Some Observations on the Temporal Responses of Ornithine Decarboxylase Activity in the Immature Rat Uterus following Estradiol 17-β or Estriol Treatment

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Table of Contents

I. Abstract

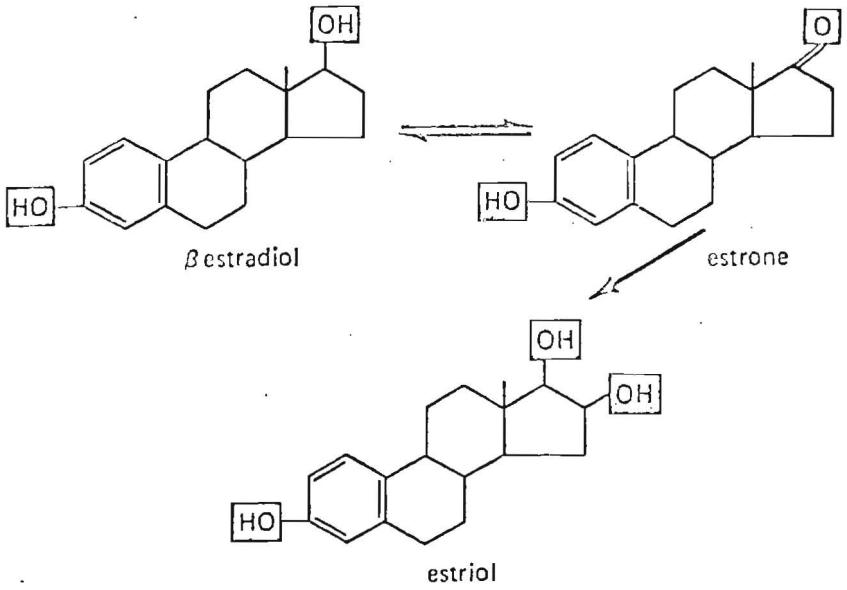
Some Observations on the Temporal Responses of Ornithine Decarboxylase Activity in the Immature Rat Uterus following Estradiol 17-β or Estriol Treatment. Jennifer C. Adler. Estradiol 17-β is known to cause growth in the immature rat uterus and cause ornithine decarboxylase (ODC) activity, an enzyme involved in polyamine synthesis which is growth related. Estriol is much less active in causing true growth. This study was designed to clarify the relative effects of estradiol and estriol on uterine ODC activity. Immature SD rats were injected (ip in corn oil) with estradiol or estriol. Following treatment, the uteri were removed, homogenized, and centrifuged. The supernatant was incubated in a sealed vial containing ornithine (DL($1^{14}C$), pyridoxal phosphate and dithiothreitol. CO_2 was trapped on hyamine impregnated paper and counted. Treatment with estradiol and with estriol resulted in an increase in ODC activity. Estradiol treatment produced a biphasic early (4-6 hour) and late (12-16 hour) increase in ODC activity, while estriol treatment produced only the early (4-6 hour) increase. Administration of cycloheximide, an inhibitor of translation, following treatment blocked all increases in ODC activity. These results support the concept that a biphasic increase in ornithine decarboxylase activity is required for true growth to occur and perhaps explain estriol's failure to induce true growth.

II. Estrogen

Some research has been done concerning the biochemical changes in the uterus which occur after treatment with estradiol 17-β, a steroid hormone which stimulates an enzyme releasing factor which in turn stimulates activity of the enzyme ornithine decarboxylase (ODC) in the immature rat uterus. Ornithine Decarboxylase activity is related to growth in many tissues. It is the rate limiting enzyme in polyamine synthesis and polyamines are believed to be necessary for "true" growth to occur, "true" refering to a significant amount of DNA synthesis, protein synthesis, and cell division. Estradiol 17β stimulates true growth whereas estriol does not. Estriol is another steroid hormone and like estradiol is one of the three natural estrogens with estrone being the third natural form. In this research my main objective was to compare these two compounds, estradiol and estriol, and their effects on ornithine decarboxylase activity in the immature rat uterus and possibly clarify somewhat their relationship to true growth in the uterus.

Estrogens are steroid hormones which are known to cause uterine growth. Estrogens are formed by the ovary, placenta, testes and possibly the adrenal cortex. The three natural estrogens produced by the thecal cells in the ovarian follicles are estradiol 17-β, estriol and estrone with estradiol 17-β being the more potent of the three. (refer to figure 1). Estrogens enter the blood stream and will travel to estrogen "target" organs. Estrogen "target" organs are tissues containing specific estrogen receptors on its cellular surfaces for the sole purpose of binding estrogen. These steroid hormones act at the level of gene expression, however, before one can determine its effect on , in our case, ornithine decarboxylase activity one must know the mechanism of action by which these hormones act.

Figure 1 **Structural formulas of the natural estrogens, estradiol, estriol, and estrone. The β estradiol is the most potent of these hormones.**



It is generally believed that estrogens stimulate transcription, RNA synthesis, translation and in turn protein synthesis (refer to figure 2). So one may assume that estradiol and estriol both follow this pathway. However, there is some evidence of effects of steroid hormones that do not require transcription and translation, called post transcriptional effects, for this reason we utilized a compound called cycloheximide (refer to figure 3). Cycloheximide is an antibiotic produced by Streptomyces griseus, that inhibits protein synthesis at the level of translation by preventing peptide bond formation. In this research we wanted to establish whether estradiol and estriol followed this mechanism of action (transcription/translation) in relation to ornithine decarboxylase activity, thus the utilization of the inhibitor of translation, cycloheximide. So, it follows if cycloheximide were able to block the effe ts, if any, produced by treatment with estradiol and estriol on ornithine decarboxylase activity in the immature rat uterus, then we would be able to state that both estrogens follow the transcription/translation pathway.

When contemplating the question of growth, one must consider the early and late effects produces by mitogenic (growth stimulating) agents. Early effects include hyperemia, H_2O inhibition,etc. Late effects include DNA synthesis, protein synthesis and cell division, etc. In order for true growth to occur both early and late effects should be produced. Estradiol is very strong in producing both early and late effects. Estriol produces early effects but is very weak in producing a significant amount of late effects. This is why it is believed that estradiol induces true growth wheras estriol does not, since estriol has been shown in vivo (in a living organism) to produce early hormone responses similar to estradiol but cannot induce the later growth phases produced by estradiol.

According to Mary E. Olson, Daniel M. Sheehan and

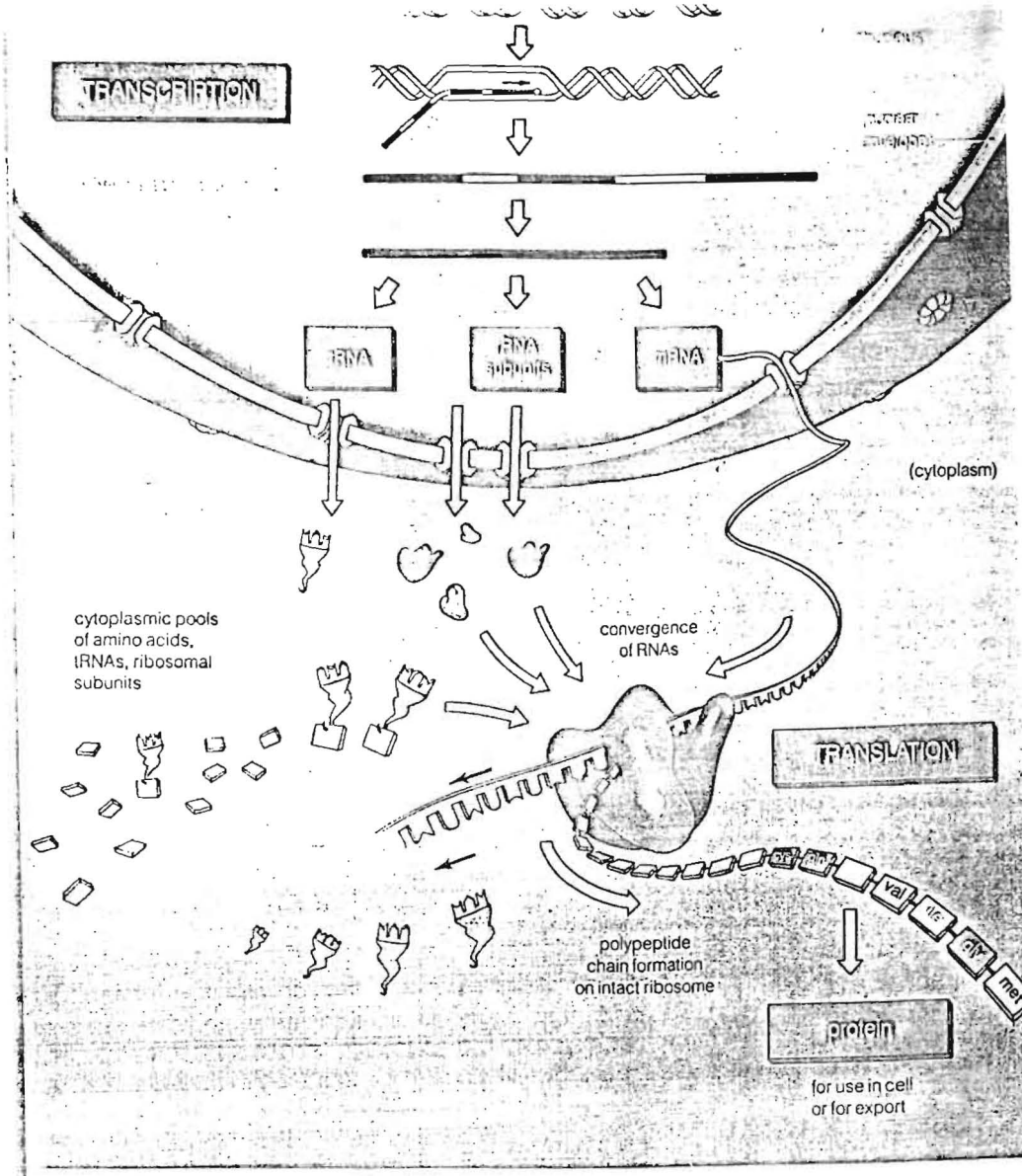
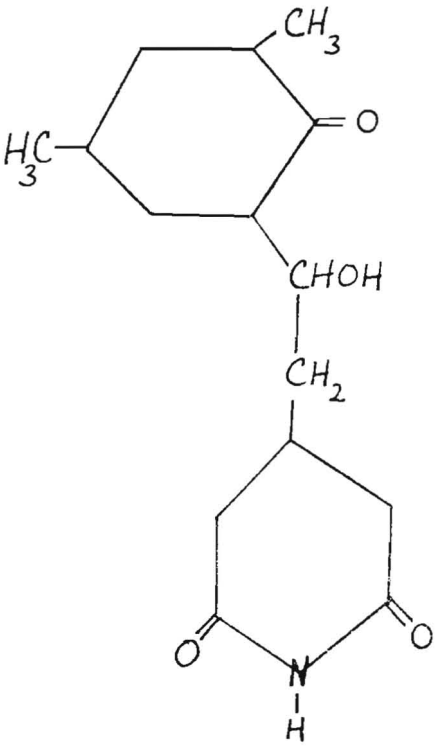


FIGURE 2: Transcription and Translation

FIGURE 3: Cycloheximide-inhibitor of protein synthesis (translation).



William S. Branham from the University of Arkansas, in immature rats a single dose of estradiol induces significant sequence of events leading to extensive uterine hypertrophy and hyperplasia. Early events (occuring up to 6 hours after estradiol exposure) include receptor binding and translocation, increased glucose metabolism and phospholipid synthesis, H_2O inhibition and increased RNA polymerase activity and protein synthesis. Late responses include net production of RNA and protein, synthesis of histones and DNA, and cell division.[1]

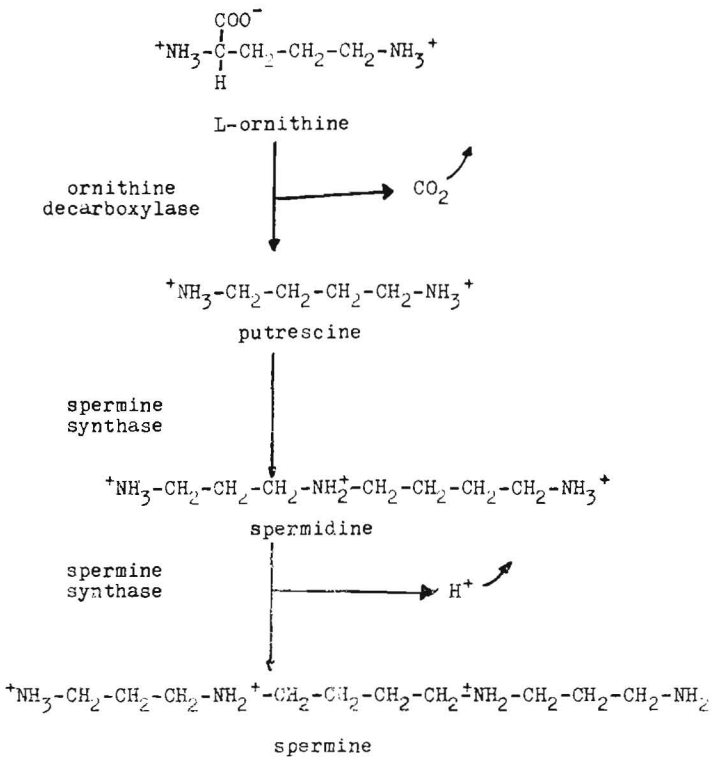
III. Ornithine Decarboxylase and Polyamine Synthesis

L-ornithine decarboxylase (ODC) is the first enzyme in the mammalian polyamine biosynthetic pathway and provides the only route to putrescine in mammalian cells.[2] Polyamines are a group of organic cations which cause cellular growth and differentiation.[3] (refer to figure 4).

Ornithine decarboxylase is also considered the rate-limiting enzyme in the synthesis of these polyamines; putrescine, spermidine and spermine. It is generally believed that polyamines are necessary for tissue growth, so obviously ornithine decarboxylase is important in understanding true growth in tissues. Many microorganisms and higher plants are able to produce putrescine from agnatine produced by decarboxylation of arginine, but all mammalian cells and many lower eukaryotes lack arginine decarboxylase. In these species, therefore, the only route[4] to putrescine is via the enzyme, ornithine decarboxylase.

The diamine putrescine and the polyamines spermidine and spermine occur ubiquitously in nearly all organisms. An elevation in concentration of these compounds is associated with a shift from a non-growing or slow growing state to one of rapid proliferation in a large number of animal tissues.[5] Because of the abundance of data relating polyamine increases

FIGURE 4: Polyamine Synthesis-believed to be related to growth in tissues.



with accelerated growth it seems likely that polyamines are in some manner intimately involved in growth regulation.[6]

According to Brigid Hogan from the University of Sussex, a large increase in the activity of ornithine decarboxylase has been observed soon after the transition of a number of cells from a non-growing state or after stimulation of tissues by growth-promoting hormones.[7] In several mammalian tissues an increase in ornithine decarboxylase activity can be produced by known mitogenic, or growth stimulating agents. However, peaks in ornithine decarboxylase activity have been shown when a non-mitogenic stimuli is applied. This seems to suggest that growth in tissues requires an increase in ODC activity, whereas an increase in ODC does not necessarily mean growth will occur.

Now the question is to determine if and how the steroid hormones, estradiol 17-β and estriol will stimulate ornithine decarboxylase activity in the immature rat uterus and then postulate on its relationship to growth in this particular tissue.

In 1971, Dr. Kaye* and his coworkers were able to determine that treatment with estradiol 17-β in the immature rat produced two peaks of ornithine decarboxylase activity in the uterus. An early increase in ODC activity was found to exist at 4-6 hours after treatment and a second, late, peak was found to exist at 12-16 hours after treatment. In doing this research we hoped to, 1). verify the existance of these two peaks in ornithine decarboxylase activity produced by treatment with estradiol as well as 2). determine if estriol produces an increase in ornithine decarboxylase activity and if so, at what time period, 3). determine the mechanism of action of estradiol and estriol in relation to the stimulation of ornithine decarboxylase in the immature rat uterus through the use of an inhibitor of translation, cycloheximide, and finally, 4). to form a hypothesis on the relationship between estrogen induced peaks in ODC activity and true growth in the immature rat uterus.

* At the Weizmann Institute of Science (Israel)

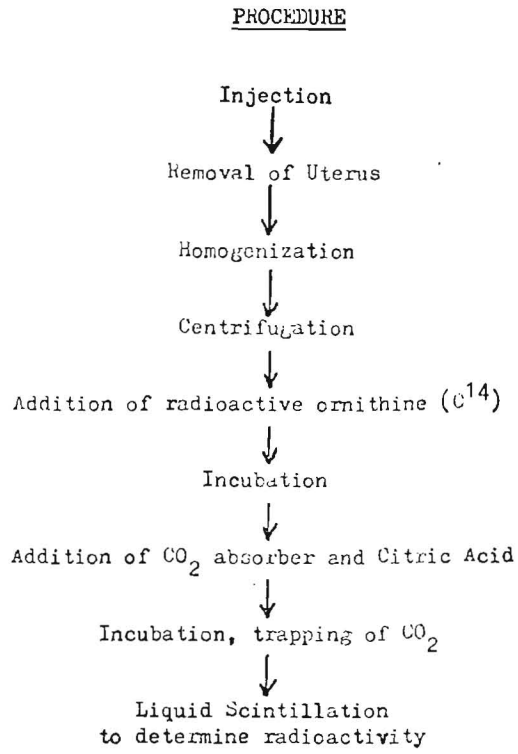
IV. Materials and Methods

The following procedure is known as an in vivo, meaning "in living", treatment followed by an in vitro, meaning "in test tube", assay involving the use of a radio-labeled ornithine, C^{14} labeled at the #1 position. Since our main objective is to record the activity of the enzyme ornithine decarboxylase we utilized this radioactive ornithine. When ornithine is converted to putrescine via ornithine decarboxylase, carbon dioxide (CO_2) is given off. The carbon atom which is radio-labeled in the molecule of ornithine is the carbon released in the form of CO_2. This radioactive CO_2 can be counted via a scintillation counter and thus corresponds to the activity of the enzyme ornithine decarboxylase. (refer to figure 5)

We must follow this in vivo, in vitro procedure since estrogens will only produce effects in vivo and not in vitro. In other words, if estrogens were injected into a test tube containing a rat uterus nothing would happen. For this reason it was necessary to utilize live animals.

Immature (approximately 22 day old) rats were used in this experiment so that the estrogen level could be measured and manipulated. Since pre-pubescent rats have not secreted any significant amount of hormone, no interference can occur with the measured amount injected. Twelve immature SD rats were divided into six groups of two rats each. The first group was labeled as "control" and injected with 5ug of corn oil. The second group was labeled estradiol 17-β and injected with estradiol dissolved in corn oil, since steroid hormones are lipid soluble not water soluble. The concentration was as follows; 10 mg of estradiol per 10 ml of corn oil. 1 ug (cc) is necessary for maximal response, in this case, we used 5 ug or .5 cc. The third group was injected with 5 ug of estriol and labeled as the estriol group. The concentration of hormone was the same as estradiol, 10 mg of estriol/ 10 ml of corn oil.

FIGURE 5: In vivo treatment followed by in vitro assay



The fourth group was injected with 5 ug of estradiol and then two hours prior to killing, injected a second time with cycloheximide, the inhibitor of translation. The concentration of cycloheximide utilized was 10 mg of cycloheximide per 25 ml of .9% NaCl. The fifth group was injected with 5 ug of estriol and then two hours prior to killing, injected a second time with 5 ug of cycloheximide. The sixth and final group was injected with 5 ug of cycloheximide two hours prior to killing. Thus the groups are as follows:

Group I-Control
Group II-Estradiol 17-β
Group III-Estriol
Group IV-Estradiol 17-β and cycloheximide
Group V-Estriol and cycloheximide
Group VI-Cyclohexomide

Since we wanted to obtain data concerning the activity of ornithine decarboxylase over a period fo 0-20 hours, this procedure was followed several times over the last two semesters (August 1986-March 1987). We chose 4 time periods to focus our experimentation on; 5, 8, 14, and 20 hour time periods. This will allow us to use the data obtained in the form of a 0-20 hour graph. The times indicated are the times allotted between injection and killing of rats. Also, several repetitions of this procedure were conducted at each time period indicated. I would like to note here that the results from this research are strictly preliminary. Due to money and time, repeated experimentation which could make this data truely "significant" were unable to be conducted.

After the specific time period was allotted for, the rats were killed and the uteri removed. The two uteri from each group were placed in 2 ml of TRIS buffer contained in centrifuge tubes (121 mg/100 ml H_2O). TRIS (hydroxymethyl aminomethane) has a pK_a value of 8.30 at 20°C and is used to prevent change in pH to ensure maximal enzyme activity. Note, our goal in this procedure is to isolate the enzyme, ornithine decarboxylase, and to measure its activity. Thus we would utilize a buffer to ensure

maximal activity of the ornithine decarboxylase. The pH we used was 7.4. Then each tube containing the TRIS buffer and the two uteri were homogenized for 10 seconds or until all tissues were successfully broken down.

After homogenization, the homogenate was centrifuged for 15 minutes at 40,000 x gravity. The purpose of this differential centrifugation is to separate the mitochondria, nuclei and large membrane fragments from the ribosomes and the cytosol, called the supernatant. Since ornithine decarboxylase is a cytosolic enzyme and our objective is to isolate this enzyme we were able to use just such a technique to accomplish this task of ODC isolation.

After centrifugation of the six tubes (corresponding to the six original groups) twelve incubation vials (two for each group) were labeled and filled with the following: .9 ml of supernatant containing the cytosolic enzyme ornithine decarboxylase, 50 ul of radio-labeled ornithine (C^{14} labeled at the #1 position). This radio-labeled ornithine will be decarboxylated (CO_2 will be released) in the conversion from ornithine to putrescine in polyamine synthesis, which requires ODC as a catalyst (refer to figure 4). The radio-labeled carbon atom will be released as CO_2 and we will be able to record the amount of CO_2 released later on in the procedure. This amount of carbon dioxide will correspond to the activity of ornithine decarboxylase. Finally, 50 ul of pyridoxal phosphate (vitamin B_6) was added to the vial. The concentration was 12.35 mg/50 ml .247 mg/ml in phosphate buffer (pH=7.2). Pyridoxal phosphate is a coenzyme necessary for the action of ornithine decarboxylase. Before the vials were sealed, hyamine impregnated paper (filter) (1.25 in. x .75 in.) was suspended in each vile for the purpose of absorbing the carbon dioxide released.

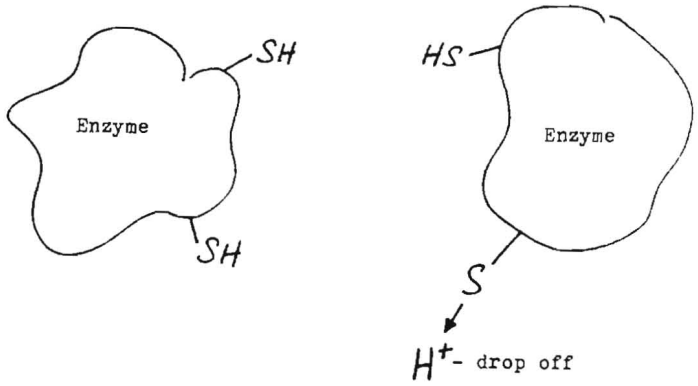
The radio-labeled ornithine was mixed with dithiothreitol which is a protective reagent for SH groups. In simpler terms, enzymes containing SH groups tend to lose the Hydrogen proton and as a result the sulfur (S) will bond to another sulfur and produce clumping of the enzyme.

Dithiothreitol prevents this from happening. (refer to figure 6). Dithiothreitol was made up as follows: 30.8 mg/20 ml made up in phosphate buffer (pH=7.2). Then 100 ul of ornithine was added to 800 ul of dithiothreitol (final concentration: Dithiothreitol=.5mM; ornithine=10uM).

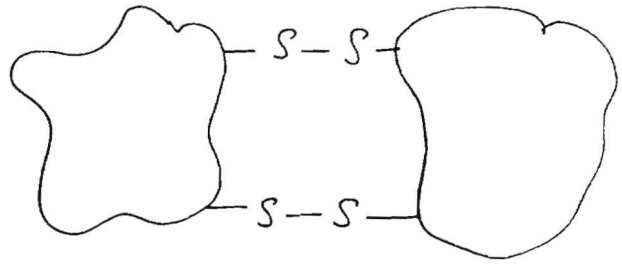
The incubation vials containing the ornithine, supernatant and the pyridoxal phosphate were then placed in the incubator for 30 minutes to allow ornithine decarboxylase to convert ornithine to putrescine and allow CO_2 to be given off. After 30 minutes, the vials were removed from the incubator. .2 ml of Methylbenzethonium hydroxide, the carbon dioxide absorber, was then injected onto the hyamine impregnated filter paper. .2 ml of citrate (citric acid) (8.4g/20 ml H_2O) was injected into the solution in the vial. Citric acid is injected for the purpose of decreasing the pH to end enzyme activity. In other words, conditions are made too acidic for enzyme activity to occur. This is then incubated for another 30 minutes to allow for carbon dioxide absorption onto the hyamine impregnated filter paper.

After this second incubation period, each filter paper (containing the absorbed CO_2) was then placed in corresponding scintillation vials containing 20 ml of econofluor and then placed in the scintillation counter. The liquid scintillation counter is able to count the radioactivity produced by the radio-labeled carbon dioxide on the filter paper. The carbon dioxide will emit particles which hit the econofluor causing light flashes to occur. These light flashes are counted by the liquid scintillation counter and correspond to the amount of carbon dioxide released in the vial and absorbed on the paper. This in turn corresponds to the activity of ornithine decarboxylase. In other words, if ornithine decarboxylase was high in activity in a particular vial, alot of ornithine was converted to putrescine and thus a lot of carbon dioxide was released (refer to figure 4).

FIGURE 6: Clumping of an enzyme as a result of S-S bonding. Dithiothreitol prevents this from happening.



RESULT:



Clumping of the enzyme

V. Results

The data obtained from the experiment support a biphasic increase in ornithine decarboxylase activity after treatment by estradiol 17-β. The first marked increase in ODC activity occured at 4-6 hours after treatment. We were able to show a second peak in ornithine decarboxylase activity following treatment by estradiol 17-β at 12-14 hours after injection. Cycloheximide blocked both estradiol induced peaks of ODC activity indicating that estradiol 17-β follows the transcription/translation pathway in relation to ornithine decarboxylase stimulation. (refer to figure 7).

Estriol produced a monophasic increase in ornithine decarboxylase activity at 4-6 hours after treatment, however, produced no second peak in ODC activity. Cycloheximide blocked the first estriol induced peak of ornithine decarboxylase activity indicating that estriol, like estradiol, follows the transcription/translation pathway in relation to ornithine decarboxylase stimulation (refer to figure 8).

As stated previously, it is known that estradiol 17-β stimulates true growth, it increases protein and DNA synthesis and is the strongest of the three natural estrogens. It is also known that estriol is very weak in its effects and although it does stimulate some protein synthesis, it is not a significant amount. Thus in comparing the two graphs (figures 7 and 8), produced by estradiol 17-β and estriol, the former producing two peaks in ornithine decarboxylase activity, the latter producing only an early peak in ornithine decarboxylase activity, and at the same time realizing that ornithine decarboxylase is the rate limiting enzyme in polyamine synthesis which is believed to be necessary for true growth one can hypothesize the following: Apparently a biphasic increase in ornithine decarboxylase activity is required for true growth to occur in the immature rat uterus. Also, this data seems to clarify estriols inability to induce true growth since it produced only a single peak in ODC activity. Whether both peaks are required or only the second peak is still unclear at this time as well as whether the peaks of ODC activity induced by estradiol 17-β continue past 20 hours (in a cycle perhaps) is also not clear.

Alot of research can still be continued in this area not only with later time periods (past 20 hours) but also with the utilization of an inhibitor of transcription, for example, acetinomycin D. Also work with the third natural form of estrogen, estrone, has yet to be conducted.

FIGURE 7:Biphasic increase in Ornithine Decarboxylase activity (Estradiol 17

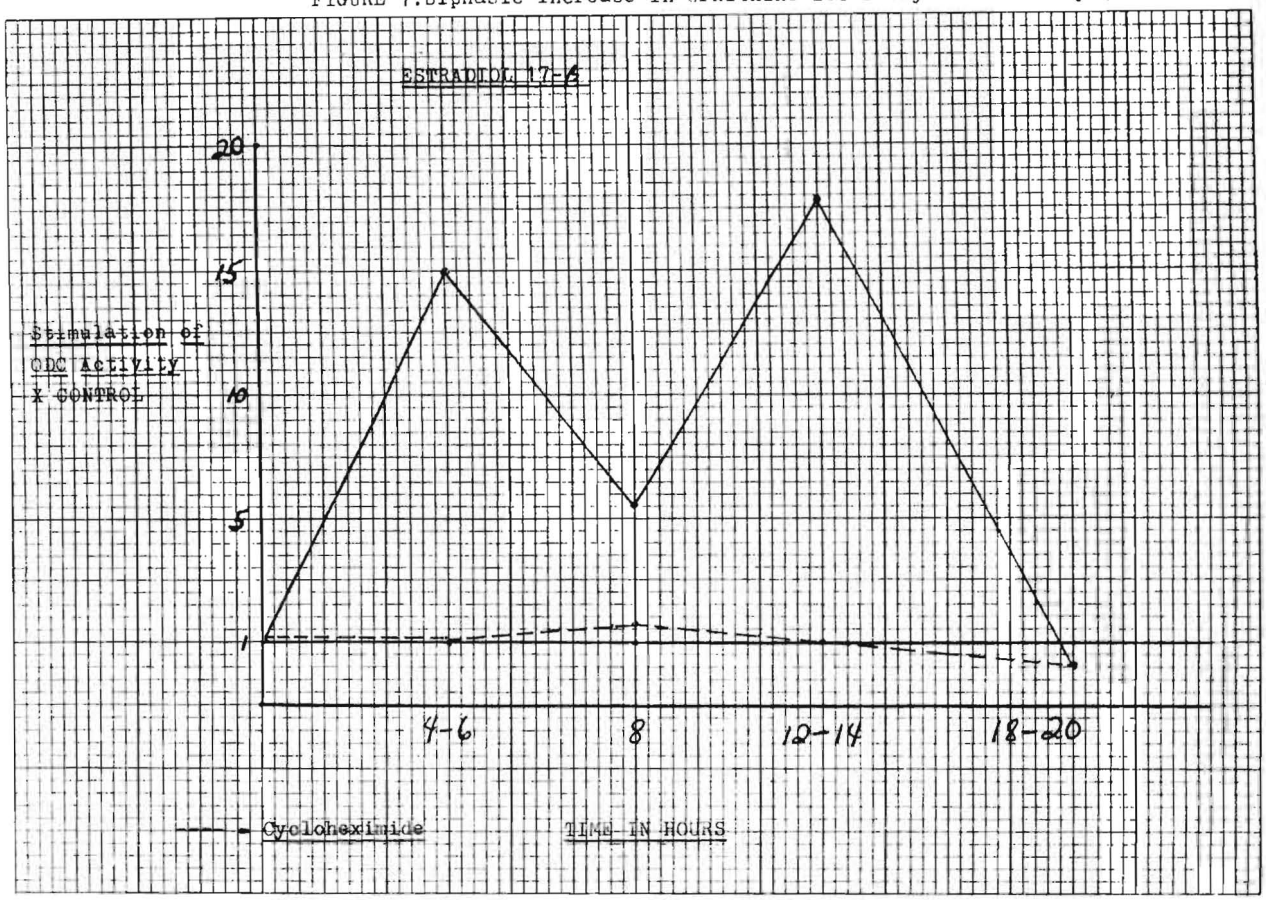
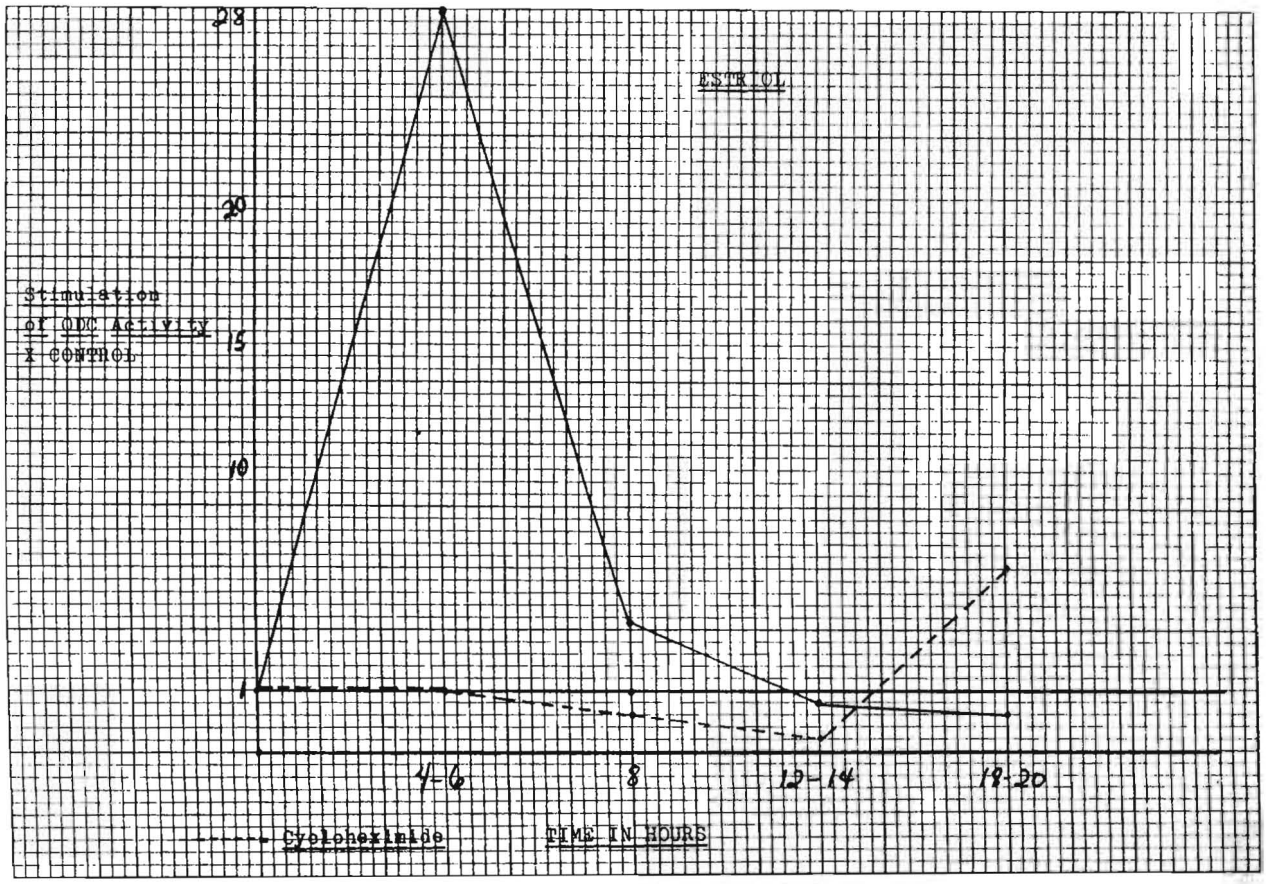


FIGURE 8: Monophasic Increase in Ornithine Decarboxylase Activity (Estriol)



VI. Final Comments

I would like to express my thanks to the Biology department here at Pembroke State University for all of their assistance concerning this research project. A special thank you to Dr. H. David Maxwell for guiding me patiently through this project, step by step, and for providing the necessary encouragement to continue forward with this research. Also thank you to Owen Martin for his assistance with the laboratory rats utilized in this experiment and most especially thank you to the MARC program for providing the funds necessary to carry out this research project.

I would like to thank the Chancellor's Scholars Program for giving me the opportunity to be a part of this prestigious program. Also, a special thank you should be extended to Dr. Jenkins for all of his assistance. And to Dr. Williams and Dr. Brown, thank you both for all of your help and patience not only during the past year while working on this project, but throughout the entire program. I will truely miss you.

One final note, throughout this research project I think the question most commonly asked pertained to the significance of this particular project. I would like to end this paper with a quote from a pamphlet distributed by the U.S. Department of Health and Human Services:
" The Researcher doing basic, untargeted studies is looking for answers to more general questions. He or she is seeking to add to the store of knowledge about how living things work. These basic researchers' experiments add pieces to the immensely complex puzzles of life. It may take time to see significant advances; "miracle cures" are not the goal of this work. Sometimes, of course, the pieces come together and a real clinical breakthrough occurs. But the scientists' main purpose is to keep following the leads that appear most likely to yield missing pieces of information even if

the exact applications of the new knowledge are not immediately evident. From the body of knowledge and understanding amassed by basic researchers, clinical investigators can construct more rational and systematic ways to approach the problems presented by the diseases plaquing us today. Untargeted basic research thus provides the fundamental theories and concepts for more disease-oriented investigations.[8]

VII. Appendix

buffer- A solution containing an acid and its conjugate base which when present in solution resists any change in pH.

centrifuge- An apparatus by means of which particles in suspension in fluid may be separated; this is done by whirling the vessel containing the fluid about in a circle, the centrifugal force throwing the particles to the peripheral part of the rotated vessel.

coenzyme- The organic molecule that functions as cofactor of an enzyme.

cycloheximide- An antibiotic produced by Streptomyces griseus, that inhibits protein synthesis (translation) by preventing peptide bond formation on 80S ribosomes. aka actidione.

cytosol- The cytoplasm minus the mitochondria and endoplasmic reticulum components.

decarboxylation- The removal or the loss of a molecule of carbon dioxide from the carboxyl group of an organic compound.

dithiothreitol- Compound used for the protection of sulfhydryl groups against oxidation to disulfides and for the reduction of disulfides to sulfhydryl groups.

enzyme- A protein, secreted by cells that acts as a catalyst to induce chemical changes in other substances, itself remaining apparently unchanged in the process.

estradiol 17-β - The most potent naturally occuring estrogen in mammals. A steroid hormone produced by the ovary, placenta, the testes and possibly the adrenal cortex.

estriol- A metabolite of, and considerably less potent than estradiol; it is usually the predominant estrogenic metabolite found in urine.

estrogen- An 18 carbon steroid that is a female sex hormone; the major estrogens are estrone, estradiol 17-β and estriol. Estrogens are formed by the ovary, placenta, testes and possibly the adrenal cortex.

homogenate- That which is made homogeneous; specifically in biochemistry, tissue ground into a creamy consistency in which the cell structure is disintegrated.

incubation- The act of maintaining controlled environmental conditions for experimentation.

in vitro- Outside a living organism.

in vivo- Within a living organism.

liquid scintillation counter- A radiation counter in which incident ionizing particles or incident photons are counted by the scintillation which they induce in a liquid fluor.

mitogen- A substance that stimulates mitosis and cell transformation-growth.

ornithine- $NH_2(CH_2)_3CHNH_2COOH$; the amino acid formed when arginine is hydrolyzed by arginase.

ornithine decarboxylase- A bacterial enzyme catalyzing the decarboxylation of ornithine to putrescine.

polyamines- Spermidine; spermine and related amines including putrescine are the bases in biological systems which are classified as the polyamines. The chemical structures reveal the multiple amino and imino groups characterizing these molecules. Appear to be growth related in tissue.

pyridoxal phosphate- The coenzyme form of vitamin B_6 that functions in the metabolism of amino acids.

steroid- A cyclic compound of animal or plant origin, the basic nucleus of which consists of 3-6 membered rings and one 5 membered ring, fused together to yield perhydrocyclopentano phenanthrene.

supernatant- The liquid above sedimented material or above a precipitate

transcription- The process wherby the genetic information of DNA is copied in the form of RNA.

translation- The process whereby the genetic information of messenger RNA is used to specify and direct the synthesis of a polypeptide chain on a ribosome.

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