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CONTENTS

Department of Biology

The effects of Estradiol-3,17 B on embryonic hamster kidney cells *in Vitro*
Cynthia Ruth Blythe

The effects of various growth regulators on *Helianthus* stem tissue.....
Jacqueline Hendrick Lavender

Department of Drama and Speech

Variations on a theme: four plays.....Shelby Archer

School of Education

A modern appraisal of the Montessori method.....Linda Cline

Department of English

The new child, a collection of poems.....Sylvia Eidam

Department of History

The philosophy of Lester Ward and its reflections on the New Deal.....
Karen Hayes

The role of Lucien Bonaparte in the *coup d'etat* of 18-19 Brumaire.....
Claudia Buchdahl Kadis

The Effects of Estradiol-3,17 B on Embryonic Hamster Kidney Cells *in Vitro*

by

Cynthia Ruth Blythe

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APPROVED BY

Laura G. Anderton
Dr. Laura G. Anderton, Director

EXAMINING COMMITTEE

Bruce M Eberhart
Dr. Bruce M. Eberhart

Hilda T. Harpster
Dr. Hilda T. Harpster

Edward McCrady III
Dr. Edward McCrady III

TABLE OF CONTENTS

INTRODUCTION 1

MATERIALS AND METHODS. 9

RESULTS. 13

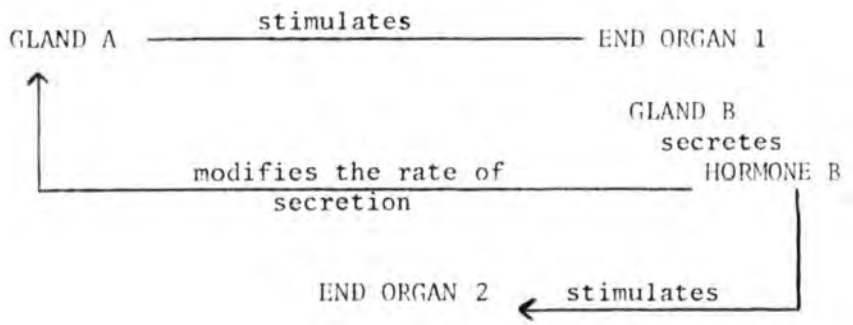
DISCUSSION AND CONCLUSION 14

SUMMARY. 20

LITERATURE CITED 21

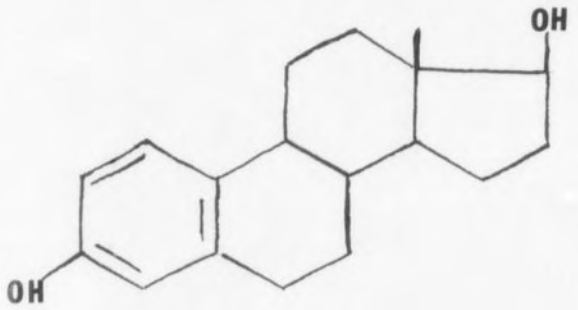
INTRODUCTION

Animals and plants possessing organs are distinguished from other cellular forms of life in having their biochemical processes regulated by hormonal control mechanisms, in addition to the fundamental principles of regulation within the cell itself. Hormones of animals, often called distance activators, are chemical substances made by the endocrine glands and secreted into the blood stream. These compounds are then carried by the blood and lymph to all parts of the body, where they modify the activity of specific genetically conditioned target organs by an intricate feedback mechanism, as illustrated below (Nalbandov, 1964).

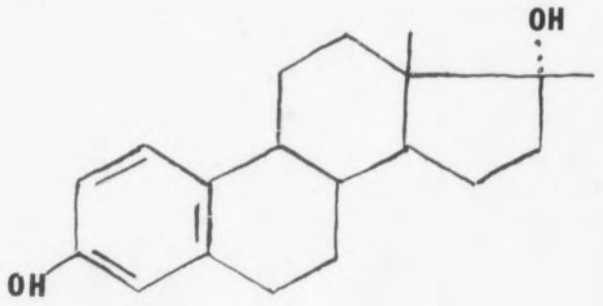


One important group of hormones is the estrogens, which are produced primarily by the ovary but which have also been isolated from the placenta, adrenals, and testes (Karlson, 1962). The ovarian follicles secrete estradiol, which is interconvertible to estrone, as designated by the following scheme:

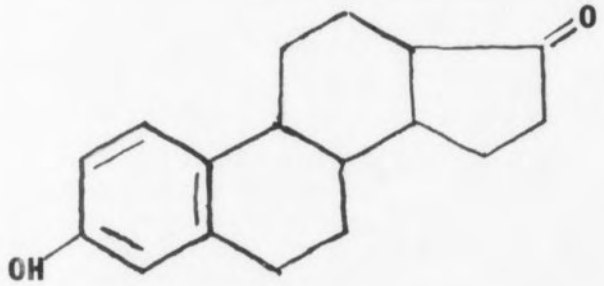
The natural estrogens differ from the other steroid hormones in that the A ring of the nucleus is aromatic and phenolic, and the methyl group at



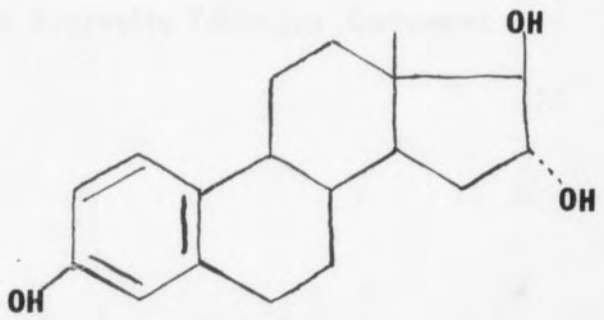
ESTRADIOL - 3,17 B



ESTRADIOL- 3, 17 α



ESTRONE



ESTRIOL- 3, 16α, 17B

FIGURE 1. STRUCTURE of the NATURAL ESTROGENS.

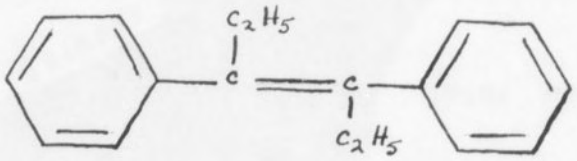


FIGURE 2. DIETHYLSTILBESTROL -- A Synthetic Estrogen Compound

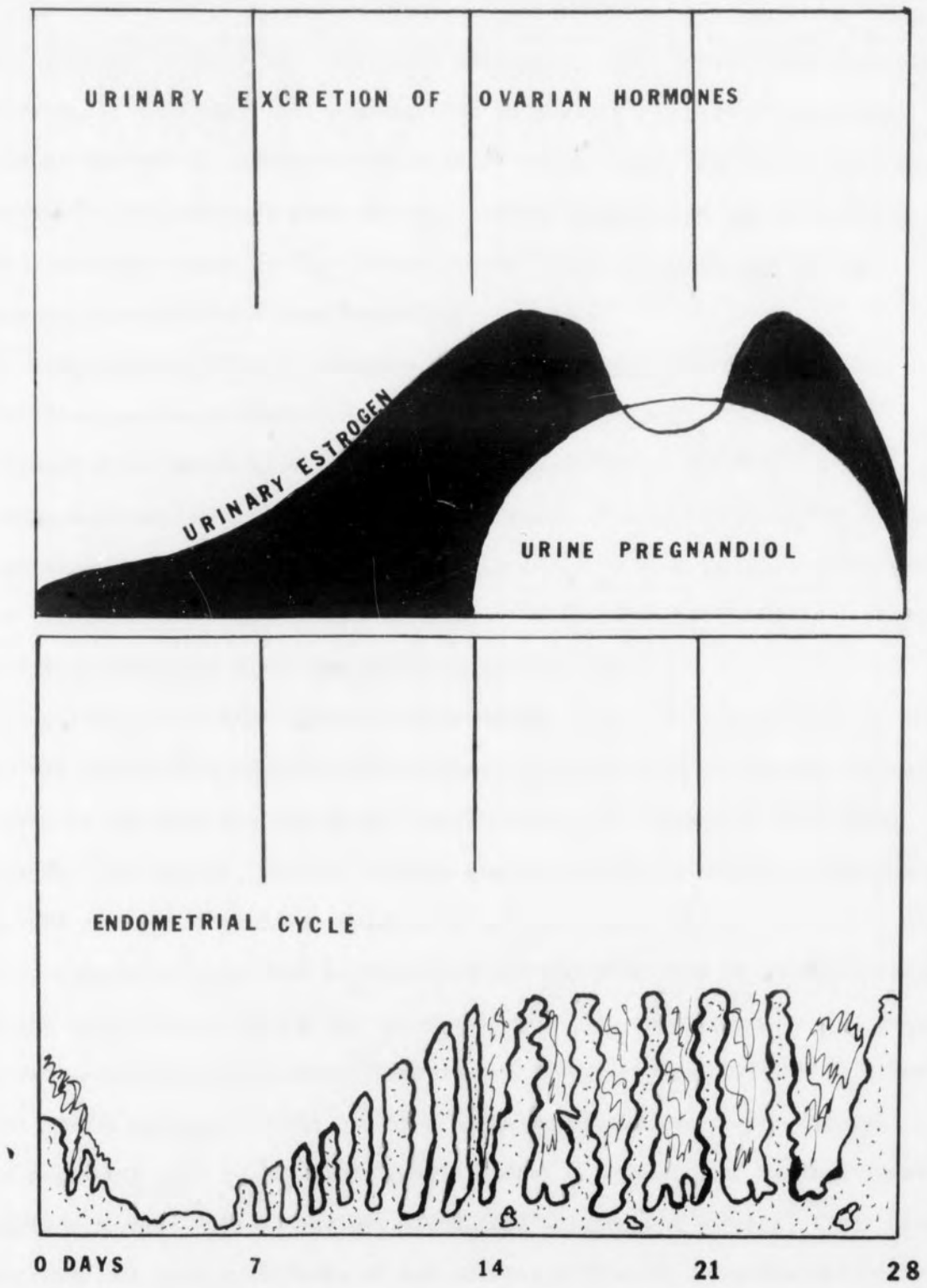


FIGURE 3. THE FEMALE SEX ENDOCRINE CYCLE
(modified from Cantarow & Schepartz, 1962)

C_{10} is absent (Figure 1). Synthetic estrogens, such as diethylstilbestrol (Figure 2), have also been demonstrated to possess estrogenic properties, such as the steric configuration of the A and B rings, the steric configuration of the hydroxyl group at C_{17}, and the presence of the characteristic hydroxyl group at C_3. The following relative activities of the natural estrogens have been observed:

Estradiol-3, 17 B $\rangle$ Estrone $\rangle$ Estradiol-3,17 $\rangle$ Estriol.

The estrogens are probably secreted into the blood in the form of free steroid, after which they are immediately bound by conjugation with glucuronate or sulfate and protein in the liver. This binding mechanism could possibly explain why estrogens are effective in smaller concentrations than the other steroid hormones and are able to antagonize the biological action of large amounts of these compounds (Nalbandov, 1964).

Evidence from many types of experiments, especially those focusing on the rat and mouse genitalia and accessory organs, has demonstrated that the estrogens increase the growth and metabolism of the following receptive tissues: the uterus, vagina, mammary glands, secondary sexual structures, thyroid and thymus glands, hypophysis, and long bones (Zarrow et al., 1964). It is generally known that estrogen induces proliferation of uterine tissue in the human female during the menstural cycle, as ascertained by the amounts of estrogen obtained in urine samples on consecutive days (Figure 3). The peak of the estrogen effect has been shown to be the fourteenth through the sixteenth days of the cycle (Masin, 1964). In addition, various investigators (Grosso, 1963; Spaziani, 1963; and Nilsson and Norberg, 1963) have described the <u>in vivo</u> effects of estradiol-3,17 B on the uterine epithelial tissue of ovariectomized rats and mice. Their results have demonstrated that

estradiol-3,17 B also stimulated the growth of the uterine tissue, resulting in increased uterine weight. Several attempts (Astwood, 1938; Nilsson and Norberg, 1963; and Spaziani, 1963) have indicated that cellular growth, occuring in interphase and resulting in the increased height of the uterine epithelium, is caused by an initial uptake of water. Of related interest are two attempts that have been made in order to study the effects of estrogen in vitro. Hardy (1953) has described the keratinizing effects of estradiol in tissue culture, and Algard (1961) has reported the cellular growth produced by estrogen in the organ culture of hamster tissue. In addition women treated for long periods with estrogen have been noted to develop uterine epitheliomas more readily than was observed with the controls. From these findings Jensen and Ostergaard (1954) concluded that continuous exogenous and endogenous estrogen stimulation is a factor in cancer of the endometrium. Finally, other investigations have shown that estrogen stimulates ductal and alveolar growth of the mammary gland in immature ovariectomized rats (Smith, 1955).

From the evidence previously cited it appears that estrogen administration to the intact animal evokes a growth response in only a limited number of different tissues. The question of what distinguishes these responsive tissues from the unresponsive tissues is an important feature of the general problem of the genetic and biochemical basis of cell differentiation. The hormones are thought to be more than mere stimulators of differentiation because often they specifically determine which of the existing developmental potentialities shall become evident. An interesting example of this phenomenon is the response of uterine epithelial cells

in vitro to an injection of estradiol. Wilson (1963) found that cuboidal epithelial cells, which are predominant when the hormone concentration is lowest (Nalvandov, 1964), become morphologically columnar four hours after the injection. In the case of insects it is only through specific hormones, such as ecdysone or the juvenile hormone of the corpora allata, that the existence of specific cell competence is revealed (Willier et al., 1955; and Wigglesworth, 1961). Exactly what endows a target organ with the inherent capacity to respond to one hormone and not to another, is a problem which remains unsolved. The dilemma is further complicated not only by the fact that almost all hormones reach the target organs via the blood stream (Robson and Adler, 1940), but also because each cell in a multicellular organism possesses the same genetic complement as every other nucleus.

A few investigators, however, have described the mitotic-stimulating effects of estrogen on tissue that is not ordinarily responsive to the hormone (Pettersson, 1962). Meir (1963) has noted that when diethylstilbestrol pellets are implanted in male Golden hamsters, renal tumors develop in the cortex. Feagans and his co-workers (1963) also described renal cell growth with the synthetic estrogens, ethinylestradiol and stilbestrol. Conflicitng reports arose from the experiments on mouse kidney tissue. Shimkin (1963) reported a decrease in the weight of intact kidneys treated with estrogen; whereas Kochakian (1947) demonstrated a small increase in kidney tissue growth.

The above evidence indicates that several workers have studied the effects of estradiol *in vivo* on uterine and renal epithelial tissue, but few have considered the possibility of similar effects *in vitro*. In addition, reading for the present investigation has not revealed a single paper on the

effect of estrogen on renal tissue in vitro. With this fact in mind, the present pilot study was attempted in order to determine the effects of estradiol-3,17 B on hamster kidney cells in tissue culture.

MATERIALS AND METHODS

Two pregnant Golden hamsters (Mesocricetus auratus) weighing approximately 150 grams were obtained from Dublin Laboratory Animals, Dublin, Virginia, and placed in separate cages where they had free access to water and hamster food. The pregnant hamster, designated as H6, was sacrificed twelve days after mating. Each of the embryos obtained from a gross dissection of the mother was removed from its amniotic sac and placed in a sterile Stendor dish containing 10 ml. of Hank's balanced salt solution (Microbiological Associates). The next operation, performed entirely under a dissecting microscope, entailed removing the tissue anterior to the liver of the embryonic hamster. Kidneys were removed from the remaining tissue immersed in the salt solution. The kidney cells were separated by alternately drawing and expelling the tissue with a fine-needled 5 ml. sterile disposable syringe containing Medium #199 (Microbiological Associates). After this mincing technique was performed several times, the contents of the syringe were expelled under aseptic conditions into a Petri dish containing a carefully cleaned slide and coverglass. The aseptic conditions used throughout this experiment included autoclaving the Petri dishes and other glassware at 121°C. for fifteen minutes, and inoculating the Petri dishes in a separate transfer room which had been surface sterilized with ultraviolet light for a period of twelve hours. Modifications of various tissue culture media (Grefer and Spence, 1963; Hsu and Zenzes, 1964; Moorhead et al., 1960; and Zakharov, 1964) resulted in the following combinations of media, each of which was tested in preliminary tissue cultures: (1) Hank's balanced salt solution (Microbiological Associates), Medium #199, and Calf serum (Microbiological Associates);

(2) the media in #1 and Chick Embryo Extract (Difco); and (3) Medium #199 and Calf serum. The third combination was selected for the greater concentration of nutrient Medium #199. The complete culture media included antibiotics and nutrient media in the following proportions:

9 ml. Medium #199 (Microbiological Associates)

1 ml. Calf serum (Microbiological Associates)

.2 ml. Penicillin-G (200 u./ml.)

.2 ml. Streptomycin (100u/ml.)

The pH was maintained at approximately 7.0, as observed from the color of the phenol red indicator in the media. After these cells were cultured for 24 hours at 37°C., a modification of the technique used by Kahn (1954) was attempted. Under sterile conditions 0.2 ml. of a 6ugm/ml solution of estradiol- 3,17 B (Nutritional Biochemicals Corporation) in Tyrode's solution were injected into each of the cultures. At the end of the 72-hour incubation period (Hardy, 1953), the slides and coverglasses covered with proliferated cells were removed from the culture media, dipped quickly in distilled water to rinse off the media, and fixed in acetic alcohol (3 methanol: 1 acetic acid). The cells were then stained with periodic Schiff's reagent, using a slight modification of the Feulgen technique (McManus and Mowry, 1963) and mounted in balsam. A period of four days was selected for the incubation period from results of preliminary tissue cultures, in which kidney cells were cultured successfully for this period of time without changing the culture medium.

The second pregnant hamster, designated as H7, was weighed and injected intraperitoneally with 1.0 ml. of Velban. The concentration of Velban (Eli Lilly & Company) used was 2 mgm/kgm body weight. Three hours after the

injection, the female hamster was sacrificed. Each of the thirteen-day old embryos obtained from the dissection was also removed from its amniotic membrane and immersed in Hank's balanced salt solution. After the kidneys were dissected from these hamsters, smears of this tissue from each of the embryos were made to serve as controls for the estrogen-injected tissue cultures. These cells were also fixed in acetic alcohol and stained, using the same modification of the Feulgen technique as previously mentioned.

Cell division was quantified by counting (at 600 and 1500 magnifications) the percentage of 200 cuboidal epithelial kidney cells in prophase, telophase, and interphase on each slide. Counts were made on four different tissue cultures and three different kidney smears. The results are expressed as the average percentage observed $\pm$ average deviation, (Table 1a and 1b).

TABLE 1a. The Effects of 3,17 B Estradiol on Mitosis in Hamster Kidney Cells in vitro.

Slide	%Interphase	%Prophase	%Telophase	Summary
H6-3b	3.0 ± .7%	93.0 ± .7%	4.0 ± 0%	Range of Cell Division:
H6-4	4.3 ± 3.0%	78.3 ± 2.4%	17.3 ± 3.1%	
H6-5a	3.9 ± .6%	95.1 ± .1%	1.0 ± .6%	78.3 ± 2.4% to
H6-5b	5.7 ± 2.9%	89.0 ± 2.0%	5.3 ± .9%	95.1 ± .1%
H6-6a	4.3 ± 2.0%	92.7 ± 1.6%	4.5 ± .3%	
H6-6b	5.7 ± 1.1%	91.7 ± 1.6%	2.7 ± .4%	

TABLE 1b. Percentages of cells in mitosis of normal hamster kidney cell smears.

Slide	%Interphase	%Prophase	%Telophase	Summary
H7-5a	98.7 ± .4%	1.3 ± .4%	0	Range of Cell Division:
H7-5b	99.3 ± .4%	.5 ± .2%	0	
H7-7a	99.3 ± .4%	.7 ± .2%	0	.2 ± .1% to
H7-7b	99.8 ± .2%	.2 ± .1%	0	2.0 ± 0%
H7-8a	99.8 ± .2%	.2 ± .1%	0	
H7-8b	98.0 ± 0%	2.0 ± 0%	0	

Key: H6-3b refers to embryo 3, slide b, of pregnant hamster H6.

RESULTS

In this investigation the culture conditions and media used were adequate for growing embryonic hamster kidney cells *in vitro* for a period of four days. Quantified results from the tissue cultures injected with estrogen indicate that estradiol-3,17 B activated epithelial cell division within the range of 78.3% to 95.1% over the basal level of 1.3% to 2.0% observed in the control smears (Table 1a and 1b). In addition the amount of cytoplasm in the cultured cells was reduced, partly because of the rounding up in preparation for mitosis.

DISCUSSION AND CONCLUSION

The results of this preliminary study of the effects of estradiol on hamster kidney epithelial cells *in vitro* indicate that the hormone elicited a mitotic-stimulating effect similar to that obtained with uterine eipthelial cells. According to Nalbandov (1964), the epithelium of the uterus is cyclically broken down and rebuilt, fluctuating between the stratified-squamous and low-cuboidal type of epithelial cells. In this study the hamster cells grown *in vitro* are also cuboidal, and have also been stimulated by estradiol to "dedifferentiate" to the extent of undergoing mitosis. Thus, it may be concluded that estradiol is capable of stimulating cell growth in renal tissue *in vitro*, even though the kidneys are not considered as target organs for the hormone. In consideration of the evidence cited in the introduction, revealing only a slight effect of estradiol on the intact kidney, the reason for the striking response of renal tissue to estradiol in this study might be attributed to the fact that chemical and physical restraints existing in the control condition may have been alleviated. It appears that the renal tissue does possess the competence to respond to estrogen but this competence is not fully realized *in vivo*. Since it is known that dispersed cells behave differently from those in close communication in the intact tissue, the response demonstrated by cells in this study could be attributed to their physical dispersal in tissue culture. However, the fact that some effect was noted previously *in vivo* seems to indicate the plausibility of an alternate explanation. The Jacob and Monod model of gene action is useful in this consideration (Figure 4). According to this theory, enzyme induction and repression operate by similar mechanisms in a hypothetical control system, consisting of a structural gene, a regulator gene, and an operator gene. The structural gene produces messenger

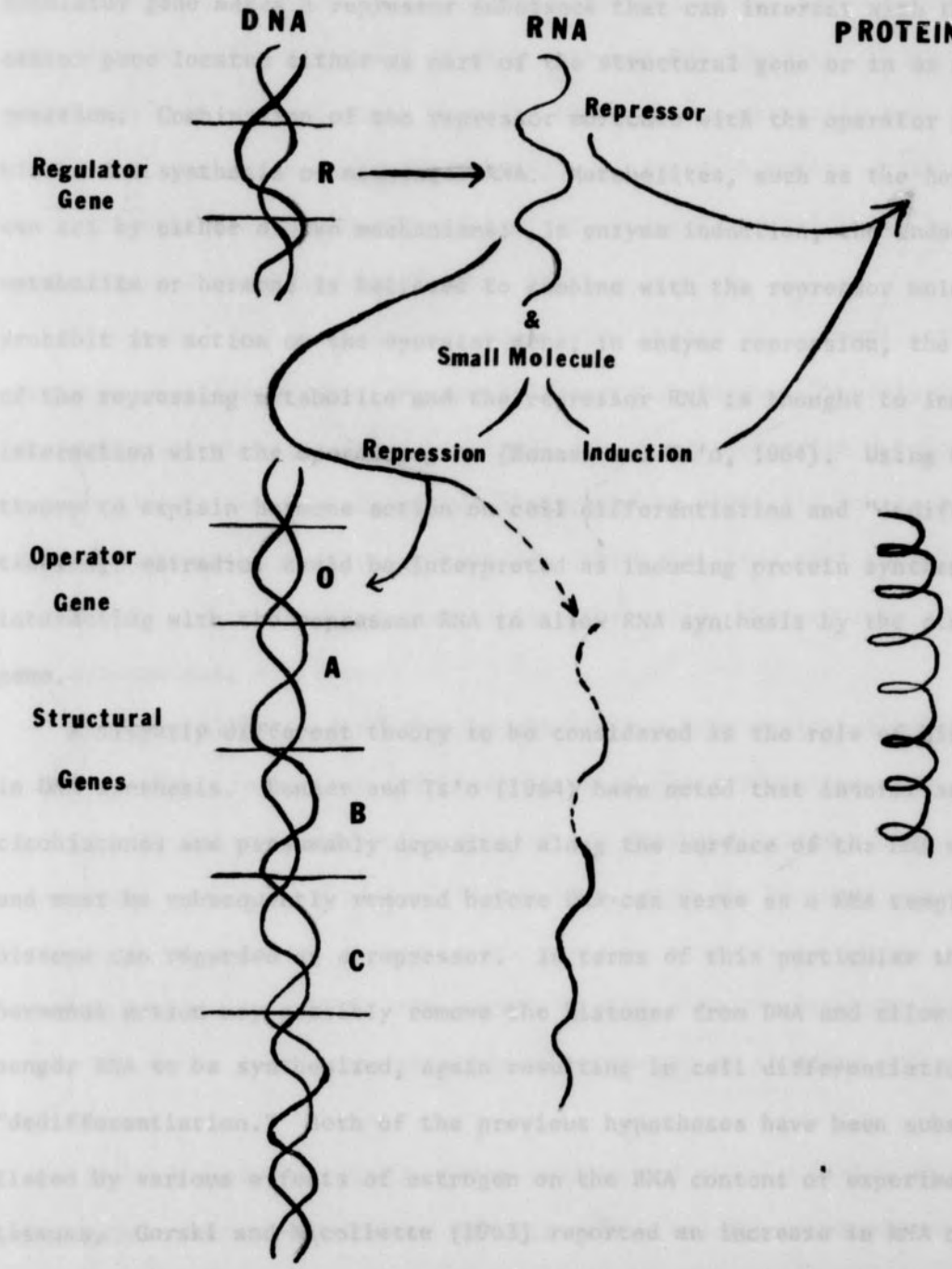


FIGURE 4. JACOB-MONOD MODEL FOR THE REGULATION OF PROTEIN SYNTHESIS.

RNA that serves as a template for the synthesis of proteins; whereas the regulator gene makes a repressor substance that can interact with the operator gene located either as part of the structural gene or in an adjacent position. Combination of the repressor molecule with the operator gene blocks the synthesis of messenger RNA. Metabolites, such as the hormones, can act by either of two mechanisms: In enzyme induction, the inducing metabolite or hormone is believed to combine with the repressor molecule to prohibit its action on the operator gene; in enzyme repression, the complex of the repressing metabolite and the repressor RNA is thought to increase interaction with the operator gene (Bonner and Ts'o, 1964). Using this theory to explain hormone action on cell differentiation and "dedifferentiation," estradiol could be interpreted as inducing protein synthesis by interacting with the repressor RNA to allow RNA synthesis by the structural gene.

A slightly different theory to be considered is the role of histone in DNA synthesis. Bonner and Ts'o (1964) have noted that insofar as nucleohistones are presumably deposited along the surface of the DNA molecule and must be subsequently removed before DNA can serve as a RNA template, histone can regarded as a repressor. In terms of this particular theory, hormonal action may possibly remove the histones from DNA and allow messenger RNA to be synthesized, again resulting in cell differentiation or "dedifferentiation." Both of the previous hypotheses have been substantiated by various effects of estrogen on the RNA content of experimental tissues. Gorski and Nicollette (1963) reported an increase in RNA content of subcellular fractions of the uteri in ovariectomized rats one hour after injection with estradiol-3,17 B. Of related interest are the experiments of

Wilson (1963) in which chromosomal RNA synthesis is enhanced by estrogen, as result of which the activities of transfer RNA and template RNA are also increased. Because of this enhanced RNA activity, it has seemed likely to some investigators that the hormones are primarily some type of enzyme regulators (Nalbandov, 1964). It is worth noting at this point that a slight increase in DNA content of estrogen-treated cells has also been observed (Tremblay and Thayer, 1964).

Increased cell permeability is a third theory of hormonal action. Although this idea is not as readily acceptable to most investigators as the other two theories (Karlson, 1962), the increased water uptake as a result of estrogen administration might tend to partially substantiate this hypothesis. In addition Bullough (1950) believes that estrogens facilitate the entrance of glucose into the cells, thereby supplying energy for mitotic cell division.

Another slightly different hypothesis has been suggested by Villee (1962). He believes that estrogens increase the ATP available for lipids and nucleic acids by diverting some of the NADP ordinarily wasted by NADP cytochrome c reductase system (Figure 5) so that the hydrogen ions are used to produce useful energy in the electron transmitter system. This idea would help to explain the growth in vivo when the hormones act normally or when a small concentration of estradiol is injected into a tissue culture.

Since hormones do act in trace amounts and since a direct relationship exists between inhibition of cell growth and the external concentration of estradiol (Grauer, 1962), it may be concluded that the concentration used in this experiment is not sufficient to inhibit DNA synthesis and cell

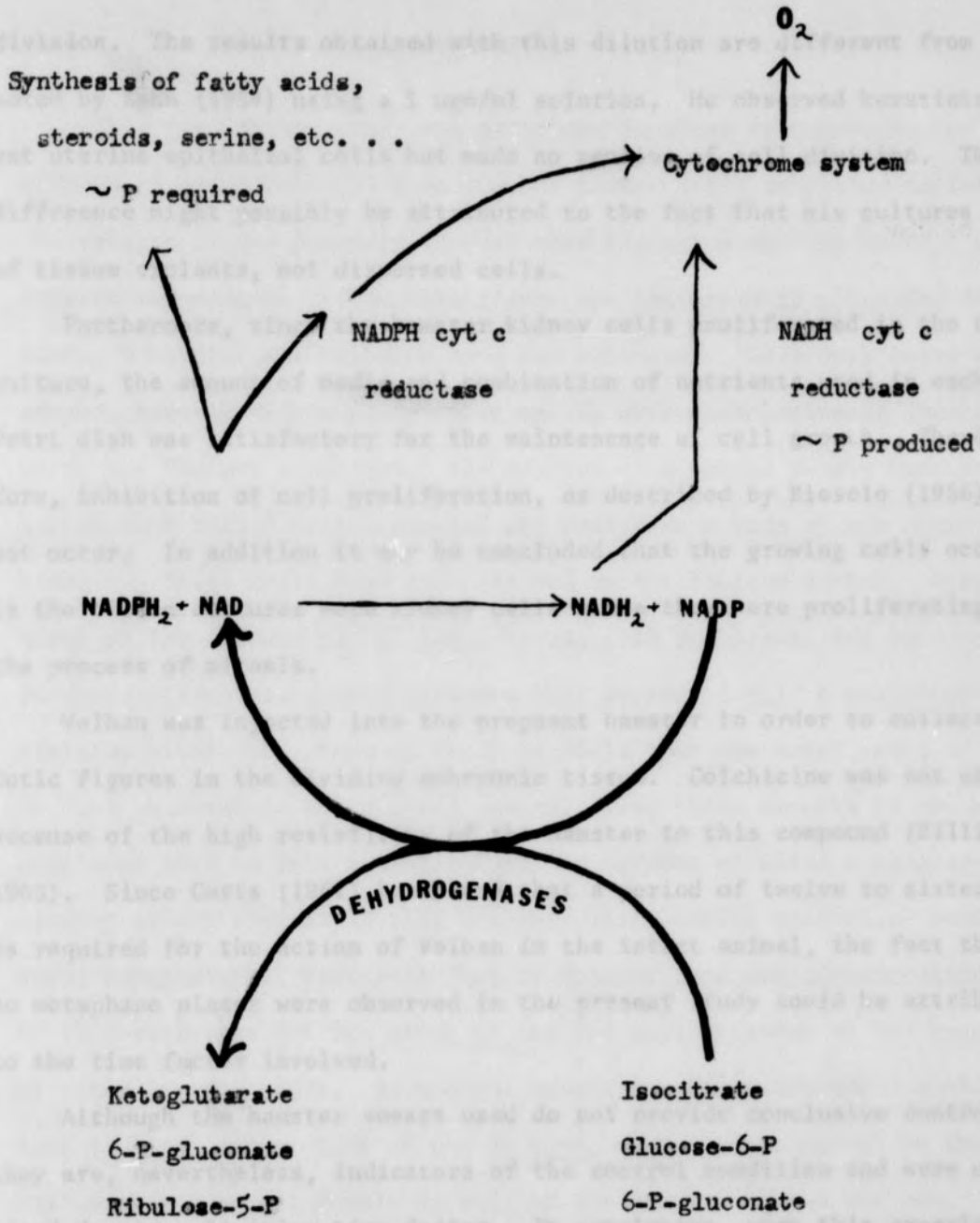


FIGURE 5. THE KEY ROLE of TRANSDEHYDROGENASE in CONTROLLING the RATE of ENERGY PRODUCTION

(modified from Allen, 1962.)

division. The results obtained with this dilution are different from those noted by Kahn (1954) using a 1 ugm/ml solution. He observed keratinized rat uterine epithelial cells but made no mention of cell division. The difference might possibly be attributed to the fact that his cultures were of tissue explants, not dispersed cells.

Furthermore, since the hamster kidney cells proliferated in the tissue culture, the amount of media and combination of nutrients used in each Petri dish was satisfactory for the maintenance of cell growth. Therefore, inhibition of cell proliferation, as described by Biesele (1956) did not occur. In addition it may be concluded that the growing cells occuring in the tissue cultures were kidney cells since they were proliferating by the process of mitosis.

Velban was injected into the pregnant hamster in order to collect mitotic figures in the dividing embryonic tissue. Colchicine was not used because of the high resistivity of the hamster to this compound (Billingham, 1963). Since Cutts (1961) has found that a period of twelve to sixteen hours is required for the action of Velban in the intact animal, the fact that no metaphase plates were observed in the present study could be attributed to the time factor involved.

Although the hamster smears used do not provide conclusive controls, they are, nevertheless, indicators of the control condition and were utilized due to a limiting time factor. In conclusion, when this experiment is repeated, more acceptable controls for the tissue cultures will be utilized and a study will be made of the <u>in vivo</u> effects of estradiol on the kidneys of the pregnant females as well as the kidneys of the embryos.

SUMMARY

This preliminary study was attempted in order to determine the effects of estradiol-3,17 B on hamster kidney cells in tissue culture. The embryos of one pregnant hamster were dissected and the kidneys were removed and minced. After this tissue was incubated *in vitro* for 24 hours, estradiol was injected into the cultures. Three days later the slides, covered with proliferating cells, were histologically prepared using the Feulgen technique. The embryos of a second female hamster injected with Velban were dissected and smears were made of the embryonic kidneys. These cells were also stained by the Feulgen method. Calculations of the percentages of interphases, late prophases, and telophases determined from each slide indicate that estradiol-3,17 B activated cell division within the range of 78.3% to 95.1% over the basal level of 1.3% to 2.0% observed in the control smears. From these results it may be concluded that in this investigation the hormone elicited a mitotic-stimulating effect similar to that obtained with uterine epithelial cells in other experiments. From this fact it follows that the concentration used in this study was not too great to inhibit cell division as has been noted by other investigators. Additional experiments will include a strict control *in vitro* and a study of the *in vivo* effects of estradiol on the kidneys of the pregnant female as well as the kidneys of the embryos.

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