose gave maximal stimulation of cytoplasmic ODC activity (Beck et al., 1972).
Cycloheximide (Actidione) was obtained from Upjohn Co. It was dissolved in distilled water for injection. The dose was 10 mg/kg, a dose determined in preliminary experiments to give a marked and sustained inhibition of protein synthesis (Figure 5).

Methylglyoxal bis(guanylhydrazone)(MBGB) was obtained from Aldrich Chemical Co. The drug was dissolved in 20% ethanol either alone or in combination with MIX, when the two drugs were administered to the same animal. A dose of 80 mg/kg was used. This dose was shown previously to effectively inhibit polyamine biosynthesis (Pegg, 1973).

Thioacetamide was obtained from Sigma Chemical Co. A dose of 150 mg/kg was administered intraperitoneally, dissolved in distilled water. This dose was shown previously to give maximum stimulation of cytoplasmic ODC (Fausto, 1969).

The 3-Methylcholanthrene was obtained from Mann Research Laboratories. It was administered at a dose of 80 mg/kg as a suspension in corn oil. This dose was previously found to significantly stimulate cytoplasmic ODC (Russell, 1971).

Aminophylline was a product of Elkins-Sinn, Inc. and was obtained in an injectable form from the University Hospital Pharmacy. The drug was used at a dose of 200 μmoles/kg or approximately 85 mg/kg (Byus and Russell, 1974).

All drug solutions were prepared so that each rat would receive between 0.5 and 0.75 ml per injection.
Cycloheximide (1, 5, and 20 mg/kg) was administered intraperitoneally at 0 hour (control). At the time points shown on the graph (thirty minutes prior to sacrifice), rats were given $^{14}$C-leucine (10 µCi/100 g body weight; 315 mCi/m mole), intraperitoneally. After the thirty minute pulse of radioactivity, animals were sacrificed, livers were perfused with 10 ml of 0.25 M STM buffer (Methods), excised, blotted and weighed. The livers were then homogenized in 10 volumes (1 g/10 ml) of 0.25 M STM buffer in a glass tissue grinder with a teflon pestle. Proteins were precipitated with equal volumes of 10% trichloroacetic acid. Samples were centrifuged and the resulting pellet was redissolved in 1 N NaOH. Aliquots of these protein solutions were then oxidized in a Packard Tri-Carb Sample Oxidizer Model #306 to prepare them for liquid scintillation counting. Protein concentration of each sample was determined and results were expressed as DPM $^{14}$C-leucine/mg protein. Final data were expressed as percent of control.
FIGURE 6

THE EFFECT OF CYCLOHEXIMIDE ON THE INCORPORATION OF

\(^{14}\text{C-LEUCINE INTO RAT LIVER PROTEIN}\)
RESULTS

Effects of 3-Isobutyl-1-methylxanthine and Cycloheximide on Ornithine Decarboxylase in the Cytoplasmic and Nuclear Compartments

It was stated above that ornithine decarboxylase activity can be stimulated by a variety of agents (Morris and Fillingame, 1975). One particularly potent class of agents are the methylxanthines (Byus and Russell, 1974; Beck et al., 1972), the most powerful of these being 3-isobutyl-1-methylxanthine (Manen and Russell, 1975). The latter agent was chosen for these studies due to its simultaneous stimulation of both ODC and RNA polymerase activity (Manen and Russell, 1975).

Figure 7 shows the effects of 3-isobutyl-1-methylxanthine (MIX; 10 mg/kg, ip) and cycloheximide on cytoplasmic ornithine decarboxylase (ODC) activity. MIX caused a rapid induction of ODC with maximal activity being attained at 4 hours after administration. By 4 hours ODC activity was 50-75 times the zero hour control value.

If cycloheximide (10 mg/kg, ip) was given 30 minutes prior to MIX the rapid induction of ODC was totally abolished. The results suggested that the rapid increase in ODC was due to new protein synthesis.
FIGURE 7

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE (MIX) AND CYCLOHEXIMIDE (CH) ON CYTOPLASMIC ORNITHINE DECARBOXYLASE ACTIVITY

All rats receiving combined treatments were pretreated for 30 minutes with cycloheximide (10 mg/kg, ip) before receiving MIX (10 mg/kg, ip). Animals receiving MIX alone were injected at 0 hour (9:00-10:00 AM). Animals were sacrificed and cytoplasmic ornithine decarboxylase activity was determined at the times indicated. See Methods for ODC assay. Each point represents the mean of 4-6 animals. Vertical bars are the standard error of the mean.
FIGURE 7

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE (MIX) AND CYCLOHEXIMIDE (CH) ON CYTOPLASMIC ORNITHINE DECARBOXYLASE ACTIVITY
FIGURE 8

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE (MIX) AND CYCLOHEXIMIDE (CH)
ON NUCLEAR ORNITHINE DECARBOXYLASE ACTIVITY

Nuclei were prepared from rats used in Figure 7. Nuclear ornithine decarboxylase activity was determined at the times indicated. See Methods for ODC activity. Each point represents the mean of 4-6 animals. Vertical bars represent the standard error of the mean.
FIGURE 8

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE (MIX) AND CYCLOHEXIMIDE (CH)

ON NUCLEAR ORNITHINE DECARBOXYLASE ACTIVITY
Figure 8 shows the effects of MIX and cycloheximide on nuclear ODC activity. The nuclei for these experiments were obtained from the same rats used in Figure 7.

The administration of MIX alone (10 mg/kg, ip) caused an increase in nuclear ODC activity with maximal activity being attained at 3 hours after drug treatment. If cycloheximide (10 mg/kg, ip) was given 30 minutes prior to MIX, ODC activity increased in a biphasic manner with maximal activities being attained at 1 and 3 hours. It is very important to realize that the increased nuclear ODC activity occurred at the same time that cytoplasmic ODC activity was unchanged. It is also important to note that the administration of cycloheximide alone had no effect on nuclear ODC, ODC activity being maintained, for the most part, at basal levels. These results indicated that the increased nuclear ODC activity was due to an effect of MIX acting alone.

The data presented in Figures 7 and 8 suggest that MIX directly effects the transport of ODC from the cytoplasmic to the nuclear compartment. The following experiments were done to more closely examine this possibility.

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4 The word "transport" will be used throughout the text to denote a movement of molecules from one subcellular location to another without regards to the mechanism of movement, except where a mechanism is specifically stated.
Effects of 3-Isobutyl-1-methylxanthine and Cycloheximide on the
Incorporation of $^3$H-Leucine into Cytoplasmic and Nuclear Proteins.

It was possible that the increased nuclear ODC activity obtained after the combined treatment with MIX and cycloheximide could have been due to synthesis of ODC by the nucleus by a protein synthetic mechanism different from that found in the cytoplasm; a mechanism resistant to cycloheximide inhibition. Though most investigators discount the likelihood of nuclear protein synthesis (Goldstein, 1970), there are those who have claimed to have demonstrated such a phenomenon (Kuehl, 1967; Zimmerman et al., 1969). To test the possibility that nuclear protein synthesis occurs, the labelling study described in Table 1 was done.

In Table 1 it can be seen that MIX treatment alone increased the labelling of protein pools in both the cytoplasmic and nuclear compartments. Cytoplasmic labelling was increased to 109% (at 4 hours) of untreated controls and nuclear labelling was increased to 140% (at 4 hours) of untreated controls (Column A; MIX). The difference in increases between the compartments (109% in cytoplasm vs. 140% in nucleus) is probably accounted for by the much larger protein pool size present in the cytoplasm (Siekevitz, 1952).

The combination of cycloheximide and MIX caused inhibition of $^3$H-leucine incorporation in both cytoplasmic and nuclear compartments as can be seen in Column B (MIX + CH) of Table 1. Incorporation of the label into cytoplasmic and nuclear protein was inhibited greater than
### TABLE I

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE (MIX) AND CYCLOHEXIMIDE (CH) ON THE INCORPORATION OF $^3$H-LEUCINE INTO PROTEINS

#### I. CYTOPLASM

<table>
<thead>
<tr>
<th>Treatment Time</th>
<th>A. MIX</th>
<th>B. MIX + CH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 HOUR (Control)</td>
<td>DPM/mg protein</td>
<td>% Control</td>
</tr>
<tr>
<td>1 HOUR</td>
<td>2433</td>
<td>100</td>
</tr>
<tr>
<td>2 HOUR</td>
<td>2141</td>
<td>88</td>
</tr>
<tr>
<td>3 HOUR</td>
<td>2117</td>
<td>87</td>
</tr>
<tr>
<td>4 HOUR</td>
<td>2603</td>
<td>107</td>
</tr>
<tr>
<td>4 HOUR</td>
<td>2652</td>
<td>109</td>
</tr>
</tbody>
</table>

#### II. NUCLEUS

<table>
<thead>
<tr>
<th>Treatment Time</th>
<th>A. MIX</th>
<th>B. MIX + CH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 HOUR (Control)</td>
<td>DPM/mg protein</td>
<td>% Control</td>
</tr>
<tr>
<td>1 HOUR</td>
<td>2061</td>
<td>100</td>
</tr>
<tr>
<td>2 HOUR</td>
<td>1752</td>
<td>85</td>
</tr>
<tr>
<td>3 HOUR</td>
<td>2046</td>
<td>99</td>
</tr>
<tr>
<td>4 HOUR</td>
<td>2123</td>
<td>103</td>
</tr>
<tr>
<td>4 HOUR</td>
<td>2885</td>
<td>140</td>
</tr>
</tbody>
</table>

Rats receiving MIX (10 mg/kg, ip) alone were injected at 0 hour. Rats receiving MIX and CH (10 mg/kg, ip) were pretreated for 30 minutes with CH before receiving MIX. $^3$H-Leucine (20 μCi/rat; 110 Ci/mmole) was administered, as a 30 minute pulse, 15 minutes before the hour indicated and the animals were sacrificed 15 minutes after the hour so that the pulse period encompassed the time point of interest. Liver fractions were oxidized and radioactivity was determined as described in Methods. Column A and Column B represent different experiments. The values expressed as DPM/mg protein represent the mean of 3 animals per time point. Individual determinations did not vary by more than 10% from the mean.
90%. The inhibition was well-sustained over the four hour time course (the same time course in which nuclear ODC activity was increased by combined MIX and cycloheximide treatment).

These data appeared to rule out the possibility that ODC was synthesized within the nucleus by a cycloheximide-resistant mechanism. The data also supported the hypothesis that MIX stimulated a nuclear membrane transport system which was responsible for the transport of proteins into the nucleus.

Effects of Dibutyryl Cyclic AMP and Cycloheximide on Ornithine Decarboxylase Activity in the Cytoplasm and Nucleus

MIX was shown previously to be a potent inhibitor of cAMP phosphodiesterase (Beavo et al., 1970). It is accepted that at least some of the effects of methylxanthine derivatives are due to the inhibition of this phosphodiesterase and the consequent increase in intracellular cAMP concentrations (Weiss and Fertel, 1977). Therefore, the possibility that the observed effects of MIX on the transport of ODC into the nucleus were mediated by cAMP was examined.

In rats treated with dibutyryl cAMP (30 mg/kg, ip) there was a rapid rise in cytoplastic ODC to a maximum at 2 hours (Table 2). The maximum ODC activity attained was approximately 9 times that found in untreated controls. The administration of cycloheximide (10 mg/kg, ip), 30 minutes prior to dibutyryl cAMP, completely abolished the rise in cytoplastic ODC activity.
### TABLE 2
EFFECTS OF DIBUTYRYL cAMP AND CYCLOHEXIMIDE (CH) ON CYTOPLASMIC AND NUCLEAR ORNITHINE DECARBOXYLASE ACTIVITY

<table>
<thead>
<tr>
<th>I. CYTOPLASM</th>
<th>Ornithine Decarboxylase</th>
<th>Dibutyryl cAMP</th>
<th>Dibutyryl cAMP + CH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment Time</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 HOUR (Control)</td>
<td>1.47 ± 0.30</td>
<td>1.07 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>1 HOUR</td>
<td>1.33 ± 0.31</td>
<td>0.26 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>2 HOUR</td>
<td>13.05 ± 2.51</td>
<td>0.19 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>3 HOUR</td>
<td>4.46 ± 1.13</td>
<td>0.28 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>4 HOUR</td>
<td>1.05 ± 0.12</td>
<td>0.75 ± 0.16</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. NUCLEUS</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.08 ± 0.13</td>
<td>2.20 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>1 HOUR</td>
<td>1.79 ± 0.13</td>
<td>2.45 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>2 HOUR</td>
<td>2.07 ± 0.10</td>
<td>2.38 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>3 HOUR</td>
<td>2.08 ± 0.28</td>
<td>2.10 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>4 HOUR</td>
<td>1.93 ± 0.26</td>
<td>2.25 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

Rats were given dibutyryl cAMP (30 mg/kg, ip) at 0 hour (9:00-10:00 AM). Rats receiving combined drug treatments were pretreated for 30 minutes with CH (10 mg/kg, ip). Animals were sacrificed and ODC activity was determined at the times indicated. See Methods for ODC measurement. Data are expressed as pmoles $^{14}$CO$_2$/hr/mg protein and represent the mean of duplicate determinations on at least 4 animals ± S.E.M.
Table 2 also shows the effects of dibutyryl cAMP and cycloheximide on nuclear ODC activity. Neither dibutyryl cAMP alone nor the combination of dibutyryl cAMP and cycloheximide had any effect on nuclear ODC activity. Even when a dose of 150 mg/kg of dibutyryl cAMP was given, no significant increase in nuclear ODC could be demonstrated. Nuclear ODC activity rose to approximately 150% of untreated control but this rise was found to be not statistically significant. The high dose of dibutyryl cAMP did not cause greater stimulation of cytoplasmic ODC than did the lower dose.

From these experiments, it was concluded that the effect of MIX on nuclear ODC activity was independent of intracellular cAMP. The following studies will further support this conclusion.

**A Cell-Free System for Studying Nuclear Membrane Transport In Vitro Effects of Various Drug Treatments**

It was desirable to test the effect of drugs on the nucleocytoplasmic transport of ODC *in vitro* so that interferences encountered with any *in vivo* experiment could be avoided. When designing this experiment there was no proven method available to specifically study the movement of proteins into the rat liver cell nucleus. However, there was a cell-free system described which had been used extensively to study transport of RNA molecules out of the nucleus (Schumm and Webb, 1972). It was felt that this system might be useful to study the transport of proteins also. The cell-free transport system was used as described in the Methods section.

Figure 9 shows the results of the *in vitro* transport experiments. The addition of $10^{-4}$ M MIX to the incubations caused an increase in ODC
FIGURE 9

EFFECTS OF MIX AND CYCLIC AMP ON THE IN VITRO TRANSPORT OF ORNITHINE DECARBOXYLASE

Incubations are carried out according to Methods. When added, MIX and cAMP were present in the incubations at a final concentration of 0.1 mM. Where cytoplasm was omitted from the incubations, 0.25 M STM buffer was added to make the correct final volume. Each point represents the mean of at least two incubations.
FIGURE 9

EFFECTS OF MIX AND CYCLIC AMP ON THE IN VITRO TRANSPORT OF
ORNITHINE DECARBOXYLASE
activity in reisolated nuclei which was linear over a 20 minute time course. If MIX was omitted from the incubations, no increase in ODC activity could be observed. Also, the addition of $10^{-4}$ M cAMP to the incubations had only minimal effects on nuclear ODC activity, supporting the *in vivo* finding that dibutyryl cAMP could not increase nuclear ODC. The increase in nuclear ODC activity with MIX was dependent on the presence of cytoplasm.

These *in vitro* experiments confirmed the *in vivo* findings. It appeared that increased ODC activity in the nucleus was due to a migration or transport of ODC into the nucleus by a direct effect of MIX on nuclear membrane transport; the cytoplasm being required to show an effect.

One finding that was not resolved in these *in vitro* studies was the slow decrease in nuclear ODC activity after 20 minutes incubation (Figure 9). The data may suggest that migration of ODC was due to an energy dependent process and that the incubation system was depleted of ATP by 20 minutes time. The nuclei were then not able to retain the accumulated ODC, allowing it to leak back to the cytoplasm; this possibility has not been tested.

It must be made clear that the cell-free transport system has not been perfected and must still be considered in the developmental stage, but this should not negate these studies.

**Effects of Various Drugs on Nuclear Membrane ATPase Activity**

Yasuzumi et al. (1966) found that there was a Mg$^{2+}$-dependent ATPase in the nuclear membrane and that the highest concentration of the enzyme
was in or around the nuclear pore complexes. Since many investigators had proposed that the nucleopores are avenues of nucleocytoplasmic exchange, it is possible that its ATPase is involved in the active transport of macromolecules between the intracellular compartments. It was thus interesting to determine whether MIX had any effect on nuclear membrane ATPase activity.

Table 3 shows that none of the drug additions affected nuclear membrane ATPase. The assays included $10^{-4}$M concentrations of MIX and dibutyryl cAMP when these drugs were added separately or in combination. Assays were also carried out in the presence and absence of $10^{-4}$M ouabain to differentiate between effects on Na:K stimulated ATPase and the Mg$^{2+}$-dependent ATPase of the nuclear pore complex. Only a small increase in ATPase activity was observed in the absence of ouabain, showing minimal Na:K stimulated ATPase activity in the nuclear membrane; which is in agreement with earlier observations (Franke et al., 1970). The possibility that MIX needed some cytoplasmic factor(s) present to affect the ATPase activity was tested. It was found that the combination of MIX and cytoplasm also gave less ATPase activity than control. ATPase in the presence of cytoplasm alone was not different from no addition.

**Effects of 3-Isobutyl-1-methylxanthine and Cycloheximide on the Activity of RNA Polymerase I and II**

The data presented above suggested the hypothesis that ODC was transported into the nucleus from the cytoplasm under the influence of MIX. As yet the importance of this migration has not been approached.
TABLE 3

EFFECTS OF VARIOUS DRUGS ON NUCLEAR MEMBRANE ATPase ACTIVITY

<table>
<thead>
<tr>
<th>Addition</th>
<th>ATPase Activity (μmoles Pi/hr/mg protein)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.76 ± 0.17</td>
<td>9</td>
</tr>
<tr>
<td>MIX&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.75 ± 0.17</td>
<td>9</td>
</tr>
<tr>
<td>DBcAMP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.31 ± 0.38</td>
<td>3</td>
</tr>
<tr>
<td>MIX + DBcAMP</td>
<td>4.61 ± 0.24</td>
<td>3</td>
</tr>
<tr>
<td>MIX - Ouabain</td>
<td>5.26 ± 0.08</td>
<td>3</td>
</tr>
<tr>
<td>MIX + Cytoplasm&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.62 ± 0.34</td>
<td>6</td>
</tr>
<tr>
<td>Cytoplasm&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.76 ± 0.21</td>
<td>3</td>
</tr>
</tbody>
</table>

All nuclear preparations were obtained from untreated animals. ATPase activity was determined by the formation of inorganic phosphate (P<sub>i</sub>) as described in Methods. Values are the average of the indicated number of experiments (N) ± S.E.M.

<sup>a</sup> Ouabain is present in all incubations at 10<sup>-4</sup> M except where indicated.

<sup>b</sup> MIX is present at a final concentration of 10<sup>-4</sup> M.

<sup>c</sup> DBcAMP (dibutyryl cAMP) is present at a final concentration of 10<sup>-4</sup> M.

<sup>d</sup> 100 μl of a 100,000 x g supernatant was added here as cytoplasm.

<sup>e</sup> mg protein represents the concentration of nuclear protein.
Manen and Russell (1975) found that MIX increased RNA polymerase activity (both I and II) in parallel with the increase in ODC activity. They suggested that ODC was intimately involved in the stimulation of RNA polymerase activity.

Studies were designed to examine increases in nuclear ODC activity in relationship to RNA polymerase activities. The data in Figure 10 shows that MIX (10 mg/kg, ip) administration stimulated both RNA polymerase I and II. RNA polymerase I and II was stimulated to 175% (at 5 hours) and 155% (at 5 hours) that of untreated controls. Stimulation in these experiments was comparable in magnitude and time course to earlier work (Manen and Russell, 1975).

The results obtained with combined MIX and cycloheximide treatment were interesting in regard to the functional significance of ODC migration into the nucleus. In Figure 11 it can be seen that the combined drug treatment (MIX + cycloheximide) caused a rapid and profound decrease in the activities of both RNA polymerases. RNA polymerase I was inhibited to a greater extent than polymerase II (80% vs. 60% inhibition). It is very important to realize that the marked decrease in RNA polymerase activity occurred at a time when ODC activity was maximal in the nucleus (see Figure 8). If indeed ODC is a modulator of RNA polymerase I, then these results are not in support of such an event. In fact, these data dissociate the increase in ODC activity from changes in RNA polymerase activity. This is in contrast to the reports of Manen and Russell (1975, 1977) in which increased ODC was accompanied by increased RNA polymerase I and II activity.
FIGURE 10

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE (MIX) ON THE ACTIVITY OF RNA POLYMERASE I AND II

Animals received MIX (10 mg/kg, ip) at 0 hour (9:00-10:00 AM). Animals were sacrificed and RNA polymerase I and II were determined in isolated nuclei at the indicated times. See Methods for the determination of RNA polymerase activity. Results were calculated first as DPM $^3$H-UTP incorporated/10 min/μg DNA and then converted to percent of control values. Each point represents the mean of duplicate determinations on at least 3 animals ± S.E.M. Control activity for RNA Polymerase I was approximately 200 DPM $^3$H-UTP/μg DNA (12,000 DPM/assay) and for RNA polymerase II was approximately 1200 DPM $^3$H-UTP/μg DNA (60,000 DPM/assay).
FIGURE 10

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE (MIX) ON THE ACTIVITY OF RNA POLYMERASE I AND II
FIGURE 11

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE AND CYCLOHEXIMIDE ON THE ACTIVITY OF RNA POLYMERASE I AND II

Animals were pretreated for 30 minutes with cycloheximide (10 mg/kg, ip) before the administration of 3-isobutyl-1-methylxanthine (MIX; 10 mg/kg, ip) at 0 hour. Animals were sacrificed and RNA polymerase I and II activity were determined in isolated nuclei at the indicated times. See Methods for the determination of RNA polymerase activity. Results were calculated as DPM $^3$H-UTP incorporated/10 min/µg DNA and then converted to percent controls. Each point is the mean ± S.E.M. of duplicate determinations on nuclei from 3 animals. Control activity for polymerase I was approximately 200 DPM $^3$H-UTP/µg DNA (12,000 DPM/assay) and for polymerase II was approximately 1200 DPM $^3$H-UTP/µg DNA (60,000 DPM/assay).
FIGURE 11

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE AND CYCLOHEXIMIDE ON THE
ACTIVITY OF RNA POLYMERASE I AND II
Effects of Spermidine and Spermine on RNA Polymerase I and II

The data above is not consistent with the idea that ornithine decarboxylase is responsible in a direct manner for the stimulation of RNA polymerase activity after treatment of animals with 3-isobuty1-1-methylxanthine. An alternative idea might be that the increase in RNA polymerase activity after drug treatment is related to levels of the polyamines, putrescine, spermidine, and spermine in the nuclear compartment. The data of Figures 12 and 13 show the effects of spermidine and spermine on RNA polymerase I and II activity in nuclei obtained from untreated rats. It can be seen in Figure 12 that RNA polymerase I was maximally stimulated by the addition of 10 mM spermidine or spermine to the incubations. Lower concentrations of spermine (10^{-3} M) were somewhat inhibitory to RNA polymerase I. A comparison of the data in Figure 12 and in Figure 10 reveals that the maximal stimulation of RNA polymerase obtained in vivo after MIX is roughly comparable to the maximal stimulation obtained in vitro by the addition of either 10 mM spermidine or 10 mM spermine.

Much different results were obtained with RNA polymerase II (Figure 13). The addition of spermidine to RNA polymerase II reactions caused no significant changes in polymerase activity at any concentration tested (10^{-3} M to 10^{-7} M). Spermine, on the other hand, significantly stimulated RNA polymerase II activity when the polyamine concentration was between 10^{-4} M and 10^{-6} M. The maximum stimulation of RNA polymerase II activity in vitro by the polyamines was less than the maximum level of stimulation of the enzyme in vivo by the administration of MIX. Compare the data in
FIGURE 12

EFFECTS OF SPERMIDINE AND SPERMINE ON RNA POLYMERASE I ACTIVITY

Nuclei were isolated from untreated animals. RNA polymerase I activity was determined in the presence of spermidine or spermine. See Methods for determination of RNA polymerase I activity. The indicated concentrations of the polyamines are final molar concentrations in the incubation mixtures. RNA polymerase I activities are the means of 5 separate experiments at each concentration of polyamines. Vertical bars represent the standard errors of the means. Statistical significance, indicated by asterisks, was determined using Student's t-test, p < .05.
FIGURE 12

EFFECTS OF SPERMIDINE AND SPERMINE ON RNA POLYMERASE I ACTIVITY
FIGURE 13

EFFECTS OF SPERMIDINE AND SPERMINE ON RNA POLYMERASE II ACTIVITY

Nuclei were isolated from untreated animals. RNA polymerase II activity was determined in the presence of spermidine or spermine. See Methods for determination of RNA polymerase II activity. The indicated concentrations of the polyamines are final molar concentrations in the incubation mixtures. RNA polymerase II activities are the means of 5 separate experiments at each concentration of polyamines. Vertical bars represent the standard errors of the means. Statistical significance, indicated by asterisks, was determined using Student's t-test, p < .05.
FIGURE 13

EFFECTS OF SPERMINIDINE AND SPERMINE ON RNA POLYMERASE II ACTIVITY
Figure 12 to that in Figure 10. These results suggest that changes in intranuclear concentrations of spermidine and spermine could be responsible for the increased RNA polymerase activity after the administration of MIX.

Effects of 3-Isobutyl-1-methylxanthine and Methylglyoxal bis(guanylhydrazone) on RNA Polymerase I and II

Methylglyoxal bis(guanylhydrazone) (MGBG) is a potent inhibitor of S-adenosylmethionine decarboxylase (Williams-Ashman and Schenone, 1972), an essential enzyme in the biosynthesis of spermidine and spermine. Administration of MGBG in vivo causes inhibition of spermidine and spermine synthesis (Pegg, 1973). MGBG was used here as a tool to study the possibility that the increases in RNA polymerase activity after treatment with MIX were mediated by spermidine and/or spermine. The data from these experiments are presented in Figures 13 and 14.

As shown earlier (see Figure 10), MIX stimulated both RNA polymerase I and II. The increase in RNA polymerase activity in these experiments was similar to that obtained in the previous experiments (Figure 10).

If MGBG (80 mg/kg. ip) was administered along with MIX, the increase in RNA polymerase activity normally seen after MIX was abolished. In fact, RNA polymerase I was inhibited by 65% (Figure 14) and RNA polymerase II was inhibited by about 40% (Figure 15). It was significant that MGBG given alone had no effect on RNA polymerase I activity and only a slight (but not statistically significant) stimulatory effect on RNA polymerase II. This latter finding ruled out a direct inhibitory effect of MGBG on the RNA polymerase reaction. Thus, the MGBG reversal of the MIX stimulation of RNA polymerase activity appeared to be an indirect
FIGURE 14

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE (MIX) AND METHYLGlyoxal BIS(GUANYLHYDRAZONE) (MGBG) ON RNA POLYMERASE I ACTIVITY

MIX (10 mg/kg, ip) and MGBG (80 mg/kg, ip) were administered either separately or in combination at 0 hour (9:00-10:00 AM). Five hours later animals were sacrificed and RNA polymerase I activity was determined in isolated nuclei. See Methods for measurement of RNA polymerase I activity. Control animals were sacrificed at 0 hour. RNA polymerase I activity values represent the means of duplicate determinations on the indicated number of animals (number in parentheses) ± S.E.M. Statistical significance of the treatments, indicated by asterisks, was determined by Student's t-test, p < .05. Control polymerase I activity was approximately 30,000 DPM/assay.
FIGURE 14

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE (MIX) AND METHYGLYOXAL BIS(GUANYLHYDRAZONE) (MGBG) ON RNA POLYMERASE I ACTIVITY
FIGURE 15

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE (MIX) AND METHYLGLOXAL BIS(GUIANYLHYDRAZONE) (MGBG) ON RNA POLYMERASE I ACTIVITY

MIX (10 mg/kg, ip) and MGBG (80 mg/kg, ip) were administered either separately or in combination at 0 hour (9:00-10:00 AM). Five hours later animals were sacrificed and RNA polymerase II activity was determined in isolated nuclei. See Methods for measurement of RNA polymerase II activity. Control animals were sacrificed at 0 hour. RNA polymerase II activity values represent the means of duplicate determinations on the indicated number of animals (number in parentheses) ± S.E.M. Statistical significance of the treatments, indicated by asterisks, was determined by Student's t-test, p < .05. Control polymerase II activity was approximately 130,000 DPM/assay.
FIGURE 15

EFFECTS OF 3-ISOBUTYL-1-METHYLXANTHINE (MIX) AND METHYGLYOXAL BIS(GUANYLHYDRAZONE) (MGBG) ON RNA POLYMERASE II ACTIVITY
Effect of the drug and might have been due to inhibition of spermidine and/or spermine biosynthesis.

Effect of Aminophylline on Cytoplasmic and Nuclear Ornithine Decarboxylase Activity

The specificity of the effect of MIX on the migration of ODC into the nucleus was investigated. To examine the specificity of the response, a close structural analog of MIX, aminophylline or 1,3-dimethylxanthine, was utilized. Aminophylline was administered to rats at a dose of 200 μmoles/kg (approximately 85 mg/kg) and ODC activity in both cytoplasmic and nuclear compartments was determined. Cytoplasmic ODC activity increased rapidly to a maximum (20-30 times control) at 4 hours after drug treatment (Figure 16). Nuclear ODC activity remained unaffected by aminophylline at all time intervals studied (Figure 16). It would appear from these data that the MIX-induced migration of ODC was a very specific effect, a close structural analog to MIX being unable to mimic the effects of MIX.

Effect of 3-Methylcholanthrene on Cytoplasmic and Nuclear Ornithine Decarboxylase Activity

Studies utilizing 3-Methylcholanthrene (3-MC) were of interest for a variety of reasons. First, it has been shown previously that 3-MC increased ODC activity in rat liver (Russell, 1971). It was later suggested that the increase of ODC activity was a preliminary step in the stimulation of hepatic drug metabolizing enzymes by 3-MC (Byus et al., 1976). Since it had also been shown that 3-MC increased RNA polymerase activity (Gelboin et al., 1967; Bresnick, 1966), there might be a
FIGURE 16

EFFECTS OF AMINOPHYLLINE ON CYTOPLASMIC AND NUCLEAR
ORNITHINE DECARBOXYLASE ACTIVITY

Aminophylline (85 mg/kg, ip) was administered at 0 hour (9:00-10:00AM). At hourly intervals, animals were sacrificed and ornithine decarboxylase activity was determined in the cytoplasm and nucleus. Control animals were sacrificed at 0 hour. See Methods for ODC activity. Each point represents the mean of duplicate determinations on 4 animals ± S.E.M.
FIGURE 16

EFFECTS OF AMINOPHYLLINE ON CYTOPLASMIC AND NUCLEAR ORNITHINE DECARBOXYLASE ACTIVITY
TABLE 4

EFFECT OF 3-METHYLCALANTHRENE (3-MC) ON CYTOPLASMIC AND NUCLEAR ORNITHINE DECARBOXYLASE ACTIVITY

<table>
<thead>
<tr>
<th>I. CYTOPLASM</th>
<th>Ornithine Decarboxylase Activity (pmoles $^{14}$CO$_2$/hr/mg protein) % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment Time</td>
<td></td>
</tr>
<tr>
<td>0 HOUR (Control)</td>
<td>1.24 100</td>
</tr>
<tr>
<td>2 HOUR</td>
<td>1.40 113</td>
</tr>
<tr>
<td>3 HOUR</td>
<td>1.21 98</td>
</tr>
<tr>
<td>4 HOUR</td>
<td>1.60 129</td>
</tr>
<tr>
<td>5 HOUR</td>
<td>2.64 213</td>
</tr>
<tr>
<td>24 hr. CONTROL</td>
<td>0.99 100</td>
</tr>
<tr>
<td>24 hr. 3-MC</td>
<td>1.99 201</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. NUCLEI</th>
<th>Ornithine Decarboxylase Activity (pmoles $^{14}$CO$_2$/hr/mg protein) % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment Time</td>
<td></td>
</tr>
<tr>
<td>0 HOUR (Control)</td>
<td>0.62 100</td>
</tr>
<tr>
<td>2 HOUR</td>
<td>0.78 126</td>
</tr>
<tr>
<td>3 HOUR</td>
<td>0.71 115</td>
</tr>
<tr>
<td>4 HOUR</td>
<td>0.74 122</td>
</tr>
<tr>
<td>5 HOUR</td>
<td>0.75 122</td>
</tr>
<tr>
<td>24 hr. CONTROL</td>
<td>0.79 128</td>
</tr>
<tr>
<td>24 hr. 3-MC</td>
<td>0.44 56</td>
</tr>
</tbody>
</table>

All rats received 3-MC (80 mg/kg, ip), as a suspension in corn oil, at 0 hour (9:00-10:00AM). Animals were sacrificed and ODC activity was determined in cytoplasm and nuclei at the times indicated. See Methods for ODC activity. The 24 hour controls are rats treated for 24 hours with corn oil. Each enzyme activity value represents the mean of duplicate determinations on 2 animals.
relationship between changes in the subcellular distribution of ODC after 3-MC and the subsequent stimulation of RNA polymerase. That is, ODC might mediate the effects of 3-MC on nuclear function.

Table 4 shows the effects of 3-MC (80 mg/kg, ip) on ODC activity in nuclear and cytoplasmic compartments. At 5 hours after 3-MC, there was a two-fold rise in cytoplasmic ODC activity. There was, however, no significant change in nuclear ODC activity. At 24 hours after 3-MC treatment, cytoplasmic ODC activity was still 200% of untreated control animals. It was surprising to find that nuclear ODC activity was actually decreased to 56% of untreated controls at 24 hours. The changes in nuclear ODC activity showed no correlation with anticipated changes in RNA polymerase activity at 5 and 24 hours after 3-MC.

Effect of Thioacetamide on Ornithine Decarboxylase Activity and RNA Polymerase I and II

It was shown previously that thioacetamide, a liver carcinogen, caused stimulation of ODC activity in the cytoplasm as well as RNA synthesis in the nucleus (Fausto, 1970). No measurement was made, however, of nuclear ODC activity after thioacetamide. Thus, the relationship of intranuclear ODC activity to changes in RNA polymerase activity remained unknown.

Thioacetamide (150 mg/kg, ip) was administered to rats. ODC activity and RNA polymerase I and II activities were measured at selected times after drug treatment. The results of this study are shown in Tables 5 and 6. In Table 5 it can be seen that thioacetamide had a
## TABLE 5
EFFECT OF THIOACETAMIDE ON CYTOPLASMIC AND NUCLEAR ORNITHINE DECARBOXYLASE ACTIVITY

### I. CYTOPLASM

<table>
<thead>
<tr>
<th>Treatment Time</th>
<th>Ornithine Decarboxylase (pmoles $^{14}$CO$_2$/hr/mg protein)</th>
<th>Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Hour (Control)</td>
<td>0.73 ± 0.14</td>
<td>15-fold</td>
</tr>
<tr>
<td>8 Hour</td>
<td>10.60 ± 3.50</td>
<td></td>
</tr>
<tr>
<td>24 Hour</td>
<td>21.40 ± 0.98</td>
<td>30-fold</td>
</tr>
</tbody>
</table>

### II. NUCLEUS

<table>
<thead>
<tr>
<th>Treatment Time</th>
<th>Ornithine Decarboxylase (pmoles/hr/mg protein)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Hour (Control)</td>
<td>1.85 ± 0.11</td>
<td>100</td>
</tr>
<tr>
<td>8 Hour</td>
<td>1.12 ± 0.20</td>
<td>61</td>
</tr>
<tr>
<td>24 Hour</td>
<td>1.51 ± 0.25</td>
<td>81</td>
</tr>
</tbody>
</table>

Thioacetamide (150 mg/kg, ip) was administered at 0 hour (9:00-10:00 AM). Animals were sacrificed at the indicated times and ornithine decarboxylase activity was determined in cytoplasmic and nuclear fractions as described in Methods. Each value represents the mean ± S.E.M. for duplicate determinations on 4 animals.
<table>
<thead>
<tr>
<th>Table 6</th>
</tr>
</thead>
</table>

**EFFECT OF THIOACETAMIDE ON THE ACTIVITY OF RNA POLYMERASE I AND II**

**RNA POLYMERASE I**

<table>
<thead>
<tr>
<th></th>
<th>DPM $^3$H-UTP</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Hour (Control)</td>
<td>10,490</td>
<td>100</td>
</tr>
<tr>
<td>8 Hour</td>
<td>8,297</td>
<td>79</td>
</tr>
<tr>
<td>24 Hour</td>
<td>12,431</td>
<td>119</td>
</tr>
</tbody>
</table>

**RNA POLYMERASE II**

<table>
<thead>
<tr>
<th></th>
<th>DPM $^3$H-UTP</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Hour (Control)</td>
<td>41,029</td>
<td>100</td>
</tr>
<tr>
<td>8 Hour</td>
<td>30,656</td>
<td>75</td>
</tr>
<tr>
<td>24 Hour</td>
<td>43,278</td>
<td>105</td>
</tr>
</tbody>
</table>

Thioacetamide (150 mg/kg, ip) was administered at 0 hour (9:00-10:00 AM). Rats were sacrificed at the indicated times and RNA polymerase I and II activity was measured. Results are expressed as DPM $^3$H-UTP incorporated into RNA/10 min. Values represent the average of duplicate determinations on 4 animals.
powerful stimulatory effect on cytoplasmic ODC activity. The ODC activity rose to a level of 15 and 30 times control at 8 and 24 hours, respectively. The stimulation was similar to previously described changes in cytoplasmic ODC activity (Fausto, 1970). Nuclear ODC activity decreased to 61% of controls at 8 hours and remained depressed (81% of controls) at 24 hours (Table 5).

Table 6 shows the effects of thioacetamide on RNA polymerase I and II activity. RNA polymerase I decreased to 80% of controls at 8 hours but was slightly stimulated (120% of controls) at 24 hours. RNA polymerase II activity was also decreased at 8 hours, to 75% of control values. At 24 hours it had returned to control levels. These changes are consistent with those described by Smuckler and Koplitz (1974).

It is readily seen that there is no simple correlation between cytoplasmic ODC activity, nuclear ODC activity, and the changes in RNA polymerase activity in the nucleus after thioacetamide. Further, these data, taken with the 3-MC data (Table 4) and the aminophylline data (Figure 16) presented above, suggest that cytoplasmic ODC activity cannot be used as an indicator of nuclear events after drug treatments.
DISCUSSION

Ornithine decarboxylase (ODC) is the first enzyme in the polyamine biosynthetic pathway (Pegg and Williams-Ashman, 1968). A rapid increase in ODC activity is one of the earliest biochemical changes noted in cells stimulated to rapid growth or proliferation (Snyder and Russell, 1970). The enzymatic products of ODC and the other polyamine synthetic enzymes (putrescine, spermidine, and spermine) have dramatic effects on many cellular processes (Bachrach, 1973) and may, in fact, be essential growth factors (Ham, 1964) mediating changes in growth rates.

One very important role suggested for the polyamines was that of acting as regulators of ribonucleic acid (RNA) biosynthesis. It appeared that the increase in ODC activity that occurred during rapid growth processes served the purpose of increasing polyamines levels and, in turn, causing stimulation of RNA synthesis. However, it was recently observed that the addition of purified ODC to isolated nuclei caused a stimulation of RNA polymerase I activity (Manen and Russell, 1975). The stimulation was due to an increased rate of initiation of new RNA chains (Manen and Russell, 1977a) and therefore a new role was suggested for
ODC; that of being an initiation factor for RNA polymerase I. Other evidence for this proposal has been discussed in the Introduction.

The role of initiation factor for RNA polymerase I presupposes that ODC has access to the intranuclear compartment. There has been evidence presented that in some tissues (chick embryos) a large proportion of total ODC activity lies within the nucleus (Snyder et.al., 1970). However, this does not appear to be the case for normal rat liver (Snyder et.al., 1970) and may not be the case in chicks (Eloranta et.al., 1976). These latter findings may be important if ODC is to be an initiation factor for RNA polymerase I. The normally low level of ODC in the nucleus could be increased greatly after application of a proper growth stimulus and RNA polymerase I activity would be stimulated. If drugs which stimulate RNA polymerase I activity could also be shown to increase the intranuclear ODC activity, then it could be suggested that ODC mediates the effects of these agents on nuclear function. It is this latter possibility that was investigated.

Effects of 3-Isobutyl-1-methylxanthine and Cycloheximide on Ornithine Decarboxylase Activity in the Cytoplasm and Nucleus

Rats given 3-Isobutyl-1-methylxanthine (MIX; 10 mg/kg, ip) showed a rapid increase in liver ODC activity of the cytoplasmic compartment (Figure 7). Cytoplasmic ODC activity increased to 50-75 times the levels found in control animals with maximal activity being attained at 4 hours after treatment. The increase in cytoplasmic ODC activity was sensitive to cycloheximide (10 mg/kg, ip) inhibition. Cycloheximide, given 30 minutes prior to MIX, abolished the MIX-induced rise in ODC activity.
The inhibition of the MIX effect by cycloheximide is evidence that the increase in ODC activity after MIX results from the de novo synthesis of enzyme molecules. This conclusion is supported by the protein labelling study discussed below.

Nuclear ODC activity also rapidly increased after MIX administration (Figure 8). At 3 hours after MIX, ODC activity was 4-6 times control levels. The most interesting aspect of the MIX-induced increase in nuclear ODC activity is the failure of cycloheximide to abolish the effect. When cycloheximide was administered 30 minutes prior to MIX, a biphasic change in nuclear ODC activity occurred. Nuclear ODC increased to 4-5 times control levels at 1 hour after MIX, decreased somewhat at 2 hours, and then increased 8-9 fold at 3 hours. The increase in nuclear ODC activity was caused by MIX alone since the administration of cycloheximide alone had no effect on nuclear ODC activity.

The finding that nuclear ODC content is regulated independently of cytoplasmic ODC content was significant. It suggested, as one possible explanation, that ODC migrated from the cytoplasm into the nucleus under the influence of MIX. Migration of cytoplasmic proteins into the nucleus was previously shown to be stimulated by phenobarbital (Savage, 1976). The time course of stimulation was approximately the same as in the present studies, suggesting that the processes involved are similar.

Of course there are other possible mechanisms by which nuclear ODC activity could be increased. One of these entails the activation of ODC by MIX. This activation would presumably not be through a direct stimulation of ODC by MIX, since MIX has no direct stimulatory effect on ODC activity in vitro (Manen and Russell, 1975).
Activation might occur by MIX-induced dissociation of ODC from its antizyme in vivo. The ODC antizyme is a small (MW = 26,000) protein which binds to ODC and noncompetitively inhibits the enzyme (Fong et al., 1976). The antizyme has been shown to be present in normal rat liver nuclei, being bound to some subnuclear particle (Heller et al., 1977). MIX could increase nuclear ODC activity by releasing bound ODC from the subnuclear particles. It is also possible that the synthesis of the antizyme is more sensitive to inhibition by cycloheximide than is the synthesis of ODC as was suggested previously (Beck et al., 1973). The interaction of MIX with the antizyme has not been thoroughly investigated in this study but data obtained in the in vitro transport study described below rules out this type of activation as being involved in the MIX-induced stimulation of nuclear ODC activity.

The possibility that ODC was synthesized in the nucleus was also considered. MIX might stimulate nuclear ODC activity by causing the nucleus to synthesize more of the enzyme by a cycloheximide-resistant mechanism.

Effects of 3-Isobutyl-1-methylxanthine and Cycloheximide on the Incorporation of 3H-Leucine into Protein

Nuclear protein synthesis has been observed by some investigators (Kuehl, 1968; Zimmerman et al., 1969). Most cell biologists, however, do not agree that the nucleus synthesizes its own proteins (Goldstein, 1970). Therefore, all nuclear proteins originate in the cytoplasm.
MIX (10 mg/kg, ip) stimulated general protein synthesis in the rat liver. Incorporation of $^3$H-leucine into cytoplasmic proteins was increased to 109% of control and labelling of nuclear proteins was increased to 140% of control at 4 hours (Table 1). The larger increase found in the nuclear compartment is explained by considering the large differences in the total protein pool sizes between the cytoplasm and the nucleus. Because the total pool is larger in the cytoplasm the label is diluted much more than in the smaller nuclear protein pool. Similar results in amino acid incorporation studies were obtained by Siekevitz (1952). The rather large (40%) increase in the labelling of nuclear proteins suggests that MIX stimulated the synthesis of proteins and transport of these proteins into the nucleus in a nonspecific manner. ODC is, then, not the only cytoplasmic protein transported into the nucleus under the influence of MIX.

In Table 1 it can also be seen that cycloheximide inhibits protein synthesis in the presence of MIX. The labelling of both cytoplasmic and nuclear compartments with $^3$H-leucine is inhibited dramatically with cycloheximide. The 3-5% of control incorporation of $^3$H-leucine into protein that remains after cycloheximide is not sufficient to account for the large increases (8-9 fold) in nuclear ODC activity observed.

These data, then, indicate that increased nuclear ODC activity is not dependent on de novo synthesis of the enzyme since the synthesis of nuclear proteins is maximally inhibited when nuclear ODC activity is maximally stimulated after the combined MIX and cycloheximide treatment.

If these data are now taken in support of the conclusion that ODC molecules migrated from the cytoplasm to the nucleus after MIX, then the
migrating ODC molecules must have been synthesized prior to the administration of MIX since the migration continues in the absence of protein synthesis. The findings are consistent with experiments in nonmammalian organisms which demonstrated that preformed protein molecules, rather than newly synthesized proteins, migrated into the nucleus after the application of a gene-activating stimulus (Holt, 1970; Helmsing and Berendes, 1971; Merriam, 1969). The findings are also consistent with work done in mouse ascites cells which demonstrated that the transfer of cytoplasmic proteins into the nucleus could not be inhibited with cycloheximide (Kawashima et al., 1971).

Effects of Dibutyryl cAMP and Cycloheximide on Ornithine Decarboxylase Activity in the Cytoplasm and Nucleus

It was previously shown that MIX is a potent inhibitor of cAMP phosphodiesterase. The degree of inhibition of cAMP phosphodiesterase correlated well with the biological effects of MIX reported by Beavo et al. (1970). A close relationship between increased cAMP levels and increased ODC activity has also been demonstrated with respect to the mechanism by which methylxanthine derivatives induce ODC activity in the rat liver and other tissues (Byus and Russell, 1974; Byus et al., 1976; Byus et al., 1977; Costa et al., 1975). Therefore, the contribution of intracellular cAMP to the MIX induced changes in nuclear ODC activity was investigated.

Dibutyryl cAMP (30 mg/kg, ip) stimulated cytoplasmic ODC activity by about 9-fold. Cycloheximide administered 30 minutes prior to the
dibutyryl cAMP completely abolished this stimulation (Table 2). Again, as with the MIX induction of ODC, the stimulation of activity appeared to be due to increased synthesis of the ODC enzyme. It was observed that dibutyryl cAMP had no effect on nuclear ODC activity. Even at a dose 5 times that which gave maximal stimulation of cytoplasmic ODC, no change in nuclear ODC activity was noted. The combination of dibutyryl cAMP and cycloheximide also gave negative results when nuclear ODC activity was evaluated. These data suggest that the intracellular cAMP fluctuations that occur after MIX are not responsible for the migration of ODC into the nucleus.

The finding that dibutyryl cAMP had no effect on nuclear ODC activity was important because it (1) demonstrated conclusively that the cytoplasmic and nuclear ODC activities were regulated independently and (2) because it strengthened the proposal that MIX acted directly on a nuclear membrane transport process without the mediation of a "second messenger".

Effects of MIX and cAMP on the In Vitro Transport of Ornithine Decarboxylase from the Cytoplasm into the Nucleus

Any in vivo experiment is subject to factors beyond the control of the investigator. One of the problems that may be involved in the experiments already described is the possibility that the drugs being tested release hormones in the body. It has been shown that the methyl-xanthine derivative, aminophylline, releases catecholamines from both

\textsuperscript{5}Ibid.
the sympathetic nerve endings (Westfall and Fleming, 1968) and the adrenal medulla (Poisner, 1973). The methylxanthine derivatives also release growth hormone from the pituitary (Hertelendy et al., 1971). Eloranta et al. (1975) demonstrated that the stimulation of ODC activity by dibutyryl cAMP was mediated to some extent by the pituitary. It is probable that dibutyryl cAMP also releases growth hormone. The effect of growth hormone on hepatic ODC activity is well documented (Russell, 1969; Jänne, 1969). It is therefore possible that the effects seen in these studies may be mediated by these hormones.

Because of these possible actions which make interpretation of in vivo data tentative, it was desirable to test the effects of drugs in an in vitro system.

Evidence from other studies suggested that nucleocytoplasmic transport could be mediated by the nuclear pore complex (Feldherr, 1962). Both RNA (Stevens and Swift, 1966) and proteins (Paine and Feldherr, 1972) have been shown to utilize this route of entry into or exit from the nucleus. A cell-free system was designed to study the transport of RNA out of the nucleus, which presumably occurred through the nucleopores (Schumm and Webb, 1972). Since proteins supposedly use the same migration route as RNA, it was felt that the cell-free system might be useful to study the effects of drugs on the transport of proteins also. With slight modifications of the previously described methods, this was shown to be the case, at least for the migration of ODC into the nucleus.

As seen in Figure 9, MIX caused a linear increase in nuclear ODC activity up to 20 minutes of incubation time. Peak ODC activity attained
in the nucleus at 20 minutes was approximately 5 times that seen in nuclei incubated for only 2 minutes. This rise is similar in magnitude to the effect seen when MIX or MIX and cycloheximide treatments are used in vivo (see Figure 8). The 5-fold increase upon addition of MIX to the incubations becomes more remarkable when the fact that the cytosol added to the incubations is prepared from untreated rats is considered. In the added cytosol, ODC activity is at an absolute minimum, yet under the influence of MIX the enzyme migrates into the nucleus. This finding indicates a rather powerful drug effect.

The addition of $10^{-4}$ M cAMP to the incubations, either alone or with MIX, had no effect on the migration of ODC, confirming the in vivo data obtained from rats treated with dibutyryl cAMP. This finding suggests again that MIX acts directly to influence a nuclear membrane transport process.

The increased ODC activity was shown to be dependent on the presence of cytoplasm. This finding was taken as evidence that MIX does not activate ODC within the nucleus. If MIX acted by dissociating ODC from its antizyme in vivo, then the same effect should be seen in vitro (see above for discussion of the antizyme).

The finding that ODC activity decreased with increasing incubation time suggested that the process of transport might be an energy dependent process. The decreased ODC activity could be due to a lack of sufficient ATP in the incubation medium to continue transport of ODC through the membrane. The nuclei failed to retain ODC against a concentration gradient in the absence of ATP. Further investigation into the problem of energy dependence was not pursued.
The cell-free system may be used as a model system in which to study the transport of proteins other than ODC between the cytoplasm and the nucleus and the effects of various drugs on this type of transport.

**Measurement of ATPase Activity in the Nuclear Membrane**

The possibility that the migration of ODC into the nucleus is an energy dependent process, led to the study of nuclear membrane ATPase. Nuclear membrane ATPase is primarily concentrated in the nuclear pore complex, a location which has suggested its importance in nucleocytoplasmic exchange (Yasuzumi and Tsubo, 1966; Yasuzumi et al., 1967). The ATPase of the nucleopore was also implicated as being involved in nucleocytoplasmic exchange by the finding that transport of RNA out of the nucleus could be inhibited by beryllium nitrate, an inhibitor of the nuclear membrane phosphatase (Cutler et al., 1974).

Though MIX, cAMP, and a combination of the two drugs were tested in the ATPase incubations, no stimulation of the enzyme could be demonstrated. The addition of MIX along with cytosol was also tested to rule out the possibility that MIX combines with a cytoplasmic factor before stimulating ATPase and transport of ODC. This experiment was deemed necessary because of the finding that the transport of ODC into the nucleus in vitro was dependent on the presence of cytosol in the cell-free system. The results of this experiment were also negative.

It was concluded that although the migration of ODC into the nucleus and the retention of the enzyme therein appeared to be an energy dependent process, stimulation of transport was due to a mechanism other than
stimulation of the ATPase of the nuclear pore complex. It may be that MIX interacts with a structural portion of the pore complex, causing a conformational change which opens the pore and allows ODC transport.

A brief summary of the findings to this point may be helpful before discussing the significance of the transport of ODC to nuclear function. The major findings are:

1. 3-Isobutyl-1-methylxanthine effects the transport of ODC from the cytoplasm into the nucleus.

2. The transport is insensitive to cycloheximide and involves preformed protein molecules rather than newly synthesized molecules.

3. The drug effect on transport is a direct one not mediated by cAMP.

4. The transport may be an active process but MIX does not act by stimulating nuclear membrane ATPase.

The problem of significance of the transport process to nuclear function, in particular RNA polymerase activity, can now be examined.

The Effects of 3-Isobutyl-1-methylxanthine and Cycloheximide on the Activity of RNA Polymerase I and II

The experiments described above provide evidence that cytoplasmic ODC gains access to the intranuclear compartment upon treatment of animals with MIX. It was shown previously that MIX also can stimulate RNA polymerase activity (Manen and Russell, 1975). If ODC is of central importance in mediating the effects of MIX on RNA polymerase, then any increase in the nuclear ODC content should be accompanied by an increase in the activity of RNA polymerase I. This should happen in every case in which nuclear ODC is increased. However, in this study increases in
nuclear ODC activity have been shown to be dissociated from changes in RNA polymerase activity.

Treatment of animals with a combination of MIX and cycloheximide resulted in a biphasic stimulation of nuclear ODC activity. Maximal activity was obtained at 1 and 3 hours after drug treatment (Figure 8). Under these experimental conditions, however, both RNA polymerase I and RNA polymerase II are greatly inhibited. These data suggest that ODC does not play a central role in the regulation of nuclear RNA polymerase activity, at least not as an initiation factor which mediates the effects of MIX on the nucleus.

ODC is the first enzyme in the polyamine biosynthetic pathway, as already discussed above. It is possible that the significance of the induction of ODC activity by MIX lies with its enzymatic products, the polyamines, spermidine and spermine, which stimulate RNA polymerases in rat liver nuclei (Moruzzi et al., 1975). Transport of the polyamines through the nuclear membrane may be stimulated by MIX as is the transport of ODC. Once in the nucleus, the polyamines then stimulate RNA polymerase activity.

The hypothesis that the polyamines spermidine and spermine mediate the MIX effects was examined by utilizing a selective inhibitor of S-adenosylmethionine decarboxylase, methylglyoxal bis(guanylhydrazone), to inhibit the biosynthesis of the two polyamines. The results are discussed in the next sections. Experiments were also carried out to determine the effects of spermidine and spermine on RNA polymerase activity in isolated nuclei.
Effects of Spermidine and Spermine on the Activity of RNA Polymerase I and II

The finding that ornithine decarboxylase content of the nucleus increased at a time when RNA polymerase activity was significantly depressed (see Figures 7 and 10) suggested that a direct interaction between ODC and RNA polymerase I was not the mechanism by which MIX stimulated the latter enzyme activity. Therefore, another possible mechanism was studied. This involved the effects of the polyamines, spermidine and spermine on RNA polymerase activity in isolated nuclei.

It was shown that spermidine and spermine, both at a concentration of 10mM, were able to stimulate RNA polymerase I activity (Figure 11). The stimulated activity was approximately 160% of control activity for both polyamines. Lower concentrations of spermine (2 x 10^{-3} and 10^{-3} M) were inhibitory to RNA polymerase I. The dual effect of spermine at different concentrations suggested that fluctuations of its intracellular concentration may function to modulate RNA polymerase I activity. Manen and Russell (1977) had previously reported that spermidine and spermine levels in rat liver do not change after MIX. However, their work was only concerned with gross, whole liver changes. No work has been done to the present time on levels of polyamines in the nucleus and how these levels might change after drug treatments. Until these studies are done, it is impossible to rule out spermidine and spermine as regulators of RNA polymerase I activity after MIX administration.

The effects of the polyamines on RNA polymerase II activity showed a somewhat different pattern. Spermidine had no significant effect on
the activity of polymerase II. Spermine was stimulatory to RNA polymerase II at low concentrations (10⁻⁴ to 10⁻⁶ M).

It was concluded from these experiments that spermidine and/or spermine may function as regulators of RNA polymerase I and II and could mediate the effects of MIX in vivo on the activity of these nuclear enzymes. To further test this hypothesis, an inhibitor of spermidine and spermine biosynthesis was used in conjunction with MIX in vivo. It was hoped that the inhibitor, methylglyoxal bis(guanylhydrazone) (MGBG) could reverse the MIX-induced increase in RNA polymerase activity, thus further implicating the polyamines as mediators of MIX action.

Effects of 3-Isobutyl-1-methylxanthine and Methylglyoxal bis(guanylhydrazone) on the Activity of RNA Polymerase I and II

Methylglyoxal bis(guanylhydrazone) is a potent inhibitor of mammalian S-adenosylmethionine decarboxylase (Williams-Ashman and Schenone, 1972). A dose of 80 mg/kg of MGBG has been shown to inhibit (greater than 90% inhibition up to 6 hours after dosing) the synthesis of spermidine and spermine in vivo although basal levels of the two polyamines are not changed greatly (Pegg, 1973). MGBG was used in the present study to investigate whether polyamines may mediate the MIX-induced increase in RNA polymerase I activity.

A dose of 80 mg/kg, ip, of MGBG was administered to rats along with 10 mg/kg, ip, MIX. With this combined drug treatment the rise in RNA polymerase activity which normally follows MIX administration was abolished. In fact, both RNA polymerase I and II activities were
significantly reduced below control levels. The reversal of the MIX effect is made more important by the finding that MGBG given alone had no effect on RNA polymerase I and only a small stimulatory effect on RNA polymerase II (116% of control). The inhibition of polymerase activity is thus obviously an indirect effect. A possible explanation follows.

During the times that liver cells can be considered in a "resting" state, polyamines are at rather low levels. However, when a proper stimulus is given the enzymes involved in the biosynthesis of the polyamines (i.e. ODC and S-adenosylmethionine decarboxylase) are rapidly induced and consequently polyamine levels also rise. These increases in polyamine concentrations are paralleled by increased in RNA synthesis (Fausto, 1970). During the resting state, then, the polyamines may not be necessary for sustaining RNA synthesis. However, when a stimulus is given which metabolically activates the liver cell, the polyamines become intracellular regulators and become important for increased activity of RNA polymerase. That the polyamines only become functional as regulators of RNA synthesis when the cell becomes "activated" by some external stimulus (e.g. hormones, drugs, etc.) would seem to make them more critical as regulator molecules.

It was concluded from these experiments that MIX-induced changes in RNA polymerase I are mediated not by ODC directly but by its eventual biosynthetic products, the polyamines spermidine and spermine. It is also possible that the stimulation of RNA polymerase II activity by MIX is mediated by spermine. Figure 17 shows the possible routes by which MIX may stimulate RNA polymerase activity.
FIGURE 17

POSSIBLE MECHANISMS BY WHICH

3-ISOBUTYL-1-METHYLXANTHINE STIMULATES

RNA POLYMERASE ACTIVITY
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Effects of Aminophylline on Ornithine Decarboxylase Activity in the Cytoplasm and Nucleus

The methylxanthines (MIX, caffeine, theophylline, aminophylline) share the same pharmacological properties, the only difference among them is their varying potencies. Therefore it was of interest to test the possibility that a methylxanthine other than MIX could mimic the effects of MIX on nucleocytoplasmic transport of ODC.

Aminophylline was chosen because of the readily available xanthines, it most closely resembles MIX structurally. Aminophylline is 1,3-dimethylxanthine and differs from MIX only at the 3 position where it has a methyl group rather than an isobutyl group. Because the structures are so close, it was thought that the two drugs would be similar in their effects on ODC migration. As shown in Figure 15, this was not the case.

Aminophylline produced a 25-30 fold stimulation of cytoplasmic ODC activity at 4 hours after treatment. There was no change in nuclear ODC activity at any of the time intervals studied. These data suggest that the MIX effect on nucleocytoplasmic transport of ODC is a specific one which may be determined by the addition of the isobutyl group at the 3 position. Most interesting also is the finding that a rather large increase in cytoplasmic ODC activity is not accompanied by a parallel change in the nucleus. Again, ODC activity of the two subcellular compartments varies independently.
Effects of 3-Methylcholanthrene on Ornithine Decarboxylase Activity in the Cytoplasm and the Nucleus

Not only is 3-methylcholanthrene (3-MC) a carcinogen in many tissues (Casarett and Doull, 1975), but it also stimulates the drug metabolizing enzymes of the liver (Conney, 1967). Russell (1971) found that 3-MC stimulated both ODC and RNA synthesis early after its administration. Later studies suggested, in fact, that the stimulation of ODC and RNA polymerase activity were responsible for the increases in the drug metabolizing capacity of the liver (Byus et al., 1976; Costa et al., 1976). In these previous studies, however, ODC activity was measured only in the cytoplasm and not in the nucleus. If ODC does mediate the effect of 3-MC on RNA polymerase I activity, then nuclear ODC content should increase at times when RNA polymerase is known to increase.

RNA polymerase activity increases as early as 3 hours after 3-MC (Gelboin et al., 1967) and this stimulation is sustained for at least 24 hours after treatment (Bresnick, 1966). Therefore, both short term (2-5 hrs) and longer term (24 hrs) effects of 3-MC on nuclear ODC activity were studied.

It was found (Table 4) that cytoplasmic ODC activity increased 2-fold at 5 hours after 3-MC and this stimulation was sustained through 24 hours. At neither of these times was there a significant change in nuclear ODC activity and, in fact, nuclear ODC activity was decreased by 50% at 24 hours after 3-MC. Since RNA polymerase activity is known to be increased at these times, there is a clear dissociation between changes in nuclear
ODC content and RNA polymerase activity that can be measured in isolated nuclei. As with MIX, it appears that ODC does not mediate the effects of 3-methylcholanthrene on RNA polymerase I.

Effects of Thioacetamide on ODC Activity and the Activity of RNA Polymerase I and II

Thioacetamide is a liver carcinogen (Casarett and Doull, 1975). The drug has been shown to stimulate both cytoplasmic ODC activity (Fausto, 1970) and nuclear RNA polymerase activity (Fausto, 1970; Smuckler and Koplitz, 1974). The drug selectively increases the activity of RNA polymerase I (Leonard and Jacob, 1977).

Another effect of thioacetamide is nucleolar swelling which occurs after treatment with the agent (Busch, 1974b). The swelling may represent the influx of proteins into the organelle, reminiscent of the protein influx into the nucleus during periods of gene activation. The migrating proteins may be responsible for activation of RNA polymerase activity and ODC might be among these migrating proteins. Earlier work by Russell (1971) linking ODC with the presence or absence of the nucleolus and therefore with RNA polymerase I activity was discussed in the Introduction.

Two other reasons for studying the effects of thioacetamide on ODC and RNA polymerase are evident. The first of these is the link which has been described in recent years between early changes in ODC activity after treatment of animals with tumor promoters or carcinogens and the carcinogenesis process itself (O'Brien, 1976). If thioacetamide could influence the migration of ODC into the nucleus, ODC might mediate some
of the effects of thioacetamide acting as a carcinogen. Also, an effect of thioacetamide on membrane transport processes has been shown previously (Schum et al., 1977; Smuckler and Koplitz, 1973). It appeared in both these reports that thioacetamide caused the nuclear membrane to become "leaky" and the transport of RNA out of the nucleus was increased. In the present study it was postulated that thioacetamide might therefore also affect the migration of proteins; in particular, ODC was examined.

As expected, thioacetamide had a powerful effect on cytoplasmic ODC activity causing a 15-fold stimulation at 8 hours and a 30-fold stimulation at 24 hours. Nuclear ODC activity, however, was decreased at both times being 60% and 80% of controls at 8 and 24 hours, respectively (Table 5). The decrease in ODC activity at 8 hours was paralleled by a decline in both RNA polymerase I and II to about 75% of control (Table 6). By 24 hours, thioacetamide had caused a slight stimulation of RNA polymerase I (120% of control). This stimulation occurred at a time when nuclear ODC activity was below control. The changes in the ODC and RNA polymerase I activities are slight but it is significant that they are in the opposite directions at 24 hours. Again, no correlation could be shown between nuclear ODC activity and RNA polymerase I activity.
SUMMARY

It is presently accepted that cytoplasmic protein molecules migrate into the nucleus prior to and during periods of increased gene activity. This migrating pool of proteins contains specific factors responsible for the gene activation. A protein molecule which has for years been linked to rapid cellular growth and which was recently shown to be a possible RNA polymerase I initiation factor is ornithine decarboxylase (ODC), the first enzyme in the polyamine biosynthetic pathway.

If ODC is an intracellular regulatory molecule, then it may mediate the effects of drugs on RNA polymerase activity by migrating from the cytoplasm into the nucleus after drug treatment. The possibility that exogenous chemical agents can influence the nucleocytoplasmic exchange of ODC has been examined in the present study. The significance of the migration of ODC into the nucleus has also been examined.

Rats treated with 3-Isobutyl-1-methylxanthine (MIX; 10 mg/kg, ip) exhibited increased ODC activity in both the cytoplasm and the nucleus in an approximately parallel fashion. Cytoplasmic ODC activity increased at 3-4 hours to levels 50-75 times that found in controls, while nuclear ODC activity rose to 5-6 times control.
The stimulation of ODC activity in the cytoplasm was sensitive to cycloheximide inhibition. Combined treatment with MIX and cycloheximide (10 mg/kg, ip) caused cytoplasmic ODC activity to drop below control levels. This same combined drug treatment could not inhibit the increase in nuclear ODC activity. Rather, a biphasic stimulation of nuclear ODC activity occurred after MIX and cycloheximide. Maximal activity occurred at 1 hour (4-5 x control) and 3 hours (8-9 x control) after the combined drug treatment. Cycloheximide given alone had no effect on nuclear ODC activity. These observations suggested that cytoplasmic ODC migrated into the nucleus under the influence of MIX. They also suggested that the migrating ODC molecules were from a preexistent protein pool rather than being synthesized de novo after MIX treatment.

In protein labelling studies, conducted after combined MIX and cycloheximide administration at dosages used above, it was demonstrated that the incorporation of $^3$H-leucine into nuclear and cytoplasmic proteins was virtually abolished (greater than 95% inhibition). It was concluded that nuclear protein synthesis could not account for the MIX-induced increase in nuclear ODC activity.

Dibutyryl cAMP (30 mg/kg, ip) was given both alone and in combination with cycloheximide and ODC activity was determined in the cytoplasm and the nucleus. Though cytoplasmic ODC rose 9-fold after dibutyryl cAMP, nuclear ODC was totally unaffected, remaining at control levels. The addition of cycloheximide (10 mg/kg, ip) to the dosing regimen prevented the rise in cytoplasmic ODC activity after dibutyryl cAMP. Nuclear activity remained the same as in controls. It was concluded that the
MIX-induced increase in nuclear ODC activity was due to a direct effect of the drug and was not mediated by intracellular cAMP.

MIX (10^{-4} M) stimulated the uptake of ODC by nuclei in vitro. cAMP (10^{-4} M) had no effect in the cell-free transport system. The increase in nuclear ODC activity after addition of MIX was dependent on the presence of cytoplasm, leading to the conclusion that MIX did not activate ODC already present in the nucleus. Energy dependence of the nucleocytoplasmic exchange of ODC was suggested by the finding that ODC activity gradually decreased in the nuclei if incubation periods longer than 20 minutes were used. The decrease suggested that ATP levels were not sufficiently high to maintain the high levels of ODC activity against a concentration gradient.

Nuclear membrane ATPase was found to be unaffected by 10^{-4} M MIX, 10^{-4} M cAMP, or the addition of a combination of MIX and cytoplasm. It appeared from these data that a stimulation of nuclear pore ATPase activity was not the mechanism by which MIX increased the flux of ODC into the nucleus.

MIX (10 mg/kg, ip) stimulated both RNA polymerase I (175% of control at 5 hours) and RNA polymerase II (155% of the control at 5 hours) activity. The stimulation was reversed if cycloheximide (10 mg/kg, ip) was administered 30 minutes prior to the MIX.

RNA polymerase activity (both I and II) was inhibited under the same treatment conditions that caused large increases in nuclear ODC activity. These findings led to the conclusion that ODC is not of central importance in the regulation of RNA polymerase I activity in vivo.
Spermidine (10^{-2} M and 5 \times 10^{-3} M) and spermine (10^{-2} M) were found to stimulate RNA polymerase I activity in isolated nuclei. Spermine at lower concentrations (2 \times 10^{-3} and 10^{-3} M) were inhibitory to RNA polymerase I. Spermidine (10^{-7} to 10^{-3} M) had no effect on RNA polymerase II activity. Spermine (10^{-6} to 10^{-4} M) stimulated RNA polymerase II. Increased rates of synthesis of these two polyamines after MIX treatment could account for the changes in RNA polymerase activity.

Methylglyoxal bis(guanylhydrazone) (MGBG; 80 mg/kg, ip), an inhibitor of spermidine and spermine synthesis, reversed the MIX stimulation of RNA polymerase activity. The combination of MIX and MGBG resulted in a decrease in RNA polymerase I and II activity to 40-60% of that found in control animals. MGBG given alone had little or no effect on either RNA polymerase. It was proposed that under conditions which metabolically "activate" the liver cell (e.g. MIX administration), the polyamines become necessary cofactors for RNA polymerase activity. In the "resting" liver cell, the polyamines are not necessary.

Aminophylline (200 μmoles/kg, ip) did not mimic the effects of its close structural analog, MIX. The MIX effect on nuclear ODC activity is a very specific one.

Neither 3-methylcholanthrene (80 mg/kg, ip) nor thioacetamide (150 mg/kg, ip) increased nuclear ODC activity although they stimulated cytoplasmic ODC activity 2-fold and 30-fold, respectively. It was significant that nuclear ODC activity was unchanged or significantly inhibited at times when RNA polymerase activity was increased. These data cast doubt on the role of ODC as a mediator of drug effects on RNA polymerase I.
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