The Effect of Estradiol 17B on Ornithine Decarboxylase and Insulin-like Growth Factor I During Growth

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by

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Abstract

There is no doubt that estrogens like Estradiol 17B are potent stimulatons of the growth process by exerting their effect in many ways and through many different processes. Ornithine Decarboxylase (ODC) and Insulin-like Growth Factor I (IGF-I) are just two of the many that Estradiol 17B stimulates. The estrogenic induction of increased ODC levels is beleived to occur at the messenger RNA level whereas it has been shown that the same mechanisms are responsible for the increased activity of IGF-I. An attempt was made to reveal with experimental evidence that indeed Estradiol 17B elevates ODC levels via mRNA; however, due to unforseen complications, a denatured probe, and the crucial element of time, the means for detecting ODC were not viable. As a result, another growth index, of interest, was used to illustrate that Estradiol 17B effects the growth process in other ways as well. A non-isotopic probe, a biotinylated oligonucleotide, was used to detect the increased activity of IGF-I. However, through our research, we were able to show that biotinylated probes are not effective enough to produce conclusive results about IGF-I activity; non-isotopic probes like the particular one we used probably perform much more efficiently when the expression of their complement is high. However, the expression of IGF-I's mRNA is minimally low and probably explains for this inconclusiveness. Other research has indicated that there is a direct relationship between IGF-I and ODC; it has been postulated that Estradiol 17B stimulates IGF-I which in turn stimulates

ODC and so on with the end result being growth. With this research laying some groundwork and opening some new doors, the possibility of a role between IGF-I and ODC can be shown by establishing a temporal relationship between these two growth related factors.

Introduction

As most people are aware, there are many different hormones found throughout the human body and a list of their functions goes on and on. Specifically speaking, I am interested in the action of Estradiol 17B on the activity of ornithine decarboxylase (ODC) in target organs such as the uterus. Estradiol 17B is a type of steroid hormone called an estrogen which is a naturally occurring substance in the human body. One of the more prominent effects of this hormone is that it induces growth and differentiation in the uterine lining, or endometrium; this induction is primarily responsible for preparing the uterus to receive and nourish an embryo (Darnell, 718). ODC is the rate-limiting enzyme in the biosynthesis of polyamines which are primarily responsible for growth regulation and differentiation in many tissues, especially the uterus (Heby, 12). In short, most tissues in response to high ODC activity demonstrate accumulation of polyamines, and consequently stimulation of growth (Pegg, C212).

Since ornithine decarboxylase is an enzyme, its composition is that of a protein, and therefore its synthesis is directed by the cell. In the first step of protein synthesis, the DNA of given cells performs a process called transcription in which the information it contains is "transcribed" or copied by another cellular constituent called messenger RNA. This, in turn, undergoes a process called translation in which the messenger RNA is "translated" into specific sequences of amino acids. These specific amino acids collect-

ively form a particular protein or, in this case, a particular enzyme. This entire process involving transcription and translation is termed protein synthesis (Lewin, 113). Since this process is responsible for the manufacture of ODC, and it has been shown that Estradiol 17B exerts a positive effect on the activity of this enzyme, then the hormone "makes its debut" either on the DNA level affecting transcription, on the messenger RNA thus affecting translation, or directly on the enzyme itself. Previous research, using metabolic inhibitors which can be used to block transcription or translation, has revealed some interesting facts. Firstly, a metabolic inhibitor called Actinomycin-D can be used to block transcription; however, even after its usage, an increase in the activity of ODC is still observed in the uteri of ovariectomized rats after the administration of Estradiol 17B (Clark and Greenspan, 253-254). From this experimental research, two hypothesis can be drawn. Either the estrogen does not effect ODC production at the DNA level or Actinomycin-D is not completely effective; more than likely, Estradiol 17B affects ODC activity through some post-transcriptional mechanisms (Clark and Greenspan, 258). Secondly, another metabolic inhibitor called cycloheximide stops translation. Under this application, the levels of ODC activity decrease dramatically which strongly suggest that Estradiol 17B exerts its effect at the messenger RNA level (Kaye, Icekson, and Lindner, 156-157).

Although Estradiol 17B affects many different elements and utilizes many different pathways to stimulate growth like

the stimulation of other growth indices like Insulin-like Growth Factor I (IGF-I) or Platelet Derived Growth Factor (PDGF), it is the point at which this estrogen intervenes in the role of ODC production and polyamine synthesis, and, consequently, the growth process which is the crucial question which is the crucial question to be addressed by my research (Murphy and Friensen, 325). Is the messenger RNA for ODC the responsive component that induces its increased activity or is it perhaps another factor, in response to Estradiol 17B, acting on ODC. Question asked are not always so easily answered, the pivotal point of any research.

Purpose/Experimental Design

With the help of Dr. Harold D. Maxwell and Dr. David Kuo, an experiment was designed to determine the point at which Estradiol 17B intervenes in the role of ODC production, polyamine synthesis, and the growth process. Through several investigative and research procedures, perhaps some insight into the mechanisms that Estradiol 17B employs will be gathered. A brief overview of these procedures and a simple explanation of each follows:

-Ten ovariectomized rats will be obtained with five receiving administration of Estradiol 17B and the other five serving as control (no estrogen). The ovaries have been removed, or ovariectomized, to ensure that the uteri are responding only to the estrogen injected and not that normally secreted by the ovaries. Although all tissues respond to estrogens in some way, the uterus is a better target organ because of its periodic and cyclic response to the hormone involved in ovulation and menustration (Eckert, Randall, and Augustine, 314).

-After sufficient time has elapsed, approximately four hours, allowing the estrogen to exhibit its influence on the uterus, both those injected with Estradiol 17B and those serving as control will be sacrificed and their uteri removed.

-Most tissues can be easily homogenized, mixed, or "blended" into a solution exhibiting the same consistency throughout; however, uterine tissue is is mainly comprised of connective-supportive tissue which resist these efforts (Marieb, 932). To homogenize the uteri, they are submerged into a liquid nitrogen bath which freezes them solid. This allows the tissue to be easily pulverized, or crushed, with a hammer into a powder-like texture so that a solution can be easily prepared; the pulverization of the uterine tissue provides a more efficient homogenization (The Fast Track..., 31).

-The powder-like, pulverized rat uteri are placed in a cold solution of guanidine isothiocyanate in the presence of 2-mercaptoethanol. These chemicals inactivate endogenous ribonucleases which are enzymes that can degrade RNA and disturb

its sequence information. The samples are then treated with ethanol which is used to precipitate the total RNA package (transfer, ribosomal, and messenger) from its deactivated RNAses (Messenger RNA Isolation System..., 3). Since the stability of messenger RNA is important, all RNAses should be removed if possible. Although there are many other factors involved in the mRNA decay process, the enzymatic activity of ribonucleases are one of the more important determinants of RNA's stability (Brawerman, 6). Successive and repeated washes with high concentrations of the chaotropic salts and ethanol are both followed each time with centrifugation. Centrifugation is a technique that is used to separate cellular components on basis of their molecular weights and densities by spinning at very high rates of speed on a gradient (Albents, Bray, Lewis, Roff, Roberts, and Watson, 168). Once this is completed, the samples that remain, for the most part, are samples of total RNA (messenger, transfer, and ribosomal).

-Since the remaining samples are that of a total RNA "package" and it is only messenger RNA that we need to be concerned with, these samples must be further purified. This is accomplished using affinity chromatography. The majority of eukaryotic mRNAs contain poly(A) tails at their 3' termini ranging from 50 to 200 nucleotides in length. In other words, at one end of most mRNAs are "tails", or sections, that are high in adenine residues. It is this feature, the poly(A) tail, which can be utilized to separate messenger RNA from the samples of total RNA. The samples of total RNA are passed over a matrix of deoxythymidine oligomers. Since thymidine is the complimentary base to adenine, and messenger RNA only possess the poly(A) tail, they hybridize with this matrix and all the other non-polyadenylated species pass through unattached. An elution buffer is the passed through the column of deoxythymidine oligomers which "dehybridize", or removes, the Poly(A) messenger RNAs from the matrix. This sample which can be collected now contains a yield of 90% or higher of mRNA (Messenger RNA Isolation System, 3-4).

-The collected messenger RNA samples are then passed through a filtration manifold where they are concentrated into a small oval-shaped well onto a piece of nitrocellulose paper. A filtration manifold is an apparatus which employs the use of suction from a vacuum hose to pull samples through the wells and onto the nitrocellulose paper. It is at this point that you have the 90% or more of

the messenger RNA concentrated in one small slot (Sambrook, Fritsch, and Maniatis, 7.53).

-The next preparation involves the labeling of the oligonucleotide to be used for this research. As staed earlier, we had intended on using a probe to label the messenger RNA for ornithine Decanboxylase; however, to our dissappointment, this probe was denatured and therefore not viable to our research. So, in the consideration of time effort, we decided to explore another growth index affected by Estradiol 17β; this growth index is better known as Insulin-like Growth Factor I. This substance's activities is increased through the estrogenic response of its messenger RNA. Therefore we decided to show that its mRNA levels increased as well

-Since the probe for ODC was not viable and a replacement could not be ordered, the oligonucleotide for IGF-I's probe preparation and the preparation kit were ordered. However, the oligonucleotide cannot be labeled because the level of radioisotopic labeling needed requires a state license, something else we did not have. It was at this point that we decided to use non-isotopic labeling. The gene specific oligonucleotide probe, Human Insulin-like Growth Factor II, 5' liver was obtained from CLONTECT Laboratories, Inc., as was the labeling systems (Product Analysis Certificate, 1). The oligonucleotide is labeled with a sulfur-containing urea derivative called biotin. When biotin is incubated with a streptavidin complex/substrate, this enzymatic reaction will produce a pink color whereever biotin is found. So, if biotin is bound to the probe and the probe is hybridized to mRNA, then the intensity of its coloration can will define how much of the mRNA that exist in each sample (Marieb, 813), (CLONTECH's Protocol for Biotin-21-dUTP..., 1)

-Once the probe has been labeled, it is then incubated with the nitrocellulose paper containing the dots of three messenger RNAs from the rats. This is done so the oligonucleotide may hybridize or bind with the messenger RNA of interest which is that belonging to IGF-I. Hybridization is allowed to occur for approximately 18-24 hours so that the mRNA and the oligonucleotide do anneal to one another (CLONTECH's Protocol for Biotin..., 10). After hybridization, detection of the biotinylated nucleic acid is performed to allow direct colorimetric determination of the amount of messenger RNA present in the samples (CLONTECH's

Protocol for Biotin..., 11). There are three dots or slots on the nitrocellulose paper: slot 1-uteri from control rats (those that have not been affected by estrogen), slot 2-uteri from injected rats (affected by estrogen), slot 3-brains from control rats (To be used for comparative analysis). If all goes as planned, slot 2 should demonstate a greater detection profile than slots one and two. Since it was the only one under the influence of Estradiol 17B, the activity of growth indices should be increased whereas the the others should reflect basal levels of activity.

Experimental Procedures/Protocols

Preparation of Rats

10 ovariectomized rats were ordered from Charles River Company. Five of these ten rats were injected with fifty micrograms of Estradiol 17B, whereas the remaining five rats were used as control meaning that they were not administered any estrogens. After a duration of four hours, the rats were then sacrificed and their uteri removed. The brains were also removed from the control rats so that some additional comparison could be made.

Isolation of Total RNA

1. Soak the probe of a power homogenizer for at least 30 minutes in fresh DEPC-H_2O to inacivate RNases that may be present. Once completed, the probe must be chilled in 250 ml of ice-cold autoclaved distilled water for 5 minutes.

2. Prepare 25 ml of guanidine isothiocyanate/2-mercapto-ethanol (GuSCN/ME) solution through the additon of 2 ml of 2-mercatoethanol to 23 ml of guanidine isothio-cyanate solution in a 50-ml sterile, disposable polypropylene centrifuge tube. Mix by inversion and chill on ice.

3. Add 15 ml of the GuSCN/ME solution to a 50-ml poly-propylene centrifuge tube and place on ice for 10 minutes.

4. Quickly place one gram of tissue in a weight boat. Freeze by submerging in liquid nitrogen. Place another weigh boat on top of the tissue. Fragment and pulverize the the tissue using a hammer. Transfer the tissue sample to the ice-cold GuSCN/ME solution in the 50 ml centri-fuge tube and immediately homogenize it with the large tissumizer until it is completely disrupted (20 to 40 seconds at maximum speed). Keep it cold.

5. Transfer the tissue homogenate to a 50 ml teflon centri-fuge tube. Add 4.5 ml of ethanol (0.3 volume), mix inversion several times and centrifuge the suspension at 16,000xg for 5min at 4°C.

6. During the centifugation, clean the homogenizer by running it in several changes of cold, autoclaved DEPC-distilled water.

7. Remove the supernatant fraction and the floating protein film that will appear after centrifugation by careful aspiration. Use a sterile glass pipette and remove the supernatant fraction carefully from the top down for it is high enriched in denatured RNases.

8. Add 1 ml of the ice-cold GuSCN/ME sol'n to the RNA pellet and resuspend it using a 1000 ul Pipetman. Add 6.5 ml GuSCN/ME and homogenize it for 10s at 70% to 100% maximum speed. This is done on the 50 ml centrifuge tube.

9. Centrifuge the solution at 16,000xg for 3 minutes at 4°C. Transfer the supernatant fraction to a sterile, 30 ml Corex centrifuge tube and discard the pellet.

10. Add 188 ul of 1M acetic acid (0.025 volume), mix, and 5.6 ml of ethanol (0.75 volume). Mix by gentle trituration with the ethanol pipet, cover with foil, and chill at -20°C for 10 minutes.

11. Prepare 25 ml of guanidine (GuHCl/ME) sol'n by adding 20 ml of 2-mercaptoethanol to 25 ml of guanidine hydrochloride sol'n in a 50-ml sterile, disposable, polypropylene centrifuge tube. Mix by inversion and chill on ice for 10 min.

12. Centrifuge the second GuSCN/ME extraction at 7000xg for 10 min at 4°C and remove the supernatant fraction by aspiration with a glass pipet.

13. Add 1 ml of the GuHCl/ME sol'n to the RNA pellet from step 12. Using a Pipetman, resuspend and emulsify the pellet. Add 9 ml additional GuHCl/ME and continue to mix by trituration (repeated expulsion of the pellet from a sterile pipet.

14. Add 0.5 ml of 1M acetic acid (0.05 volume) and 5 ml of ethanol (0.5 volume). Mix by trituration and cover with foil. Chill at -20°C for 10 minutes

15. Centrifuge at 7000xg for 10 minutes at 4°C and aspirate the supernatant fraction.

16. Add 1 ml of GuHCl/ME sol'n to the RNA pellet, resuspend with a Pipetman, add an additional 6ml of GuHCl/ME and dissolve by trituration with a sterile pipette.

17. Add 0.35 ml of 1M acetic acid (0.05 volume) and 3.5 ml of ethanol (0.5 volume). Mix by trituration and chill at -20°C for 10 minutes.

18. Centrifuge at 7000xg for 10 minutes at 4°C and aspirate the supernatant fraction.

19. Add 1 ml of GuHCl/ME sol'n to the RNA pellet. Resuspend with a Pipetman, add 4 ml additional GuHCl/ME and mix by trituration with the pipette.

20. Add 1 ml of 1 M acetic acid (0.05 volume) and 2.5 ml of ethanol (0.5 volume). Mix by trituration with the ethanol pipet, cover with foil, and chill at -20°C for 10 min.

21. Centrifuge at 7,000 x g for 10 min at 4°C and aspirate the supernatant fraction.

CAUTION: It is extremely important to observe all precautions designed to prevent introduction of RNase into the sample from this point until the end of the procedure.

22. Add 1 ml of TEN (DEPC-treated) (at room temperature) to the RNA pellet and resuspend with your Pipetman. Add an additional 2 ml of TEN and dissolve by trituration. TEN is 10 mM Tris, pH 7.5, 100 mM NaCl and 1 mM EDTA.

23. Remove a 50 ul aliquot to a microcentrifuge tube with 950 ul TEN. Read the OD_{260} blanked against TEN and calculate the amount of RNA recovered.

The RNA concentration in ug/ml can be determined by the formula:

$$\text{total RNA} = A_{260} \times \text{dilution factor} \times 40\text{ug/ml}/A_{260} \times 3\text{ml}$$

where A_{260} is the absorbance of the solution at 260nm, 40ug/ml/A_{260} is the conversion factor relating absorbance to concentration, 20 is the dilution factor, and 3ml is the total volume.

24. Add 160 ul of (DEPC-treated) 5M NaCl, mix well. Add 8 ml (2.5 volumes) of ethanol. Mix by trituration several times, cover with foil, and chill at -20°C for 10 min.

25. Centrifuge the RNA in ethanol at 7,000 x g for 10 min at 4°C and aspirate the supernatant fraction. Drain on a layer of sterile Kimwipes for 5 min. Cover with parafilm, poke several small holes with a needle and dry in Speed Vac (without rotor) for 10 min.

Isolation of mRNA by Oligo-dT Cellulose Chromatography

A. Preparation of Oligo-dT column

1. Remove the end cap from the Oligo-dT column and clamp the column to a ring stand. Always keep the column covered when it is draining.

2. Load 1 ml of 0.1 M NaOH onto the column and let it drain completely.

3. Equilibrate the column with 4 ml of binding buffer in 1 ml aliquots. The column now is ready to use.

B. RNA Sample Preparation

1. Add 3 ml of binding buffer to the RNA pellet and dissolve by trituration using a sterile pipette.

2. Heat the RNA solution in a 70°C water bath for 5 min and chill on ice for 5 min. If the SDS in the buffer precipitates, warm it to room temperature only and swirl it until the SDS goes back into sol'n.

3. If there are any particulates that do not go into solution, remove them by centrifugation at approximately 1,000 x g for 5min at room temperature and retain supernatant fraction. A small, table-top clinical centrifuge is sufficient for this purpose.

4. Dilute a 10 ul aliquot from the clarified solution with 990 ul of binding buffer. Read absorbance at 260 nm and calculate amount of total RNA. If the amount of RNA exceeds 20 mg. the column must be ran more than once to prevent overloading..

5. Load the dissolved RNA onto the column under gravity flow and wash with 4 ml of binding buffer to elute non-messenger RNA.

6. Elute the messenger RNA with 1.5 ml of elution buffer and collect the eluate as one fraction in a 15-ml sterile, disposable centrifuge tube.

7. Reequilibrate th Oligo-dT cellulose column with 4 ml of the binding buffer.

8. Heat the RNA from step 6 in a 70°C water bath for 5min and chill in an ice-water bath for 5 min.

9. Place the RNA solution at room temperature for 20 min, then add 90 ul of 5M NaCl. Immediately load the RNA onto the column and wash it with 4ml of binding buffer.

10. Elute the messenger RNA with 1.5 ml of elution buffer and collect the eluate as one fraction in a 15-ml sterile, disposable centrifuge tube.

11. Add 90 ul of 5M NaCl and 3 ml of ethanol to the RNA. Place the tube at -20°C overnight, or until needed.

12. Centrifuge at 7000 x g for 20 min at 4°C and remove the supernatant fraction.

13. Carefully add 1 ml of ethanol to the tube and centrifuge at 7000 x g for 2 min at 4°C.

14. Remove the supernatant fraction and dry the pellet at room temperature until the ethanol has evaporated completely, approximately 30 min. This can be hastened by using a heating block at 50°C for approximately 10 min.

15. Dissolve the pellet in a minimal volume of DEPC-treated EDTA solution (5 to 50 ul is recommended) and store at -70°C if available; if not, store at -20°C.

Preceding procedures were adapted and taken from the "Messenger RNA Isolation System Instruction Manual" which is provided by Bethesda Research Laboratories out of Bethesda, Maryland.

Dot Hybridization of Messenger RNA

1. Wet a piece of nitrocellulose (0.45-micron pore size) briefly in water and soak it in 20X SSC for 1 hour at room temperature. Meanwhile, clean the manifold carefully with 0.1 N NaOH and then rinse it well with sterile water.

2. Place two sheets of heavy, absorbent paper, previous-wetted with 20X SSC, on top of the vacuum unit of the apparatus. Place the wet nitrocellulose on the bottom of the sample wells cut into the upper section of the manifold. Smooth away any air bubbles trapped between the upper section of the manifold and the nitrocellulose. Clamp the two parts of the manifold together, and connect the vacuum unit to a vacuum line.

3. Fill all of the slots with 10X SSC, and apply gentle suction until all of the fluid has passed through the nitrocellulose filter. Turn off the vacuum and refill the slots with 10X SSC.

4. Mix the RNA (dissolved in 10 ul of H_2O) with:
   20 ul of 100% formamide
   7 ul of formaldehyde (37%)
   2 ul of 20X SSC

5. Incubate the mixture for fifteen minutes at 68°C, and cool the samples on nice.

6 Add 2 volume of 20X SSC to each of the samples.

7. Apply gentle suction until the 10X SSC has passed.

8. Load the samples into the slots, and then apply gentle suction. After all of the samples have passes through the filter, rinse each of the slots twice with 1ml of 10X SSC.

9. After the second rinse has passed through the filter continue suction for five minutes to dry the nitrocellulose.

10. Remove the nitrocellulose filter from the manifold, and allow it to dry completely at room temperature. Bake the filter for 2 hours at 80°C in a vacuum oven.

Preceding procedures were adapted and taken from "Dot and Slot Hybridization of RNA," Molecular Cloning: A Laboratory Manual. pgs 7.53-7.54.

Dot Hybridization of Messenger RNA with Biotinylated Probe

A. Biotin-21-dUTP OLigonucleotide 3' End Labeling

List of Components

Solution 1---500 mM potassium cacodylate, pH 7.2
1 mM DTT, 10mM $CoCl_2$

Solution 2---50 ng/ul Control 16-mer oligonucleotide

Solution 3---0.5 mM Biotin-21-dUTP
50 mM Tris-Cl, pH 7.5

Solution 4---8 mM dCTP

Solution 5---Sterile, distilled water

Solution 6---25 units/ul Terminal deoxynucleotidyl transferase (TdT)

Solution 7---200 mM EDTA

Protocol

1. Lyophilize 100 pmol of oligonucleotid (or 10 ul of Solution 2 control) in a 1.5 microcentrifuge tube.

2. Add the following components in order:

10 ul Solution 1 (5X buffer)
20 ul Solution 3 (Biotin-21-dUTP)
5 ul Solution 4 (dCTP), 14 ul Solution 5 (H_2O)
1 ul Solution 6 (TdT

Mix by gentle vortexing.

8. Load the samples into the slots, and then apply gentle suction. After all of the samples have passed through the filter, rinse each of the slots twice with 1 ml of 10X SSC.

9. After the second rinse has passed through the filter continue suction for 5 minutes to dry the nitrocellulose filter.

10. Remove the nitrocellulose filter from the manifold, and allow it to dry completely at room temperature. Bake the filter for 2 hours at 80°C in a vacuum oven.

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Solution 7---200 mM EDTA

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2. Add the following components in order:

10 ul Solution 1 (5X Buffer)
20 ul Solution 3 (Biotin-21-dUTP)
5 ul Solution 4 (dCTP)
14 ul Solution 5 (H_2O)
1 ul Solution 6 (TdT)

Mix by gentle vortexing.

3. Incubate for 3 hours at 37°C.

4. Add 5 ul Stop Solution (Solution 7).

5. Continue with Purification of Biotin Labeled Nucleic Acids and Hybridization Protocols.

B. Hybridization Conditions for 3' Biotin-21-dUTP Tailed Oligonucleotide Probes

1. Prehybridize filter 1 hour at 42°C in:

5X SSC
0.5% nuclease-free BSA
0.5% PVP
1% SDS
50 ug/ml yeast tRNA
100 ug/ml sheared and freshly denatured salmon sperm DNA

2. Add biotinylated probe to give a concentration of 5 pmol/ml probe. Hybridize at 42°C for at least 2 hours. Overnight hybridization will give better results.

3. Wash filter with 1X SSC, containing 1% SDS twice at room temperature and twice at 42°C (5 minutes each wash).

4. Perform two final washes with 1X SSC at room temperature.

C. Detection of Biotinylated Nucleic Acids with CLONTECH's GENE-TECT Detection System

List of Components

1. Streptavidin-Alkaline phosphatase conjugate (0.05ug/ml) in: 30 mM Tris-HCl (pH 8.0), 0.05% sodium azide, 250 mM NaCl.

2. NBT (Nitro Blue Tetrazolium); 50 mg/ml in 70% methanol; 1.8 ml.

3. BCIP (5-Bromo-4-chloro-3-indolyl phosphate); 50ug/ml in Dimethylformamide; 900 ul.

4. Biotinylated lambda DNA control (500 pg/ul) in 6X SSC with 0.1 0.1 ug/ul sheared salmon sperm DNA; 250 ul.

5. DNA dilution buffer (6X SSC with 0.1 ug/ul sheared salmon sperm DNA); 1.0 ml.

6. Buffer A---0.2 M NaCl
   0.1 M Tris-HCl, pH 7.5
   0.05% Triton-X-100

7. Buffer B---3% BSA in Buffer A

8. Buffer C---0.1 M NaCl
   0.1 Tris-HCl, pH 9.5
   50 mM $MgCl_2$

Preparation of Test Strips

1. Prepare dilutions of the bitinylated DNA (control with kit) to 100, 50, 25, 10, 5, 2 and 0 pg/ul. Use 6x SSC for the dilutions.

2. Spot 1ul spots sequentially onto 0.5 X 4cm nitrocellulose strips.

3. Dry filtens in a vacuum oven at 80°C for 1 hour.

4. Detect the bound biotinylated DNA as described below.

Detection Protocol

1. Block filtens for 30 minutes at room temperature in Buffer B. Use 5 ml per 100 cm^2 filter.

2. Incubate filtens for 25 minutes with streptavidin-alkaline phosphatase conjugate. The conjugate solution is prepared by adding 2.5 ul of conjugate per 1 ml of Buffer A.

3. Wash three times with 50-100 ml of Buffer A (10 minutes each wash).

4. Wash once with Buffer C

5. Prepare 5 to 10 ml of dye solution by adding 32 ul of NBT and 16 ul of BCIP to 5 ml Buffer C.

6. Incubate filter in dye solution for 30 minutes under reduced illumination. You may wish to extend the incubation period for as long as 3 hours in which case a stronger signal may occur, together with a higher background.

7. Terminate the reaction by washing in 1 mM EDTA. Dry filtens and store in the dark.

Preceding procedures were adapted and taken from "CLONTECH's Protocol for Biotin-21-dUTP Labeling Systems and GENE-TECT Non-Isotopic Detection Systems" provided

Results, Data, and Calculations

-Four hours after the administration of Estradiol 17B, removal of the uteri from the five injected rats exhibited a very definite response to the influence of estrogen. One obvious characteristic commonly seen was an increase in size of the uterus; with comparison to control, they are almost twice as large. Although a large percentage of this increased size is due to the absorption of water, it is still evident that Estradiol 17B affects growth in the uterus. Another prominent characteristic seen as well is increased blood flow, or hyperemia; the number of blood vessels increase and the existing vessels enlarge (Marieb, 637). In the rats that we sacrificed, the estrogen-injected uteri all illustrated hyperemic qualities.

-As indicated before, there were three samples of tissue obtained from the rats: uteri from control rats who did not receive any Estradiol 17B, uteri from rats that did receive administration of the estrogen, and the brains from the control rats. Spectrophotometric analysis comparing and relating absorbance with concentration revealed that indeed the total RNA levels for the uteri of the estrogen-injected rats was a three-fold higher than the other samples.

Spectrophotometric Data

Sample #	Absorbance	Total RNA
1-Control (no estrogen)	.133	1.58ug/ml
2-Inj. w/Estradiol 17B	.403	4.83ug/ml
3-Brain (control-like)	.146	1.75ug/ml

-The biotinylation of our oligonucleotide is shown to be successful after incubating it with the streptavidin-alkaline-phosphatase conjugate. Microliter dot blots on nitrocellulose are clearly visible after detection protocol.

-The dot-hybridized nitrocellulose filter paper after hybridization with the biotinylated oligonucleotide is not greatly illuminated nor easily seen by the detection protocol. Some non-specific binding is probably responsible for some daekening of the wells; however, no concrete details can be shown about IGF-I's activity although we know that Estradiol 17B does act on this growth factor in some way.

Conclusion

Past experiments have shown that Estradiol 17B does indeed cause an increase in the activity of ornithine decarboxylase and Insulin-like Growth Factor. It has also been revealed that Estradiol 17B increases IGF-I's activity through its messenger RNA which leads many to believe the same mode of operation is responsible for ODC. In an attempt to reveal this mode, our research was able to demonstrate some other important facts that may be worthy of knowing in future research. Biotinylated probes do not seem to be effective enough to determine the messenger RNA for IGF-I. However, biotinylated probes may be of some significance in detecting the mRNA for ODC since its expression is much greater than that of IGF-I's mRNA.

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Reflections

When I first started this research, everything was seemingly clear and straightforward. I remember saying, "How hard can this be?" But this was all an illusion. Research does not always provide one with answers but it always raises questions. Research is a challenge to those who accept it. The deeper I dug, the more I realized that this was a learning experience and not just a search for answers; it taught me how to be creative and ingenious as well as rational and analytical. Research is a journey with many roads to choose and your destination is not always reached, but it is the distance you travel that will be remembered.

Although this research did not and will not win any Nobel Prizes, the benefits that I have reaped from it and the foundation that it lays for those who will follow have certainly made it worth the hard work and the "long journey". This research project has also ejected me into an area to which now my interest is drawn. If all do possible, I hope one day I will be able to continue this research.

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