THE SUBCELLULAR DISTRIBUTION OF UTERINE ORNITHINE DECARBOXYLASE FOLLOWING ESTROGEN TREATMENT

A Thesis

Presented to

the Chancellor's Scholars Council

of Pembroke State University

In Partial Fulfillment

of the Requirements for Completion of

the Chancellor's Scholars Program

by

Tony Jones

April 29, 1985

Table of Content

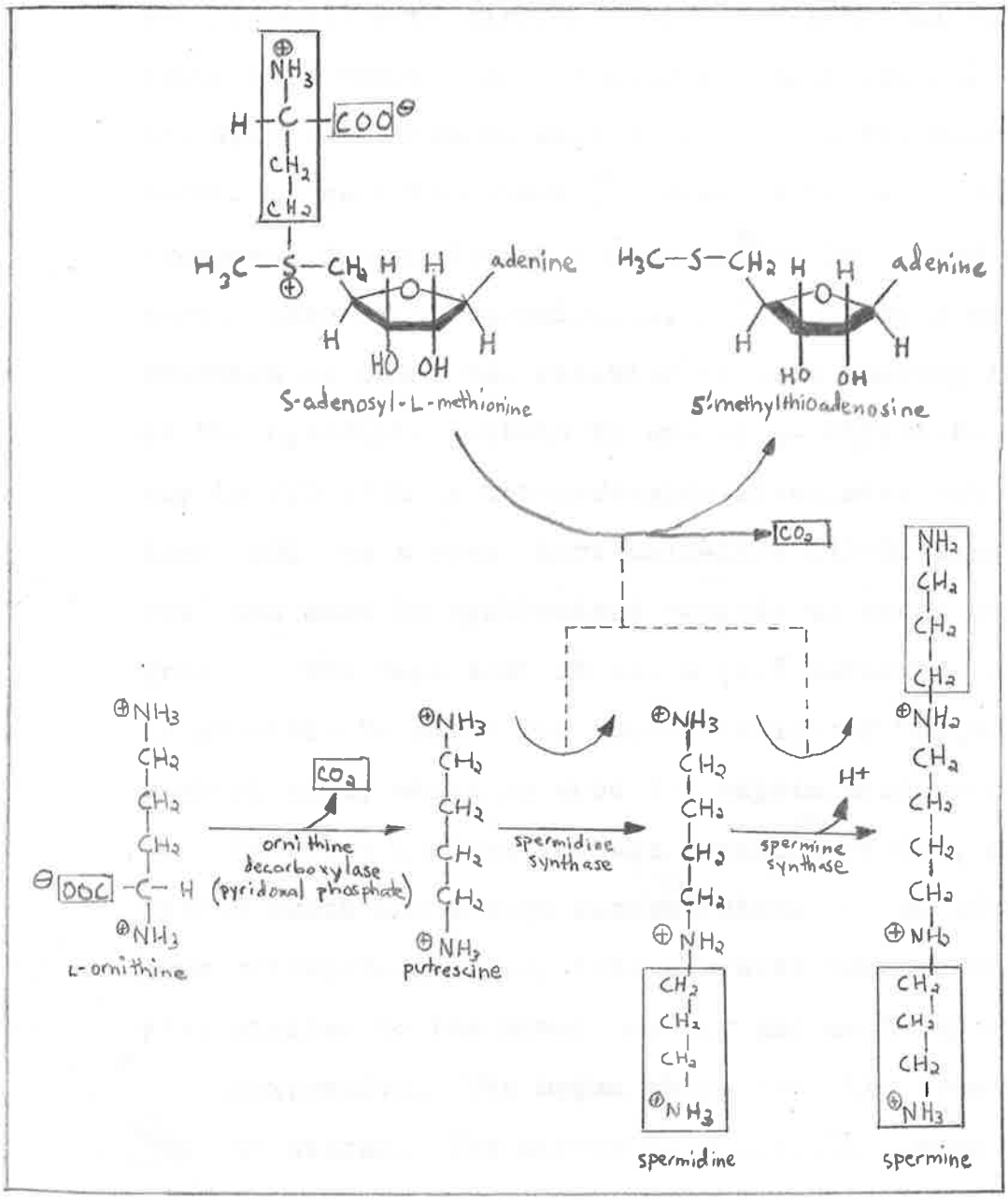
THE SUBCELLULAR DISTRIBUTION OF UTERINE ORNITHINE DECARBOXYLASE FOLLOWING ESTROGEN TREATMENT

Introduction

Within a cell, the basic structure and functional unit of all living things, many substances contribute to its continuing existence. Ornithine decarboxylase, an enzyme, is one of these substances. This paper deals with finding the subcellular distribution of ornithine decarboxylase within the uteri of immature rats after they have been injected with estrogen. In other words, this project is to determine whether the enzyme is located in the nucleus, in the cytoplasmic components (supernatant), or possibly in both these cellular parts of the uteri of immature rats, following estrogen injection. First of all, however, an explanation of ornithine decarboxylase, estrogen, and similiar research of this nature must be given.

According to Ian S. Zagon and others from the Milton S. Hershey Medical Center at Pennsylvania State University, "L-ornithine decarboxylase (ODC) is the first enzyme in the mammalian polyamine biosynthetic pathway and provides the only route to putrescine in mammalian cell."[1] (This can be seen in figures 1.) Polyamines are a group of organic cations which are required to stimulate cellular growth and differentiation.[2] This means that without polyamines and ODC there would be no cellular growth. The polyamines include putrescine, spermidine and spermine. As figure 1 indicates,

Figure 1.



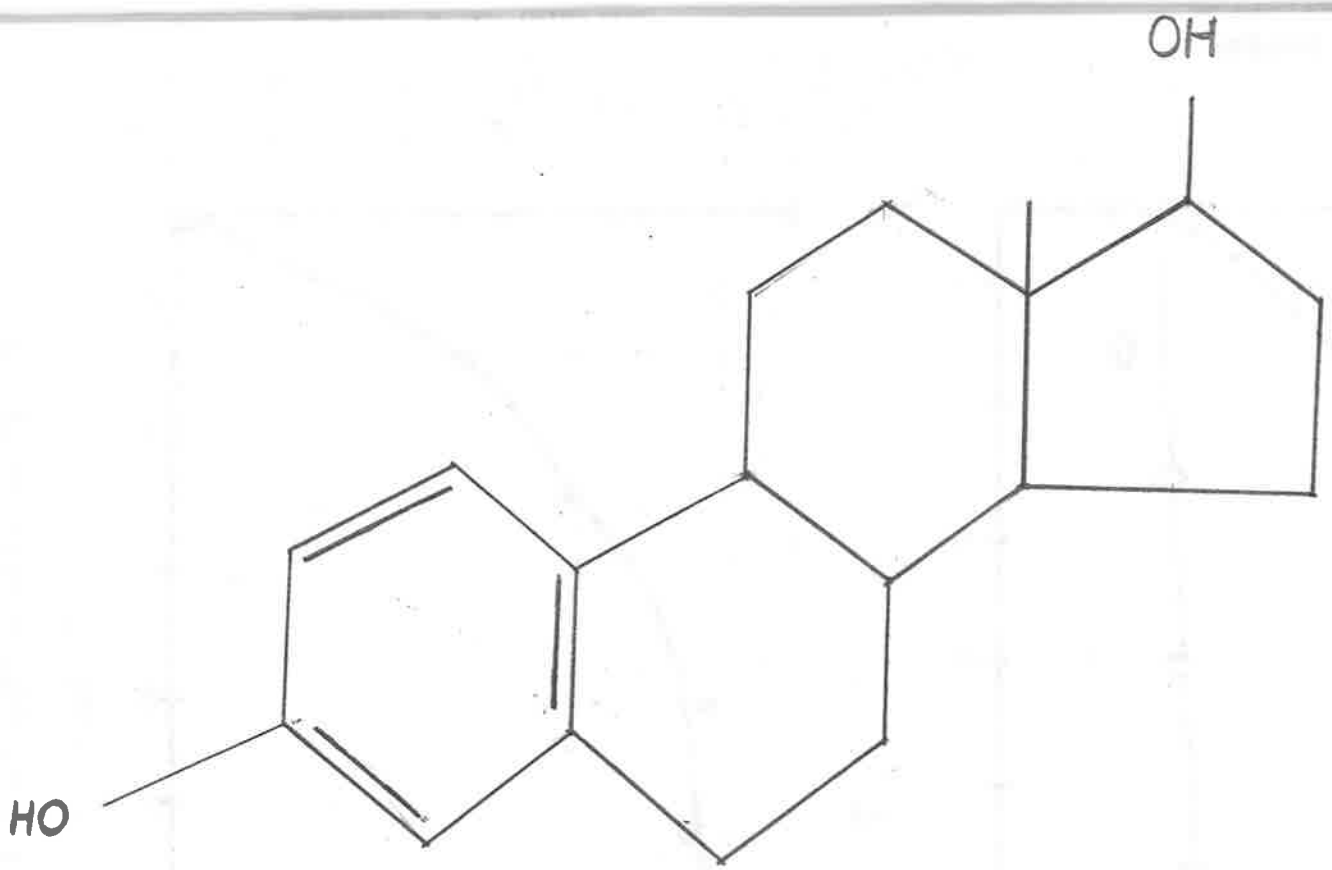
The synthesis of putrescine, spermidine, and spermine. The figure indicates the greater dissociation of one of the ammonium groups in spermine.

ornithine decarboxylase (ODC) acts upon ornithine, which is not one of the twenty-one naturally occurring amino acid, in the presence of pyridoxal phosphate (a coenzyme) causing the production of carbon dioxide and putrescine. It is present in "very small amounts in quiescent cells, and its activity is increased many fold after a few hours of exposure to certain stimuli."[3] Some of these stimuli include hormones, drugs, tissue damage and or loss, and growth factors. Even after stimulation, ODC makes up a very small fraction of the total cellular protein ranging from 0.01% of the cytosolic protein in androgen- stimulated mouse kidney to 0.00012% in thioacetamide-stimulated rat liver.[4] Also, ODC has a very short half-life (10-20 minutes in the rat) and must be synthesized rapidly to cause cellular growth. The fact that it has a fast turnover rate makes it possible to alter the concentration of the enzyme in a short time, which is good for expermintal purposes.[5]

In order to test for the location of ODC, an organ is needed which has a high concentration of ODC after it has been stimulated. Also, rats are used because they are somewhat similar to the human anatomy and most importantly they are inexpensive. The organ which has this requirement is the rat uterus. The uterus is a muscular organ designed to enclose and provide a pathway of food for the developing fetus. To increase ODC activity in this uterus, the stimulant,

estrogen, must be used. Estrogen is a steroid hormone and is normally produced by thecal cells in the ovarian follicles. Mainly three estrogens are produced by the ovaries: β-estradiol, estrone, and estriol. (The β means beta.) The most potent of the three is β-estradiol.[6] (See figure 2.) Also, the female rats must be immature to ensure that the amount of uterian ODC is low. For example, in an immature rat, one less than thirty days old, the production of estrogen is insignificant. Thus the presence of a small amount estrogen causes very little ODC production in the uterus. This low ODC concentration is needed so the researcher can either raise its amount by injecting estrogen, or lower it by injecting cycloheximides, an inhibitor of protein synthesis. However, in adult rats the concentration of estrogen is not easily controlled because of the menstrual cycle. Hence for experimental purposes, it is best to use an immature rat whose estrogen level and ODC concentration are low or approximately constant.

When β-estradiol is injected into a female rat, it eventually reaches the uterus. ODC concentration and activity increases; thus, the cells begin to grow and the rate of cellular division increases. Daniel Sheeman in figure 3 shows uterine weight gain of 17-β-estradiol injected rats as opposed to those not treated. The treated uteri were again compared to the nontreated control in the form of percentages.[7] P.J. Stewart and his colleagues from the Department of Biological Sciences at the University of



Estra-1,3,5(10)-triene-3,17β-diol
(Estradiol-17β, from OVARY)

Figure 2.

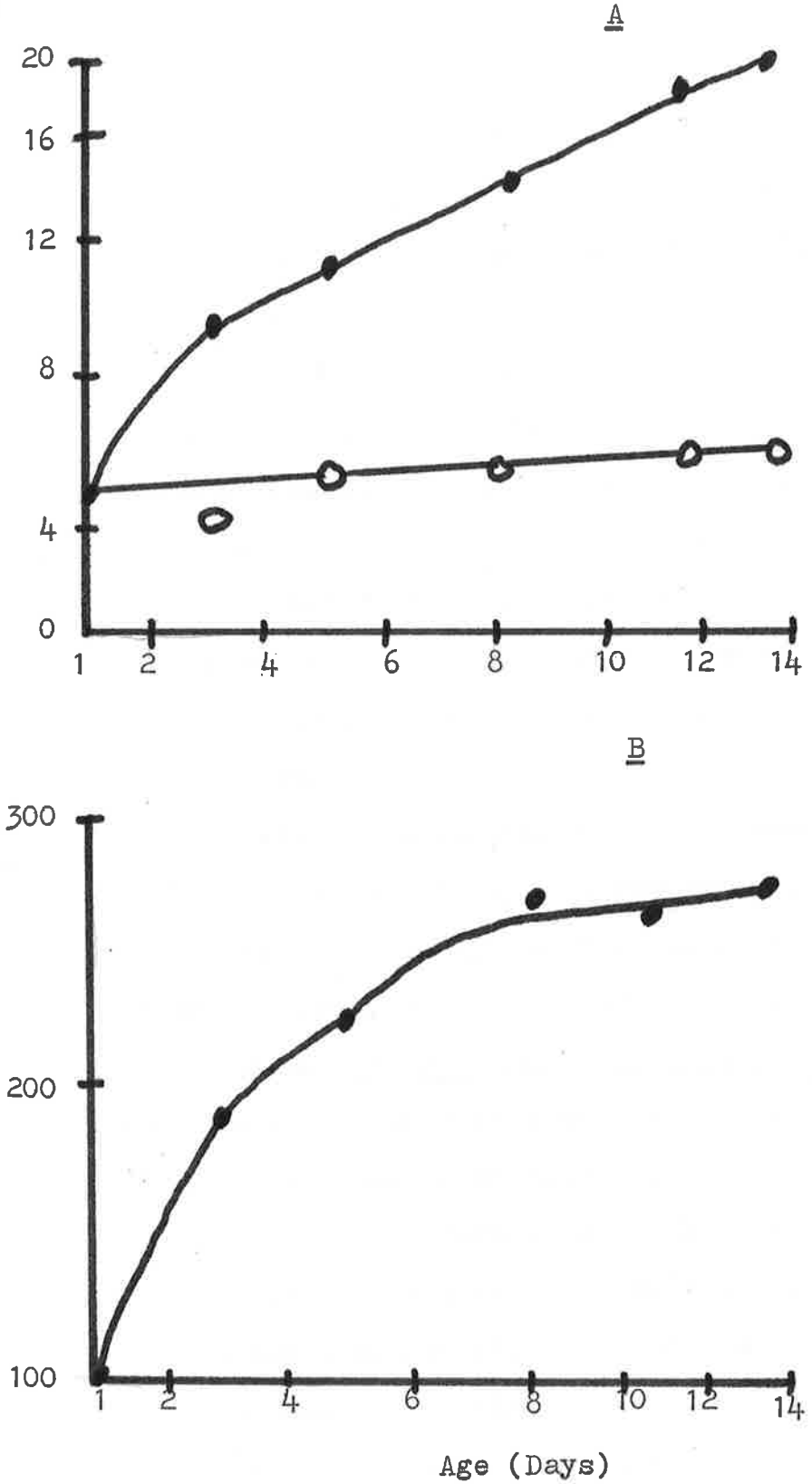


Figure 3. Uterine weight gain in response to estradiol. A, Animals received 10 mg estradiol daily (●—●) or no treatment (○—○). B, weight gain in estradiol treted animals as a percent of the control value.

Illinois, report (figure 4) that there was an increase of ODC activity in rat uteri four hours after an 17-β-estradiol injection. Also, they show in the figure the rats which were treated with dexamethasone (DEX), an inhibitor of "estrogen-stimulated uterine wet weight and ODC activity."[8]

It can now be seen that ODC concentration and activity is increased in a rat uterus by estrogen, but what has other research shown? First of all, B.J. Murphy concluded in 1976 that ODC in a rat liver was only found in the cytoplasm.[9] Several other researchers concluded the same.[10] Second, M.H. Goyns collected data about hormones known to stimulate ODC activity in animal tissues which is found in Table 1, and suggested that ODC was located in the rat liver nucleus.[12] Third, Hadar Emanuelsson, from the Department of Zoophysiology at the University of Lund/Sweden, proved by the use of electron microscope autoradiography that ODC was located in the nucleus, especially the nucleolus, of a polychaete or sandworm (Ophryotrocha labronica), as well as in the cytoplasm. He used tritium labeled alphadifluoromethylornithine which is an enzyme-activated irreversible inhibitor of ODC, forming a covalent bond with it.[13] Fourth, Ian Zagon used the same procedure as Emanuelsson on the mouse kidney and found ODC mainly in the cytoplasmic proximal convoluted tubules, but also to a level of 10-15% ODC in the nuclei, especially the nucleolus.[14] Finally, Alan Bitonti, from the Department of Pharmacology at Ohio State University, discovered that cytoplasmic ODC may migrate into the nucleus of rat liver after treatment with

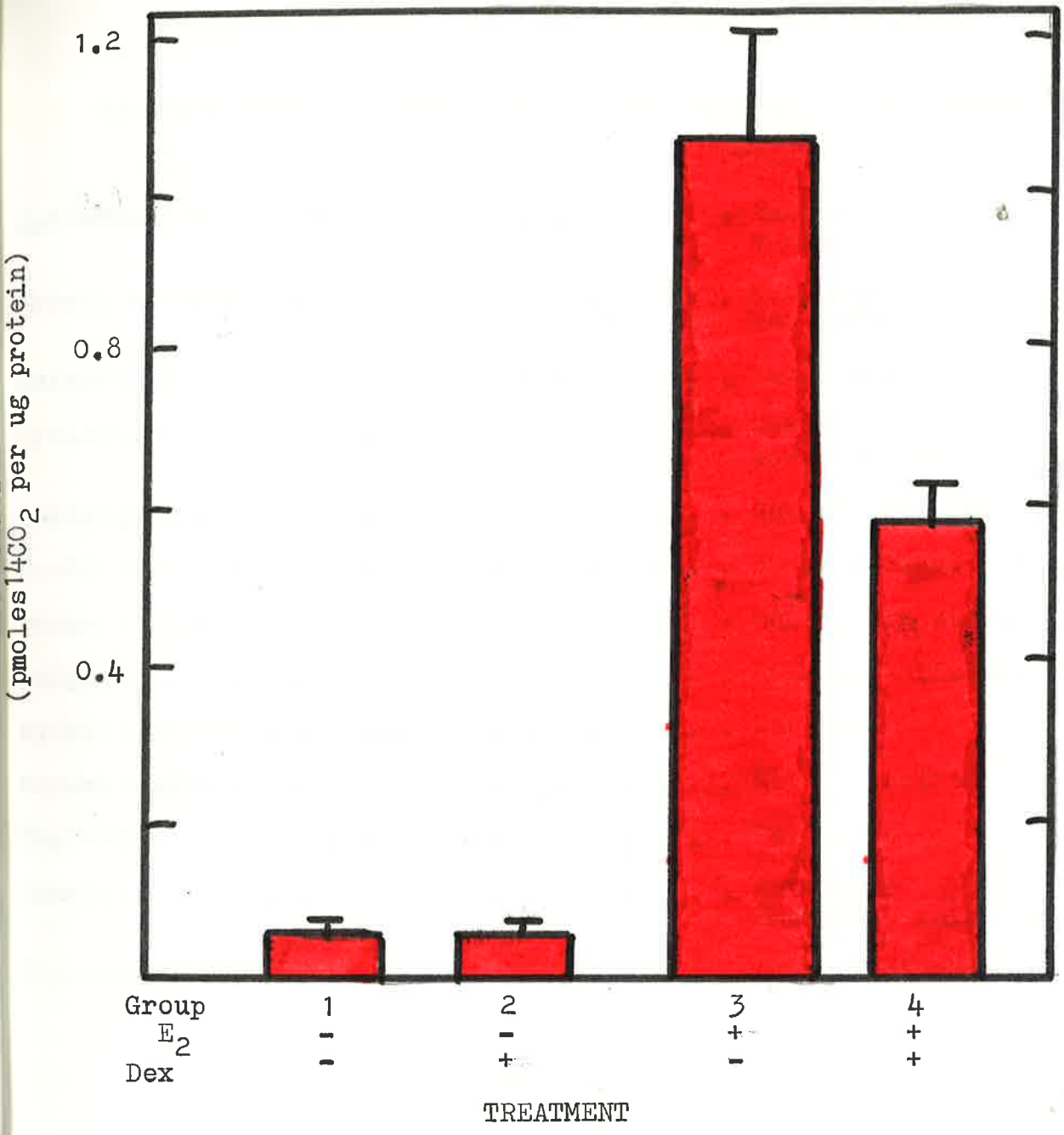


Figure 4. Ornithine Decarboxylase (ODC) activity 4 hr. After treatment with Dex (an inhibitor of ODC) or nothing (-) and with estradiol (E_2) or nothing (-). The bars represent the mean $\pm$ s.e.m. for 9 separate animals.

HORMONES KNOWN TO STIMULATE ODC ACTIVITY IN ANIMAL TISSUES

HORMONE	TISSUE
Epidermal growth factor	Embryonic chick epidermis mouse skin
Growth hormone	Rat heart Rat liver
Luterinizing hormone	Rat ovary
Prolactin	Rat tissues mouse mammary epithelium
Oestrogens	Rat uterine hormogenate
Testosterone	Human seminal plasma
Progesterone	Guinea pig uterus
Ecdysone	Silk moth tissues
Hydrocortisone	Rat liver
Dexamethasone	Rat HTC cells
Thyroxine	Rat heart
Insulin	Rat liver Embryonic chick epidermis
Glycagon	Adrenalectomized rat liver

Table 1.

1-methyl-3-isobutylxanthine (MIX).[15] (MIX causes rapid increases in cytoplasmic and nuclear ODC activity in rat liver.)

Materials and Methods

Animals

Forty-eight Albino Sprague-Dawley rats were ordered from Charles River Company, Wilmington, Delaware. They arrived 21 days old and the first experimental group was killed at 24 days old. The second experimental group was killed at 25 days old. They were fed dog food and water.

Biochemicals

There were several chemical agents to be mixed. One was phosphate buffer, for which 300 mg of $NaH_2PO_4 \cdot 7H_2O$ was added to 100 ml of water. Its pH as well as the following solutions were adjusted to 7.2 by using sodium hydroxide or hydrochloric acid. Second, 12.35 mg of pyridoxal phosphate was added to 50 ml of this phosphate buffer. Third, 121 mg of TRIS buffer was added to 1000 ml of water to prepare a 10 mM solution. Fourth, to make the cycloheximide solution, 10 mg of cycloheximide was added to 25 ml of .9 percent NaCl. Sixth, 81 g of NaCl was added to 90 ml of water. Seventh, one mg of 17-β-estradiol was added to 10 ml of corn oil. Finally, a radioactive ornithine, dithiotheitol solution was made 200 µl of (1-^{14}C) ornithine was added to 1600 µl of the dithiotheitol solution.

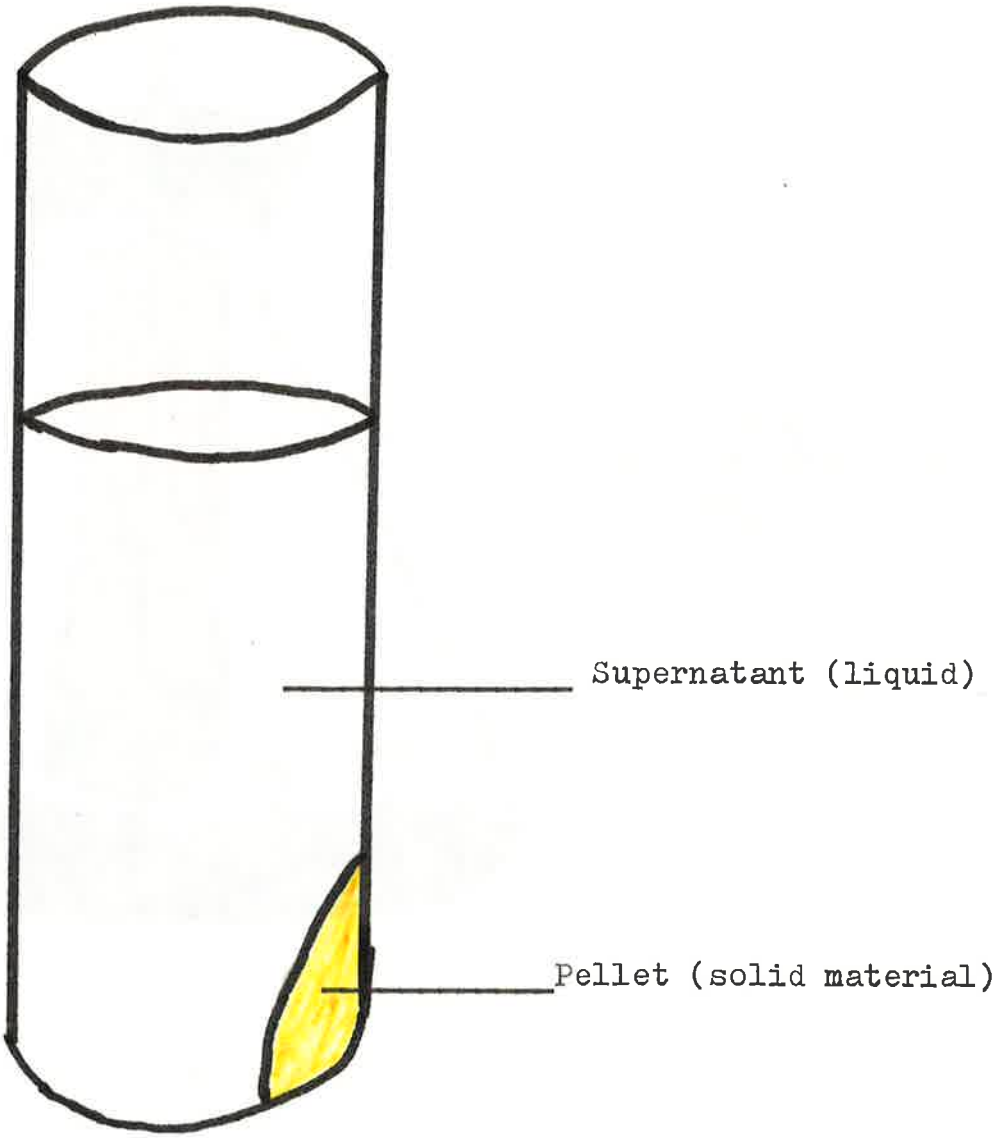
Twelve rats were used per experiment. At 8:30 am, five rats were injected with .5 ml of corn oil and labeled control group. Five more were injected with .5 ml of 17-β-estradiol.

At 10:45 am, two rats were injected with .5 ml cycloheximide. (Cycloheximide inhibits the production of protein in the rat uterus. Thus, there should be little to no ODC activity as compared to the other groups.)

Four hours later (12:30 pm) the rats were decapitated in groups of three: estrogen injected rats, control rats, and cycloheximide injected rats. The uterus was removed and placed in one ml of TRIS buffer and kept on ice. Each uterus was then homogenized for about 10 seconds and placed within centrifuge tubes. They were centrifuged for 10 minutes at 2,500 RPM. When the centrifuge stopped, the supernatant liquid (see figure 5) was pipetted into twelve more tubes and recentrifuged for 10 minutes at 19,000 RPM. The remaining nuclear pellet was resuspended by adding 1 ml tf TRIS buffer and stirring with a glass rod. This was added to a 25 ml flask (figure 6) and 50 µl of the pyridoxal phosphate wolution was added to activate the ODC. (ODC does not react with ornithine unless pyridoxal phosphate is present.)

Then 50 µl of the (1-^{14}C) ornithine (radioactive), dithiothreitol solution was added to each flask and immediately sealed gas tight with a polypropylene center well, into which a 1.5 inch piece of filter paper was suspended from a rubber serum bottle stopper. These twelve flasks were incubated for 30 minutes.

Figure 5.



1. The Nuclear Pellet contains- whole cells, cytoskeletons, and mostly ruptured nuclei.

2. The High Speed Supernatant contains microsomes, small vesicles, ribosomes, viruses, and large macromolecules.

3. The High Speed Pellet has mitochondrin, lysosomes, and peroxisomesin it.

THE 25 ml SEAL FLASK

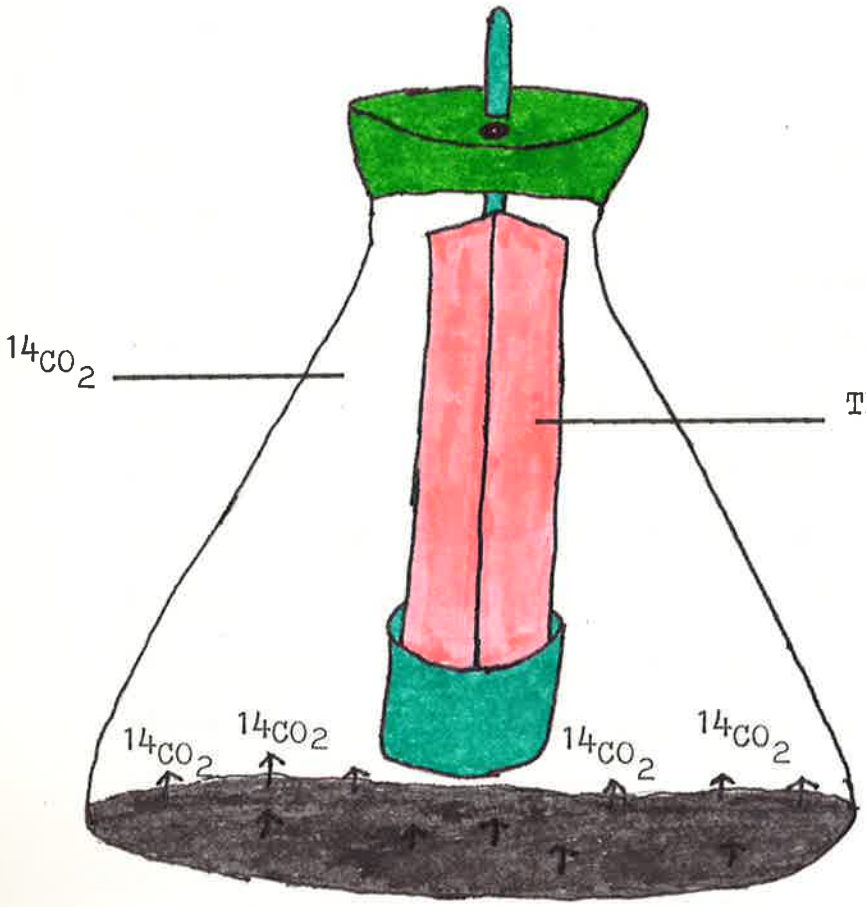


Figure 6

After the centrifugation of the supernatant liquid was completed, they were removed from the centrifuge, and the high speed supernatant pipetted off each one, and placed in individual flasks. The procedure which was performed to the nuclear pellet was performed to these flasks. (This is found on page 6.)

One ml of TRIS buffer was placed in the high speed pellet-the solid remaining in the centrifuge tubes- and stirred with a glass rod. This solution was then placed in the 25 ml flask, and the same procedure performed on page 6 to the nuclear pellet was repeated.

When the nuclear pellet had incubated for 30 minutes, the flasks were removed and each one injected with .2 ml of citric acid into the solution through the rubber stopper. (When the flasks were incubating the first time, the ODC from the uteri converted the radioactive ornithine into putrescine. As this was done, the $^{14}CO_2$ (radioactive carbon dioxide) was released from the ornithine into the atmosphere of the flask. (See figure 6) The citric acid which was added inactivated ODC from producing further $^{14}CO_2$ and putrescine.)

Then .2 ml of methyl benzothonium hydroxide was injected into the flasks on the 1.5 inch piece of paper-figure 6- and allowed to incubate for 30 minutes. This chemical absorbs the radioactive ($^{14}CO_2$) carbon dioxide from the atmosphere.

After the high speed supernatant had incubated for 30 minutes, the same procedure was performed on it and on

the high speed pellet. Both were allowed to reincubate for 30 minutes.

When the nuclear pellet had incubated the second time for 30 minutes, the rubber stopper was removed. The paper was taken out and placed in a small vial with 15 ml of scintillation fluid (econofluor-2) and capped.

The high speed supernatant and high speed pellet in turn was treated the same way.

These vials were taken to the Research Triangle Park in Raleigh for a liquid scintillation counter count of the radioactivity of the paper. The radioactivity of the paper was measured for one minute. This information can be seen in table 2.

RESULTS AND DISCUSSION

The experimental and averaged data are shown in tables 2 and 3. In experiment one, the numbers which were obtained from the radioactive counter are used to calculate the relative ODC activity of each group of rats. The relative ODC activity of the injected rats' nuclear pellet was 1.33. To arrive at this, the average of all the nuclear control pellets from experiment one was taken, yielding 2072. An average of all the estrogen injected nuclear pellets was taken, yielding 2521. Of the two cycloheximide nuclear pellets, one was too high to be considered so it was discarded. Since even the controla rats have ODC activity, the average of the injected cycloheximide rats is subtracted from both the control and estrogen group averages. Therefore, the average of the control nuclear pellet group is 1354, while the estrogen injected rats' nuclear pellet is

1803. By dividing the control group into the estrogen group, the relative ODC activity of the injected rats' nuclear pellet is obtained as 1.33. Another way of stating this is that there was a .33 fold increase or 33% increase in ODC activity in the estrogen injected rats' nuclear pellet as compared to the control rats' nuclear pellet. (The .33 was abtained by substracting 1, which was the constant value of the control rats, from 1.33.) However, in experiment two, the average of the control nuclear pellet minus the cycloheximide injected group is 197. The average estrogen injected rats' muclear pellet minus the cycloheximide group is 2442. (One of the cycloheximide injected rats' nuclear pellet was discarded because of human error.) This shows that the relative ODC activity in the estrogen injected rats' nuclear pellet is 12.40 or a 11.40 fold, (1140%), increase as compared to the control rats' nuclear pellet.

By averaging 1.33 and 12.40, a relative nuclear ODC activity of 6.87 was found. (Seen on table 2) The graph of this is shown in figure 7. The fact that there were only two experiments performed, and a wide variation in the ODC activities in each experiment indicates that there is little chance to validate statistically the data without more experimentation. But no further research can be done at this time because of the lack of time and money. However, since in both cases the ODC activity in the estrogen injected rats was higher than the control, there is an indication the ODC activity does occur in the nucleus of immature uterine rats.

	EXPERIMENT 1			EXPERIMENT 2			TOTAL AVERAGE
NUCLEAR PELLET	CON 3672 2651 896 796 2344	EST 1790 2036 4009 2784 1987	CY 718 CON-CY= 1354 ES-CY= 1803	CON 724 434 446 ? 852	EST ? 3543 3471 1422 2999	CY 417 CON-CY= 197 EST-CY= 2442	12.40 + 1.33
	Average 2072	Average 2521	EST/Con=1.33	Average 614	Average 2859	EST/CON=12.40	6.87
HIGH SPEED SUPERNA-tant	CON 736 1738 1557 2878 ?	EST 9117 11608 8575 ? 15608	CY-576 894 735 CON-CY= 992 EST-CY= 10492	CON 753 579 ? 1309 571	EST 8229 11862 ? 16729 13960	CY 431 CON-CY= 372 EST-CY= 12264	33.00 10.58
	Average 1727	Average 11227	EST/CON=10.58	Average 803	Average 12695	EST/CON=33.00	21.79
HIGH SPEED PELLET	CON 773 1106 950 1220 ?	EST 1063 893 766 ? ?	CY 654 754 707 CON-CY= 305 EST-CY= 200	CON 437 418 467 792 534	EST 762 472 645 777	CY 501 CON-CY= 29 EST-CY= 163	5.62 .66
	Average 1012	Average 907	EST/CON = .66	Average 530	Average 664	EST/CON= 5.62	3.14

Table 2.

	EXPERIMENT 1	EXPERIMENT 2	AVERAGE
RELATIVE NUCLEAR ODC ACTIVITY	1.33	12.40	6.87
RELATIVE HIGH SPEED SUPERNATANT ODC ACTIVITY	10.58	33.00	21.79
RELATIVE HIGH SPEED PELLET ODC ACTIVITY	.66	5.62	3.14

Table 3

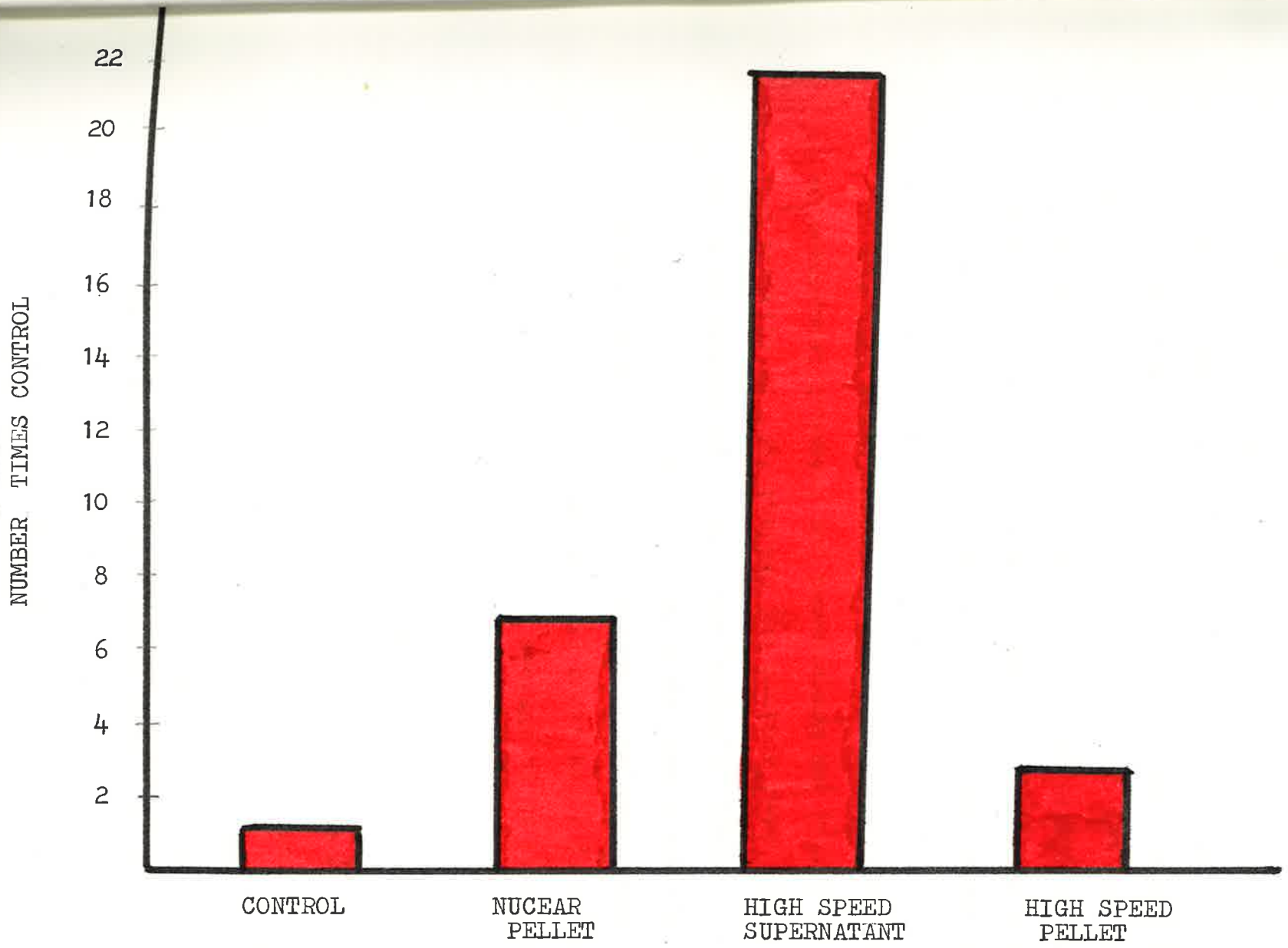

NUMBER TIMES CONTROL
22
20
18
16
14
12
10
8
6
4
2
CONTROL
NUCEAR PELLET
HIGH SPEED SUPERNATANT
HIGH SPEED PELLET

Figure 7

In the high speed supernatant, the first experiment's average control ODC activity is 992. (One of the controls had to be discarded due to human error.) The estrogen injected rats' average ODC activity is 10492. By dividing 10492 by 992, the estrogen injected rats' supernatant has an ODC activity 10.58 times that of the control rats. In the second experiment, the average estrogen ODC activity was 33.00 times that of the control rats. The average of 10.58 and 33.00 is 21.79 which indicates that the supernatant ODC activity is higher than the nuclear pellet as seen in figure 7. Again however, there is little chance to validate this from statistical analysis.

The high speed pellet in the first experiment includes mitochondrin, lysomes, and peroxisomes has relative average ODC activity of .66, whereas the control rats' ODC activity is on an average of 1.00. This indicates that ODC activity does not increase in this part of the cytoplasm when stimulated by estrogen. In the second experiment, however, the relative average ODC activity is shown to be 5.62 times that of the control rats. The average of the high speed is 3.14.

CONCLUSION

The experiment shows that there is an indication of increased ODC activity in the nucleus after estrogen treatment. There is an even stronger indication that ODC activity increases after estrogen stimulation in the supernatant of the cytoplasm. (Some microsomes, small vesicles, ribosomes, viruses, and large macromolecules.) This of course is consistent with findings of previous researchers. Also, the

high speed pellet which includes mitochondrin, lysosomes, and peroxisomes shows almost conclusively that ODC activity increases very little if any after estrogen treatment. Further experiments will allow statistical verification of these data.

Hence, by learning more detailed effects of the subcellular location of ODC activity, scientists can learn more about the embryonic development in the uterus because it is stimulated to grow by ODC. In this way, the inhibition of ODC could be used as a form of birth control.[16] ODC is also responsible for the replication of some viruses in host cells. Therefore, by gaining more knowledge about its activity, it is possible to isolate this viruses and stop their growth.[17]

REFERENCES

[1]Ian S. Zagon, Patricia J. McLaughlin, James E. Seely, Greg W. Hocksema, and Anthony E. Pegg, "Autoradiographic Localization of Ornithine Decarboxlyase In Mouse Kidney By Use of Radiolabeled Alpha-Difluoromethylornithine," Cell And Tissue Research, 235 (1984), p. 371.

[2]Anthony E. Pegg, and Peter P. McCann, "Polyamine Metabolism And Function," The American Physiological Society (1982), p.4.

[3]Ibid.

[4]Ibid.

[5]R.W. McGilvery, Ph.D. Biochemistry, A Function Approach, sec. edition, (W.B. Saunders Company, Philadelphia, PA, 1979), p. 628.

[6]Alvin Silverstein, Human Anatomy And Physiology, sec. edition; (John Wiley And Sons, NY, 1983), p. 604.

[7]Daniel M. Sheehan, William S. Branham, Kevin L. Medlock, Mary E. Olson, and David R. Zehr, "Uterine Response To Estradiol In The Neonatal Rat," Endocrinology, 109 (1981), p. 77.

[8]P. J. Stewart, C. J. Zaloudek, M. Murphy Inman and R. A. Webster,"Effects of Dexamethasone And Indomethacin On Estrogen-Induced Uterine Growth," Life Sciences, 33 (Sept. 21, 1983), p. 2352.

[9]Hadar Emanuelsson, Olle Heby, "Nuclear Ornithine Decarboxylase. Electron Microscope Autoradiographic Identification Of The Active Enzyme Using Alpha ($5-^3H$)Difluoromethylornithine," European Journal Of Cell Biology, 30 (Feb. 12, 1983), p. 293.

[10]Ibid.

[11]M. H. Goyns, "The Role Of Polyamines In Animal Cell Physiology," Journal Of Theory Biology, 97 (Dec. 10, 1982), p. 579.

[12]Ibid., p. 585.

[13]Emanuelsson, p. 288.

[14]Zagon, p. 371.

[15]Alan J. Bitonti, and Daniel Couri, "Drug-Induced Migration Of Cytoplasmic Ornithine Decarboxylase Into Rat Liver Nucleus Is Not Related To Increased RNA Polymerase Activity," Biochemical And Biophysical Research Communications, 99 (April 30, 1981), p. 1040.

[16]Pegg, p. 219.

[17]Ibid., p. 217.

BIBLIOGRAPHY

Bitonti, Alan J. and Couri, Daniel. "Drug-Induced Migration of Cytoplasmic Ornithine Decarboxylase Into Rat Liver Nucleus Is Not Related To Increased RNA Polymerase Activity." Biochemical And Biophysical Research Communications, 99 April 30, 1981), p. 1040-1044.

Emanuelsson, Hadar and Heby, Olle. "Nuclear Ornithine Decarboxylase. Electron Microscope Autoradiographic Identification Of The Active Enzyme Using Alpha (5-^{3}H)Difluoromethylornithine." European Journal Of Cell Biology, 30 (Feb. 12, 1983), 288-294.

Goyns, M.H. ."The Role Of Polyamines In Animal Cell Physiology." Journal Of Theory Biology, 97 (Dec. 10, 1982), 577-589.

Kaye, Alvin M. and Icekson ."Stimulation By Estrogens Of Ornithine And S-Adenosylmethionine Decarboxylases In the Immature Rat Uterus." Biochemistry And Biophysiology, 252 (June 7, 1971), 150-159.

McGilvery, R.W. Biochemistry, A Function Approach, sec. edition, (W.B. Saunders Company, Philadelphia, PA, 1979), p. 628.

Pegg, Anthony E. and McCann. "Polyamine Metabolism And Function," The American Physiological Society (1982),

Sheehan, Daniel M. and Branham, Medlock, Olson, Sehr. "Uterin Response To Estradiol In The Neonatal Rat," Endocrinology, 109 (1981), p. 77.

Silverstein, Alvin. Human Anatomy And Physiology, sec. edition; (John Wiley And Sons, NY, 1983), p. 604.

Stewart, P.J. and Zaloudek, Inman, Webster. "Effects of Dexamethasone And Indomethacin On Estrogen-Induced Uterine Growth," Life Sciences, 33 (Sept. 21, 1983), p. 2352.

Tabor, Celia White and Tabor. "Polyamines," Annual Rev. Biochemistry 54 (1984), pp. 749-790.

Turner, Donnell C. and Bagnara, Josrph. General Endocrinology, sixth Edition, (W.B. Saunders Company, Philadelphia, PA, 1976), p. 327.

Zagon, Ian S. and McLaughlin, Seely, Hocksema, Pegg. "Autoradiographic Localization of Ornithine Decarboxlyase In Mouse Kidney By Use of Radiolabeled Alpha-Difluoromethylornithine," Cell And Tissue Research, 235 (1984), p. 371.