A COMPARATIVE STUDY OF SPECIFIC NUCLEAR BINDING OF ESTROGEN IN SOME TARGET AND NONTARGET ORGANS OF RATS

by

ALFREDA JONES

A thesis submitted to the
Chancellor's Scholars Program
in partial fulfillment of
the requirements for graduation
with honors

PEMBROKE STATE UNIVERSITY
1982

Primary Advisor	Secondary Advisor

ACKNOWLEDGMENT

I am indebted to a number of individuals for their assistance in this project.

First, my primary advisor, Dr. Harold David Maxwell, whose patience, understanding and expertise in hormone research sustained me throughout this research. Many times, were it not for his encouragement and expectations, I would have given up on myself and on this project. Also, I wish to thank the Biology Department of Pembroke State University, both staff and students. All were extremely supportive of my endeavours and sought to lend assistance in every way possible.

Secondly, I wish to thank Dr. John Reissner, secondary advisor to this project. Dr. Reissner's mental and moral support were indeed instrumental in the completion of this work.

Thanks goes also to the Chancellor's Scholars Council for their patience and support.

Next, I wish to make a special acknowledgment to Dr. Donald E. Smith of North Carolina State University for his generosity to Pembroke State University, and to me in particular by allowing me to use the liquid scintillation counter in his lab at N. C. State.

Last--not least, but most--I wish to thank my parents, Mr. and Mrs. James A. Jones; my sister, Mrs. Kathy Dudley; and my brother, Mr. James A. Jones, Jr. My family gave me every kind of support possible, including their very gracious financial support. When I became particularly frustrated during the course of this research

I looked to my family for understanding and help, and always they seemed to know just what to do to ease my situation. My parents were indeed the people who truly made this research possible, and I am deeply grateful. Special thanks goes to my mother who stayed with me all those gruelling nights in lab.

TABLE OF CONTENTS

LIST OF FIGURES

FOREWORD

When this research project was initiated, my advisor, Dr. David Maxwell, said to me, "Freda, good research answers one question and asks ten more." Thus, we prepared to test our hypothesis regarding specific nuclear binding of estrogen in four organs of the laboratory rat. Based on information obtained from studies involving estrogen binding in the uterus, spleen, liver and large intestine, we hypothesized that the uterus, a well known target organ for estrogen, would have a high degree of specific nuclear binding. The large intestine, for which no evidence was found that implicated it as a target organ, would characteristically have a small amount of specific nuclear estrogen binding. The liver and spleen are two possible target organs. More substantial research has been found that implicates the liver as a target organ than has been found implicating the spleen, so we expected to find a degree of specific binding in the liver somewhat less than the uterus, but significantly greaterthan the large intestine. The spleen, while it is a controversial organ does not seem to be as good a candidate for a target organ as the liver--at least not at the present--so we expected specific nuclear binding to be less than the liver but still significantly greater than the large intestine.

This states the hypothesis of this paper and what we expect to see in the results. The remainder of the paper concerns itself with giving background information about estrogen and estrogen binding, with a statement of the procedure used to test the hypothesis and the results of that test.

INTRODUCTION

17β-estradiol, sometimes referred to as the "female hormone", is an eighteen carbon compound having a basic steroid nucleus that is one in the group of hormones known as the estrogens. Two other naturally occurring forms of estrogen are estrone and estriol, but is is estradiol that is most potent in estrogenic biological roles and so is used in this particular study.

Estrogen is characteristically thought of as one of the hormones that regulates the menstrual cycle and female sex behavior and which functions in the development and maintenance of the sex organs and of the secondary sex characteristics. In organs that respond to estrogen, its mode of action is manifested on two levels; cellular and organismal. On the cellular level, estrogen's action is primarily anabolic. In organs such as the uterus, a major "target" organ of estrogen, RNA and protein synthesis are increased as well as carbohydrate synthesis. There is also an increase in mitosis which results in increased growth. Catabolic activities such as carbohydrate glycolysis also show an increase in response to estrogen. On an organismal level in target organs, estrogen increases glycolysis, respiration, H_2O permeability, hyperemia, and releases histamine (uterus); potentiates and stimulates thyrocalcitonin in calcium bone deposition; causes development of female characteristics; causes growth of primary and secondary sex organs; regulates the menstrual cycle and sex behavior and maintains secondary sex characteristics (14).

Estrogen Target Tissues

Estrogen, a steroid having no apparent barrier to cellular permeability (11), diffuses freely across the plasma membrane until equilibrium is established between cytoplasm and extracellular fluid. However, hormone researchers found that in some cells the hormone accumulated creating a greater concentration of hormone within the cell (4). These cells were called "target" cells. Further study of target tissues (tissues which respond to a given hormone) yielded specific characteristics of target tissues and a mechanism by which the hormone enters the target cell nucleus and thereby elicits its particular response.

Target tissues of steroid hormones are classically characterized by the following:

1. After in vivo exposure of the tissue to the hormone, the hormone appears in the tissue within minutes and is retained there long after it has left the nontarget cells.
2. An agent is found in target cells called a "receptor", that binds to the hormone in the cytoplasm, and is responsible for its accumulation in the nucleus and moreover, its retention in the nucleus that is vital to hormone response.
3. The movement of the hormone into the nucleus is extremely rapid, preceding all other observable changes in the target cell.
4. The receptor molecule must be present in the target cells of the hormone but absent in all other cells.
5. The receptor molecule has a high affinity for its particular hormone but a low affinity for other compounds of similar structure but different biological activity (18).

Estrogen target tissues as listed by Dr. Roman J. Kutsky are: uterus, mammary gland, vagina, ovary (corpus luteum), secondary female sex organs, skin, CNS, thyroid, thymus, long bones, anterior pituitary and hypothalamus. As Dr. Kutsky states, conflicting

data exists on such topics as hormone-target tissues. There is evidence that suggests that some tumors (20) and other organs such as the liver (7) are target tissues. However, whether these tissues meet the full requirements for classification as an estrogen target organ is yet to be decided.

Mechanism of Estrogen Entry into Nuclei

Estrogen entry into the nucleus of target cells is accomplished by a "two-step" mechanism: transformation and translocation.

The model of the two-step mechanism begins with the entry of the steroid, estrogen in this case, into the cytoplasm of the target cell. In the target cell cytoplasm two types of estrogen receptors exist, nonspecific and specific. Therefore two types of binding exist. The first is a high affinity, low capacity binding characteristic of specific receptor-steroid complexes. The second is a low affinity, high capacity binding characteristic of nonspecific receptor-steroid complexes. Nonspecific binding usually follows the polarity rule whereby increasing the number of polar substituents in the steroid decreases the protein binding potential. The binding is of a hydrophobic type and is somewhat influenced by the spatial arrangement of the steroid substituents but not nearly to the extent that specific binding is influenced. With specific type binding, the polarity rule is not applicable and the spatial arrangement of the steroid substituents is of major importance (11, p. 25).

The binding of estrogen to the specific receptor causes a modification of the receptor. This process is known as transfor-

mation and precedes entry of the estrogen-receptor (E-R) complex into the cell nucleus, a process called translocation. (8) Translocation of the E-R complex into the nucleus is supported by much evidence. Numerous reports found that injection of estradiol into immature rats followed by in vitro assay of the amount of cytoplasmic receptor indicates that there is a progressive loss of receptor up to four hours after the injection (maximum uptake of estrogen at one hour) and the amount lost is dependent on the amount of estradiol injected. The decrease in cytoplasmic receptors is accompanied by a comparable increase in the amount of bound estrogen in the nucleus (6). In countless similar studies examination of cytoplasmic and nuclear fractions of immature estrogen injected rats established translocation of cytoplasmic receptor as a fact.

When the specific E-R complex enters the nucleus, it attaches to a nuclear component which has been called the "acceptor". The two candidates for the role of acceptor are DNA and a nonhistone protein (12). When the E-R complex binds to the acceptor, transcription of mRNA is increased. The mRNA migrates to the ribosomes where it is translated into proteins that mediate the target tissue response to the hormone (18).

RATIONALE FOR THE STUDY

The uterus has long been proven to be a major estrogen target organ and in this experiment was used as a basis for comparison of specific nuclear binding of three other organs: liver, spleen, large intestine. The major reason for selecting these three organs for this study is that the liver and spleen may be regarded as

possible estrogen target organs while the large intestine is generally accepted as an estrogen nontarget organ. The establishment of the liver and possibly the spleen as target organs is a subject of much controversy.

Estrogens are metabolized to less active substances mainly in the liver and at least in rats--in the spleen. The liver and spleen inactivate estrogens, and presumably the liver also converts certain synthetic proestrogens to more active estrogenic hormones. The liver secretes the estrogens to the biliary tract, where they may be recirculated and, in rats, but also to some extent in man, excreted by way of the feces. The liver also conjugates the estrogens with glucuronic and sulfuric acid prior to their elimination by the kidneys (9).

Conflicting evidence exists regarding the establishment of the liver as a target organ. There is much research being conducted on hormone action in the liver of various animals. Gschwendt and Kittstein in 1973 proposed that the liver of male chicks might be considered a target tissue of estrogen. However, as they stated in their paper, there is disagreement in the literature regarding the existence of a specific cytoplasmic estrogen receptor in chicken liver. Arias and Warren reported on a high affinity cytoplasmic receptor for estradiol in chicken liver (2), whereas Mester and Baulieu, while confirming that estradiol injection does cause a rapid increase in the number of estradiol binding sites in the nucleus found no receptor in the liver cytosol fraction under any physiological conditions (17). Their evidence indicated a soluble

nuclear receptor. Lebeau *et al* (16) reported on a direct binding of estradiol to nuclear binding sites *in vitro*. However, Catherine B. Lazier did find a cytosol binder in the cockerel liver that is at a much lower concentration than the classic cytosol steroid receptors found in chick oviduct or rat uteri. Lazier further cited several reports that indicate that the high-affinity estradiol binding site concentration in rat liver cytosol is of the same order of magnitude (3,5,21). It is known that estradiol can also increase the concentration of lipoproteins in the rat liver (10). Powell-Jones, Davies and Griffiths reported their findings of a cytosol protein receptor in liver having a concentration of 50-100 fmol/mg protein and suggested that the role of an estradiol-receptor complex in liver might be related to plasma protein synthesis (19). King and Mainwaring reported that while liver has been used in many rat experiments as non-target organs, this is probably not justified; it does respond to estrogen. They added that liver nuclei accept more uterine estradiol receptors than spleen nuclei although neither are as effective as uterine nuclei (11).

Two target organs of estrogen are long bones and the thymus. Estrogen is shown to affect antibody properties and while lymphocytes arise in bone marrow and some travel to the thymus to become T cells (where they acquire the capacity to respond to certain antigens by facilitating their destruction), the spleen is a major lymphocyte storage organ important in both humoral and cell-mediated immunities (15). As stated earlier, the spleen, at least in rats is a site of estrogen metabolism to less active substances. This is not

to say that any kind of estrogenic correlation can be made between the long bone, thymus and spleen; however, reports do exist that show that the spleen is being studied as a possible target organ (11), and in light of this it seemed of interest to take a look at specific nuclear binding in the spleen.

No evidence for a cytosol receptor specific for estrogen in the large intestine was found so this made a study of the specific nuclear binding in this nontarget organ particularly interesting for two reasons. One reason was to see if the results of this study were concurrent with the acceptance of the large intestine as a nontarget organ. The second reason was that if indeed the large intestine did exhibit binding characteristic of a nontarget organ, it would serve as an interesting "nontarget" basis of comparison for the controversial liver and spleen in contrast to comparison with the uterus, a classical estrogen target organ.

MATERIALS AND METHOD

Animals used for this study were female Holtzman rats, 21-26 days old, which were maintained in an environmentally controlled laboratory and fed Purina Lab Chow and water ad libitum. Four rats were used per experiment and a total of six experiments were conducted, all employing the same procedure. One hour prior to sacrifice, the rats received a 5μg intraperitoneal injection of unlabeled 17β-estradiol. The rats were sacrificed by delivery of a sharp blow to the head followed by rapid decapitation. Tissue samples from four organs (uterus, spleen, liver, large intestine) of each rat were removed, freed of fat and connective tissue and immediately

weighed. (In the case of the uterus, the entire organ was used.)

Separation of the two classes of bound steroid is one of the most practical aspects of steroid-receptor studies. With the exception of specific precipitants/adsorbants and sucrose gradient analysis, all of the methods used to separate high and low affinity binding depend either on their differences in affinity or number of sites. Distinction between nonspecific and specific binding sites cannot be made by washing because washing is a very ineffective way of removing nonspecific binding. A superior method is to measure the binding of a labelled steroid in the presence and absence of excess, unlabelled competitor. Labelled steroid is displaced from the low capacity sites by the competitor whereas the high capacity sites are unaffected; the difference between the two experiments is a measure of the high-affinity, low capacity (specific) binding. The ratio of labelled agonist:unlabelled antagonist in such experiments is governed by their relative association constants. This is a technique that can be advantageously applied to any type of tissue preparation provided a method is available for separating the two classes of binding (11,, p. 21).

In this study, the technique just described has been employed using the ^{3}H-estradiol exchange assay of Anderson, Peck, and Clark (1).

Procedure

Excised, weighed tissue samples were homogenized in 3 mls of Tris buffer solution (ph 7.4) in a Kontes all-glass homogenizer and

kept on ice when possible. The homogenate was then centrifuged at 800 x g for ten minutes and the supernatant discarded. The pellet was resuspended in 3 mls of buffer and again centrifuged for ten minutes. This washing process was performed a total of three times discarding the supernatant each time to remove the unbound, unlabelled 17β-estradiol. After the final washing the pellet was re-homogenized in 2 mls of buffer, and a .5 ml aliquot placed in each of two tubes, A and B. Tube A contained .2 ml of a solution of 13nM ^{3}H-estradiol, 1.3M diethylstilbesterol (100 fold excess) and buffer. The tubes with .5 ml aliquots of homogenate and .2 ml of their respective solutions were then placed in a 37° shaking water bath and incubated for one hour to allow for "exchange". In Tube A, tritiated estradiol exchanged with unlabelled estradiol on all the estrogen receptors, both specific and nonspecific. Thus a radioactivity counting of Tube A would result in a measure of total binding. In Tube B, two different exchanges took place. Unlabelled diethystilbesterol, a competitor for the specific binding sites, exchanged with the 17β-estradiol that was bound specifically. The tritiated estradiol therefore exchanged with the unlabelled estradiol bound to nonspecific receptors. A radioactivity counting of Tube B would result in a measure of the nonspecific binding. Specific binding would be equal to Tube A counts minus Tube B counts.

RESULTS AND DISCUSSION

In analysis of the data $p < .05$ confidence level was used unless otherwise indicated.

Figure 1 illustrates specific nuclear binding of estradiol in the rat uterus, liver, spleen and large intestine. These results indicate that specific nuclear binding in the liver and spleen was significantly lower than that of the uterus (student's t test). The results further indicate that specific nuclear binding in the large intestine is not significantly different from that of the uterus. This is a surprising result in light of the fact that the large intestine is classically not regarded as as estrogen target organ.

The relationship between total, nonspecific and specific binding in the uterus, liver, spleen and large intestine of the estradiol treated rats is illustrated in Figure 2. Total binding of estradiol was significantly different from the uterus in the liver, the spleen and the large intestine. As stated earlier, specific nuclear binding in the spleen and liver was significantly different from the uterus, but specific binding in the large intestine was not significantly different. Importantly, nonspecific binding which was expected to show no significant difference in any of the organs did show a significant difference in the liver and spleen but not in the large intestine.

Figure 3 illustrates the ratio of specifically bound estradiol to the total amount of estradiol bound in the uterus, liver, spleen and large intestine. The liver showed significant difference with respect to the uterus. However, the spleen and large intestine did not show a significant difference.

It must be said that the results of this study are indeed

surprising. Much of that surprise results from the high amount of specific nuclear binding found in the large intestine. Inevitably one must ask, "Why would the large intestine exhibit estrogen target organ characteristics? What would be the advantage in having estrogen accumulate in the nuclei of the cells of the large intestine?"

This paper cannot answer those questions; it can only raise those and more. At this point, any attempt to explain what happened in this experiment would be pure speculation and faced with these astonishing results regarding the liver and large intestine, even speculation is not easy.

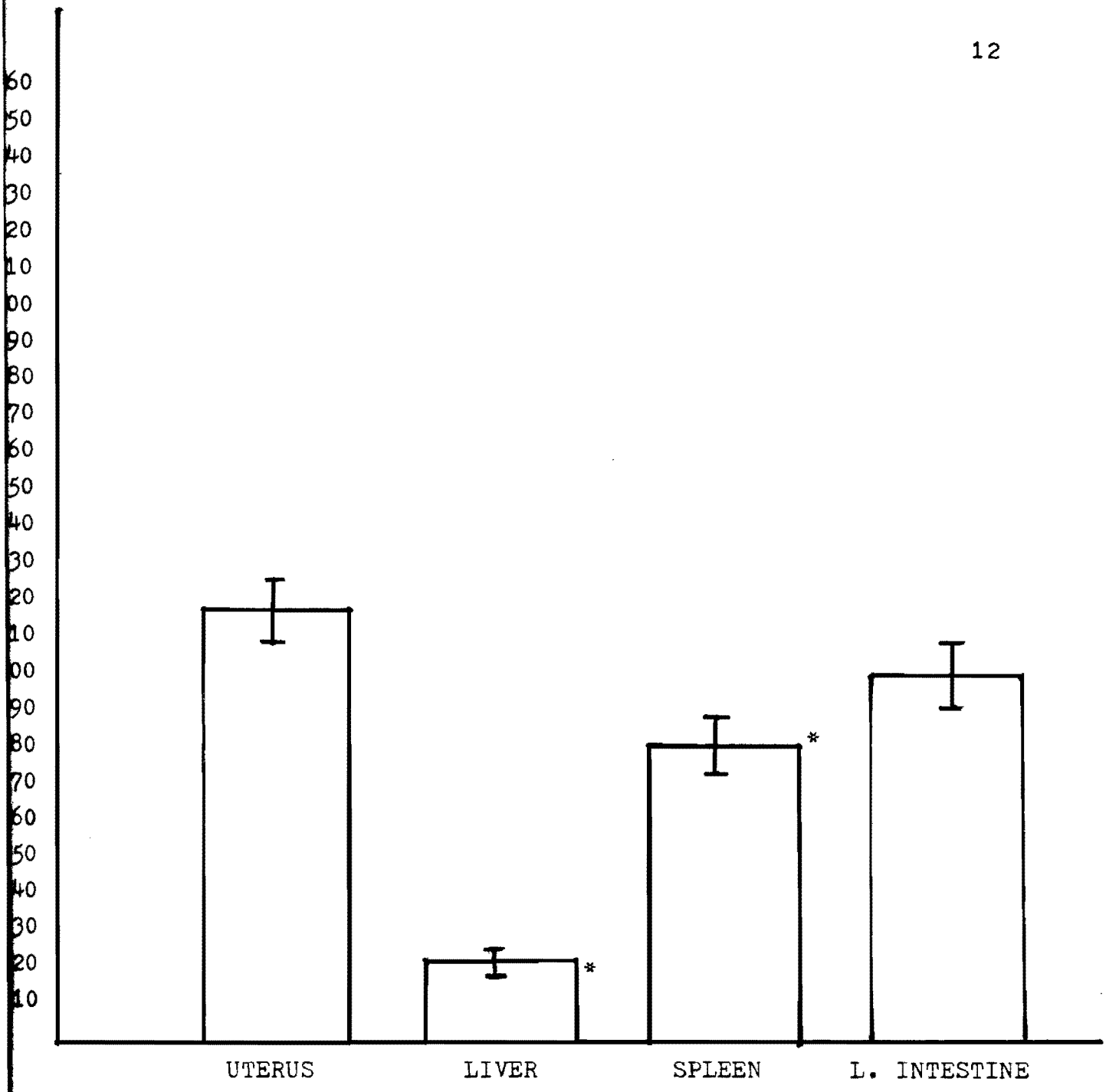

Figure 1. Specific nuclear binding of estradiol in the rat uterus, liver, spleen and large intestine. Each value is the mean (± S.E.) based on six experiments. Values are expressed as specific counts per minute per milligram of tissue,

* = significantly different from uterus at $p < .05$.

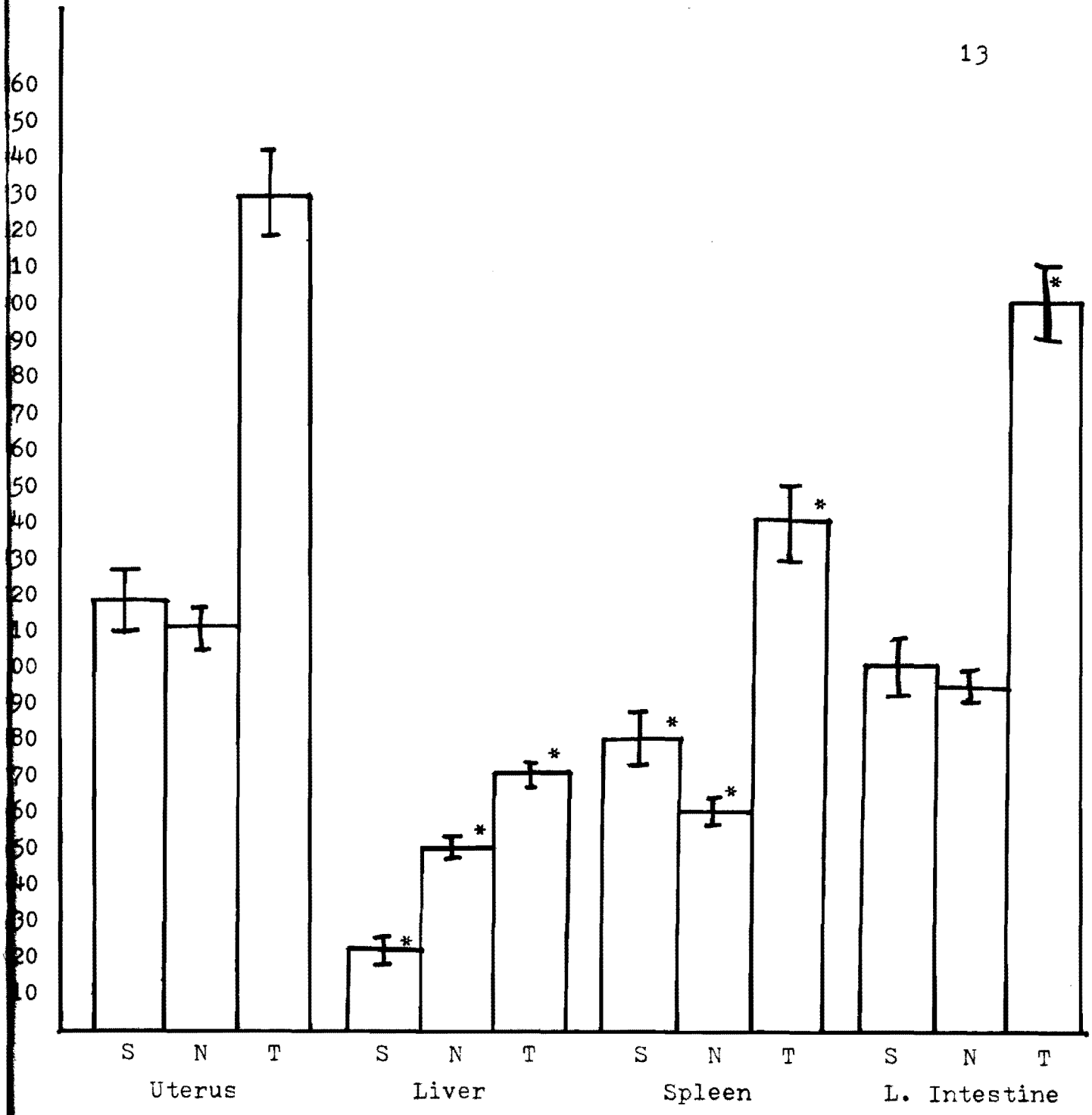

Figure 2. Total, specific and nonspecific binding of estradiol in the uterus, liver, spleen and large intestine of estradiol treated rats. Each value is the mean (± S.E.) based on six experiments. Values are expressed as counts per minute per milligram of tissue.

* = significantly different from uterus at $p < .05$.
S = specific; N = nonspecific; T = total

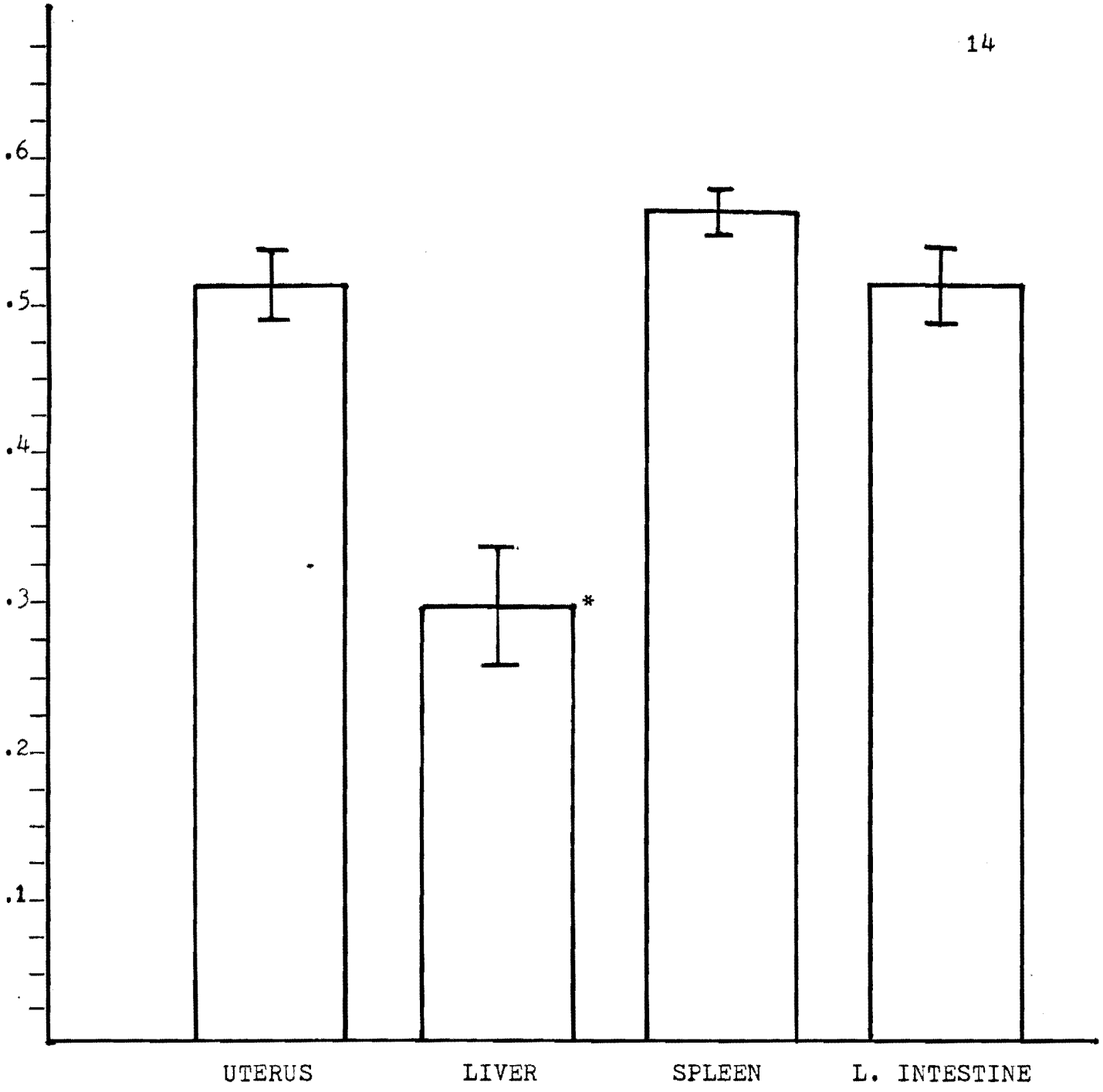

Figure 3. Ratio of specific nuclear counts to total number of counts in the uterus, liver, spleen and large intestine of rats treated with estradiol. Each value is the mean (± S.E.) based on six experiments. Values are expressed as counts per minute per milligram of tissue.

* = significantly different from uterus at $p < .05$.

CONCLUSIONS

1. The large intestine exhibits no significant difference to the uterus in the specific nuclear binding of 17β-estradiol at the $p < .05$ level. Therefore, the large intestine may be regarded as an estrogen target organ.

2. The liver shows significantly less specific nuclear binding of 17β-estradiol.

3. The spleen shows significantly less specific nuclear binding of 17β- estradiol.

4. Further research is needed to confirm the surprising findings of this study. That research might begin with a repeat of this study using, in addition to the estrogen injected rat, an uninjected control rat and making a determination of the specific nuclear binding for the purpose of basal comparison. Also a time sequence study on estradiol treated rats could be conducted. Other suggested projects would be those that attempt to answer such questions as, "Does the increase in nuclear binding in the large intestine of the rat correlate to an increase in metabolic rate, and if so, how is this change in metabolic rate manifested? What is the mechanism by which estrogen enters the cell nucleus of the liver, spleen and large intestine? Is it the two-step method of translocation and transformation, some other method or a combination of methods?" Lastly, a question that is very thought provoking and is intimately related to all that we have talked about in this paper: While virtually all tissues that contain estrogen receptors are estrogen responsive, the very important question of whether all responsive cells contain receptors is a question that still remains to be answered.

A SELECTED BIBLIOGRAPHY

1. Anderson, J., J. H. Clark, and E. J. Peck. "Oestrogen and Nuclear Binding Sites. Determination of Specific Sites by ^{3}H-oestradiol exchange." Biochemical Journal. 126 (1970), pp. 561-567.

2. Arias, F., and J. C. Warren. "An Estrophlic Macromole in Chicken Liver Cytosol." Biochimica et Biophysica Acta. 230:3 (1971), pp. 550-559.

3. Chamness, G. C., M. E. Costlow, and W. L. McGuire. "Estrogen Receptor in Rat Liver and its Dependence on Prolactin." Steroids. 26 (1975), pp. 363-371.

4. Clark, J. H., T. H. Hamilton, and W. A. Sadler. Ontogeny of Receptors and Reproductive Hormone Action. New York: Raven Press, 1979, p. 5.

5. Eisenfeld, A. J., R. F. Aten, G. K. Haselbacher, and K. Halpern. "Specific Macromolecular Binding of Estradiol in Mammalian Liver Supernatant." Biochemical Pharmacology. 26 (1977), pp. 919-922.

6. Gorski, J. and M. Sarff. "Control of Estrogen Binding Protein Concentration Under Basal Conditions and After Estrogen Administration." Biochemistry. 10:13 (1971), pp. 2557-2563.

7. Gschwendt. M. "Specific Estrogen Binding Sites on the Liver Chromatin of Estrogen-pretreated Roosters." Zeitschrift Fuer Physiologische Chemie. 354:12 (December 1972), pp. 1642-1644.

8. Gschwendt, M. and T. H. Hamilton. "The Transformation of the Cytoplasmic Oestradiol-receptor Complex into the Nuclear Complex in a Uterine Cell-free System." Biochemical Journal. 128:3 (1972), pp. 611-616.

9. Heftmann, E. and E. Mosetling. Biochemistry of Steroids. New York: Reinhold Publishing Company, 1960, pp. 159-161.

10. Hill, P., D. Dvornik, and M. N. Cayen. "Agents Affecting Lipid Metabolism." Canadian Journal of Biochemistry. 42:2 (1968), pp. 189-191.

11. King, R. J. B. and W. I. P. Mainwaring. Steroid Cell Interactions. Baltimore: University Park Press, 1974, pp. 1.

12. King, R. J. B., J. Gordon and A. W. Steggles. "The Properties of a Nuclear Acidic Protein Fraction that Binds 6,7,-^{3}H-Oestradiol 17β. Biochemical Journal. 114:3 (1970), pp. 649-657.

13. Korach, L. S. "Diethylstilbestrol Metabolites and Analogs: New Probes for the Study of Hormone Action." Journal of Biological Chemistry. 254:18 (September 1979), pp. 8963-8968.

14. Kutsky, R. J. Handbook of Vitamins and Hormones. New York: Van Nostrand Reinhold Company, 1973, pp. 215-221.

15. Landau, Barbara R. Essential Human Anatomy and Physiology. Dallas: Scott, Foresman and Company, 1980, pp. 450-451.

16. Lebeau, M-C., N. Massol, and E-E. Baulieu. "An Insoluble Receptor for Estrogens in the 'residual' Nuclear Proteins of Chick Liver." European Journal of Biochemistry. 36:1 (1973) pp. 294-300.

17. Mester, Jan and Etienne-Emile Baulieu. "Nuclear Estrogen Receptor of Chick Liver." Biochimica et Biophysica Acta. 261:1 (1972), pp. 236-244.

18. O'Malley, B. W. and W. T. Schrader. "The Receptors of Steroid Hormones." Scientific American. 234:2 (February 1976), pp. 32-43.

19. Powell-Jones, W., P. Davies and K. Griffiths. "Specific Binding of ^{3}H-oestradiol by Cytoplasmic Protein Components of Female Rat Liver." Journal of Endocrinology. 69:2 (1976), pp. 167-168.

20. Sluyser, M. "Hormone Receptors in Mouse Mammary Tumors." Biochimica et Biophysica Acta. 560:4 (May 1979), pp. 509-529.

21. Viladin, P. C. Delgado, J. Pensky, and O. H. Pearson, "Estrogen Binding Protein in Rat Liver." Endocrinology Research Communications. 2 (1975) pp. 273-280.