PEMBROKE STATE UNIVERSITY

THE MECHANISM OF KEPONE-INDUCED ESTROGENIC RESPONSE

by

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This thesis is recommended for approval by the student's major professor.

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Introduction

Kepone and other organochloride insecticides have been shown to exert estrogenic activity on estrogen target cells (17). In this experiment we have attempted to determine the mechanism by which Kepone exerts this extrogenic effect.

The physiological Effects of Estrogen Binding

Estrogen is a female sex hormone and is responsible for the stimulation, development and functioning of the accessory sex organs (the uterus, uterine tubes, and vagina) and for the development and maintenance of the secondary sex characteristics. Naturally occuring estrogens are steroids characterized by 15 carbon atoms and three double bonds in the first ring.Three compounds, estradiol, estrone, and estriol, found in the female bloodstream, have been shown to produce estrogenic activity, but estradiol only acts on certain cells called, "target cells". These target cells contain receptor proteins within the cytoplasm which are specific for estrogen. These estrogen receptors constitute a family of macromolecular complexes which are composed of dissimilar subunits. The macromolecular association state is altered by the binding of the hormone with accompanying release of a small molecular component of the original receptor complex (15). The estrogen in combination with its receptor modulates the interaction between the two

molecular capacitor systems of the cell; the nucleochromatin and the cytoplasmic membranes. The cytoplasmic protein receptor binds to the hormone (a process called transformation) and carries it to the nucleus of the cell (a process called translocation) (see figure 1). When estradiol enters the uterine it combines with the receptor to form a non-covalent, high affinity complex (15). When the steroid-receptor complex moves to the nucleus of the cell it is bound to acceptor sites on nuclear chromatin. A new equilibrium is then acheived in which up to 90 percent of the estrogen bound to the receptors is in the nuclear position. When the estradiol is bound to nuclear chromatin it promotes the synthesis of mRNA (messenger RNA) which codes for cell-specific proteins. Transport of mRNA to the cytoplasm results in the synthesis of new proteins on cytoplasmic ribosomes which then produce the specific steroid-mediated functional response which is characteristic of that particular target tissue.

The inference that estradiol binds to receptor substances and initiates uterine growth without itself undergoing chemical conversion has proved consistent with the results of Jensen and DeSombre (13). Upon homogenization of uterine target tissue, a soluble protein capable of binding tritiated-estradiol (^{3}H-estradiol) was shown to exist in the cytoplasm (16). This binding protein was considered to be a receptor because of its limitation to estrogen target

tissue, its high binding affinity for estrogens, and its specific attraction for only biologically active estrogens.

After exposure of uterine tissue to (^{3}H)-estradiol, two intracellular sites of hormone binding were noted; one in the cytoplasm and another in the nucleus. Subsequently nuclear binding appeared to predominate (16). Jensen and DeSombre (13) noted that as estradiol reacts with uterine cells to become localized in the nucleus, the extranuclear receptor protein temporarily disappears. It was found that the total receptor content of rat uterine cytosol is less after a large dose of estradiol than after a small one and after administration of physiologic amounts of hormone there is a progressive fall in cytosol receptor content for about four hours. Quantitative studies (13) on the nature of this transfer reaction have shown that it is at the expense of the cytoplasmic estrogen-receptor complex and that the rate of depletion of the cytoplasmic receptor is dependent on the concentration of estradiol in the incubation medium.

It has been found (13) that when uterine nuclei are incubated with tritiated estrdiols, the nuclei take up more radioactivity when the incubation is done with uterine cytosol rather than with buffer solution alone. These findings indicate that the receptors in the cytosol are necessary for the maximum uptake of the steroid.

Because it has been reported by O'Malley *et al* (16) that the deoxyribonuclease releases bound estradiol from

uterine nuclei, DNA is implicated in the nuclear binding of the hormone-receptor complex. Both cytosol and nuclear receptor forms of the complex were shown to bind DNA. The DNA molecules within the cell nucleus have only a limited number of high affinity binding sites for the steroid-receptor complex. O'Malley et al (16) found that when (^{3}H)-estradiol is incubated directly with preparations of target tissue chromatin in vitro, very little of it becomes bound to the chromatin. However, incubation of the preformed (^{3}H)-estradiol receptor complex from the uterus with uterine chromatin results in significant retention of the complex on the chromatin. These findings indicate that the receptor complex is necessary to bind the steroid to the nuclear DNA.

Formation of the nuclear complex is a temperature dependent phenomenon that proceeds at an appreciable rate between 25° and 37°C (13). After immature rat of calf uteri are exposed to diluted solutions of tritiated estradiol at 37°C, the intracellular distribution pattern of uterine radioactivity is similar to that seen after hormone administration in vivo. But if the tissue is treated with hormone at 2°C, the major portion (70-75%) of the radioactive steroid appears as a complex in the cytosol fraction. When such uteri, rich in extranuclear complex, are warmed briefly to 37°C, redistribution of the steroid takes place within the tissue to yield predominately nuclear bound steroid. These observations imply that the radioactive estradiol

can associate with the extranuclear binding site by a second process that does not proceed readily at low temperatures.

Available data (16,13) strongly suggests that estrogen acts in the nucleus to promote the synthesis of mRNA's which are necessary for the subsequent actions of this steroid on growth and differentiation. In vivo RNA metabolism has been extensively studied in the mouse and the rat uterus (11). After administration of estradiol, an increase in total uterine RNA has been observed (17). after six hours of hormone treatment.

After incubation at 25°C for 30 minutes with uterine cytosol containing stradiol, subsequent RNA synthesis is increased nearly threefold in uterine nuclei (13) whereas there is no enhancement of kidney of liver nuclei after incubation with the hormone and their own cytosols. These nuclei from the hormone-dependent tissue appear to possess a specific defeciency in RNA synthetic capacity that can be alleviated by the estradiol-receptor complex of uterine cytosol.

In order to test the hypothesis that estrogen acts in the nucleus to promote the synthesis of mRNA which codes for specific cell proteins, O'Malley and Means (16) incubated chick ovalbumin in RNA which represents 60 percent of the protein synthesized in oviduct gland cells under the influence of estrogen with Avain DNA. Their results showed

that 90 percent of the labeled DNA formed a stable complex with the mRNA indicating that the (^{3}H)-labeled DNA was indeed a complementary copy.

The binding of estradiol to receptor proteins of uterine tissue is remarkably strong (13). This tight binding appears to result from a slow rate of dissociation; once formed the complex does not readily lose estradiol.

Estrogenic Effect of Kepone and Other Organochlorides

Both Kepone (decachlorotetracyclodecane) and DDT have been studied (5, 12, 4, 7) as to their effects on estrogen target cells. These insecticides are members of the chlorinated hydrocarbon group and tend to accumulate in the tissue lipids of animals. Both Kepone and DDT are present in the tissue lipids of people in the general population of the United States (19). The routes of entry are primarily alimentary and respiratory. These organochloride insecticides are stored primarily in the adipose tissues of humans and it has been reported (19)that these compounds crooss the placenta allowing the fetus to engage in metabolism of the organochloride compounds. Various concentrations of Kepone inhibited reproduction of bobwhite quail and altered feather pigmentation of male pheasants, quail and ducks to resemble the plumage of adult females (19). Kepone reduced reproduction in mice and decreased egg production in laying chickens (5). Kepone is also reported (4) to

cause hepatic carcinoma in experimental animals. In humans Kepone has been seen (19) to cause tremors, irritability, memory loss, ataxia, sterility, and was shown to change the structure of schwann cells which wrap around the nerve cell axons. In 1974 Kepone residues were found in almost all commercial foodstuffs and in 99.5 percent of all human tissue analyzed (2)!

The similarity of DDT to synthetic estrogen has prompted investigation of the estrogenic activity of DDT in mammals and birds (5). Bitman et al (14) have examined two different isomers of DDT. o-p'-DDT, which composes 20 percent of the total DDT, was found to be estrogenic, while p-p'-DDT, which comprises the remaining 80 percent of DDT, is only weakly estrogenic.Glycogen content increased after administration of o-p'-DDT in a manner very similar to its reaction to estradiol, while p,p'-DDT exhibited only slight actvity. It was found (5) that a 100 percent increase in oviduct weight and 150 to 175 percent increase in glycogen content occurred, along with increases in water content, and RNA content 24 hours after administration of o,p'-DDT. Bitman et al (4) report that over 1 billion pounds of DDT is presently available in the biosphere. Since o,p'-DDT is present in technical DDT to the extent of 15 or 20 percent, approximately 200 million pounds of an active estrogen may be present in the environment. Palmiter and Mulvihil (17) report that Kepone's ability to compete with (^{3}H)-estradiol uptake into the nuclei of in vitro oviducts indicates that Kepone is 1/20th as effective as estradiol in competing with estradiol for oviduct nuclear receptor sites (17). Palmiter and Mulvihill

report that Kepone mimics the estrogen steroids by binding to and activating the receptor molecules that normally mediate estrogenic activity and increase the concentration of other active steroids by altering the normal synthetic or degradation pathways in steroid metabolism leading to altered serum concentrations of the active steroids. Palmiter and Mulvihil (17) isolated oviduct nuclei from estrogen-treated chickens and incubated the nuclei in (^{3}H)-estradiol plus varying concentrations of unlabeled estradiol or Kepone. Kepone acts in vivo by binding to estrogen receptors, although with a weak affinity, thereby causing the migration of those receptors into the nucleus and promoting specific RNA production. Eight hours after the chicks received intraperitoneal injections of Kepone (30mg. per bird), the concentration of ovalbumin RNA in the oviduct showed a 25 fold increase which is similar to the effect that estradiol would have on these target cells (17).

Since Kepone has been shown to exert an estrogenic effect on estrogen target cells of the body (17) this sudy will investigate the hypothesis that Kepone causes the same protein receptor transformation and translocation as is seen in the specific binding of estrogen.

DISCUSSION OF THE TECHNIQUE

The procedure used in the experiment was first developed by Anderson, Clark, and Peck (3). The method is based on the exchange of (^{3}H)-estradiol with non-labelled estradiol that is bound to nuclear binding sites. The depletion of cytoplasmic binding sites is accompanied by a parallel increase in the quantity of nuclear binding sites. The determination of specific nuclear binding may be the only accurate way to detect the amount of estradiol bound in the uterus. When the procedure was first developed portions of a nuclear fraction from rat uteri were dispensed into two series of tubes, A and B. Series A contained (^{3}H)-estradiol and was used to determine the total amount of (^{3}H)-estradiol exchange. Series B contained the same amount of (^{3}H)-estradiol as in A plus a 100-fold excess of diethylstilbesterol which competes for the specific binding sites but not for the non-specific sites. The number of specific sites is obtained by subtracting the total number of sites found in series B from the total number of sites found in series A. The difference between the two series indicates the number of specific sites which were bound by the diethylstilbesterol.

Because a 100-fold excess of diethylstilbesterol results in maximal competition for nuclear binding of (^{3}H)-estradiol, the (^{3}H)-estradiol present in the nuclear fractions that were incubated with diethylstilbesterol was non-specifically bound. The quantity of specifically-bound estradiol was found

by subtracting the nonspecific fraction in series B from the total nuclear binding sites in series A.

The amount of bound (^{3}H)-estradiol in the abscence of diethylstilbesterol indicates the total number of nuclear binding sites. The number of non-specific binding sites was determined by incubating an identical nuclear fraction with (^{3}H)-estradiol in the presence of an unlabeled competitor

MATERIALS AND METHODS

Animals: Immature (21-26 days of age) female Holtzman rats were maintained in the laboratory and were fed Purina Lab Chow and water.

1. Injection of animals.

   a) Control group was injected intraperitoneally with .02ml. of vehicle (dimethylsulfoxide).

   b) Kepone-injected group received 1mg. of kepone in .02ml. of vehicle.

If kepone acted in the same fashion as estrogen, the receptor transformation and translocation into the nuclei of target cells should have occurred at this time.

   c) A third group was injected with 1 mcg. of estradiol per rat in .5ml. of vehicle.

Estrogen incorporation into the uterus is markedly dependent upon the route of administration and the type of injection vehicle used. Maximal estrogen levels were observed by Bitman et al (4) within one-quarter to one hour after intravenous and saline subcutaneous (s.c.) injections, but were not attained until two to six hours with s.c. oil injections.

2. I waited one hour.

3. The animals were sacrificed by decapitation.

4. The uteri were removed.

5. The uteri were homogenized in order to separate the nuclei from the surrounding cytoplasm.

6. The homogenate was centrifuged to isolate the nuclear fraction.

7. The nuclear fraction was divided into two series: Series A

measured the total bound nuclear receptor sites and series B measured the non-specifically bound receptor sites. All three groups were divided into series A and B.

8. The totally bound nuclear group (series A) was incubated in (^{3}H)-estradiol for one hour to allow the (^{3}H)-estradiol to bind to the receptors in the nucleus.

9. The non-specific fraction (series B) was incubated in (^{3}H)-estradiol plus a 100-fold excess of competitor (diethylstilbesterol).

10. The homogenate was washed in buffer solution to remove excess unbound (^{3}H)-estradiol.

11. The homogenate wassgiven a final washing using 100% ethanol which removed the (^{3}H)-estradiol from the homogenate and left it suspended in solution.

12. The (^{3}H)-estradiol suspended in the ethanol was then placed in scintillation vials and the amount of radioactivity was determined.

RESULTS AND CONCLUSIONS

Table of Group Radioactivity Levels (c.p.m.)

Group	Series A	Series B
Control	58591	130447
Estrogen	96332	206123
Kepone	53538	114445

The numbers represent mean c.p.m. (counts per minute) on the basis of seven trials.

As the above table indicates, the mean series A radioactivity for the control is 58591 and for series B is 130447. The mean series A estrogen was found to be 96332 and the series B mean was 206123. The Kepone group showed a mean 53538 for series A and an mean of 114445 for the B series. A comparison of the Kepone and control series A means indicates that Kepone is probably not having any estrogenic effect on the uterine target cells. This data however; cannot be used to draw any definite conclusions due to the wide variability in the results and the unexpectedly high levels of radioactivity found in the B series. Possible explanations for the high B series are:

1) The B solution was incorrectly prepared.

2) The A and B series were mislabeled.

3) Steps in the procedure were omitted.

4) Not enough buffer solution was used to wash off the (^{3}H)-estradiol.

5) The time of centrifugation was not long enough to separate the nuclear portion from the cytosol.

One of several of the above factors may have contributed to the excessively high B series radioactivity. After the first trial another B solution was prepared but the results again showed a high B series. Each series was labeled as the Aand B solutions were added so the chances for mislabeling the series were very small. The procedural steps were checked with experiments similar to this one and no steps were found to be omitted. Quite possibly the amount of buffer solution was not adequate and the time of centrifugation should have been prolonged from 7 to 15 minutes in order to insure the separation of the cytosol and nuclear fractions. In the event of future experiments of this type I would suggest using at least 15 mililiters of buffer solution per washing combined with at least 15 minutes of centrifugation.

GLOSSARY

Diethylstilbesterol- a crystalline synthetic estrogenic substance which competes with estrogen and kepone for specific nuclear binding sites.

Estradiol- a naturally occuring female sex hormone.

Estrogenic- having the same effects as estrogen such as increased glycogen production, increase in weight, increase in water content, and increase in RNA content.

(^{3}H)-Estradiol- estradiol which has been labelled with tritium (^{3}H), a radioactive isotope.

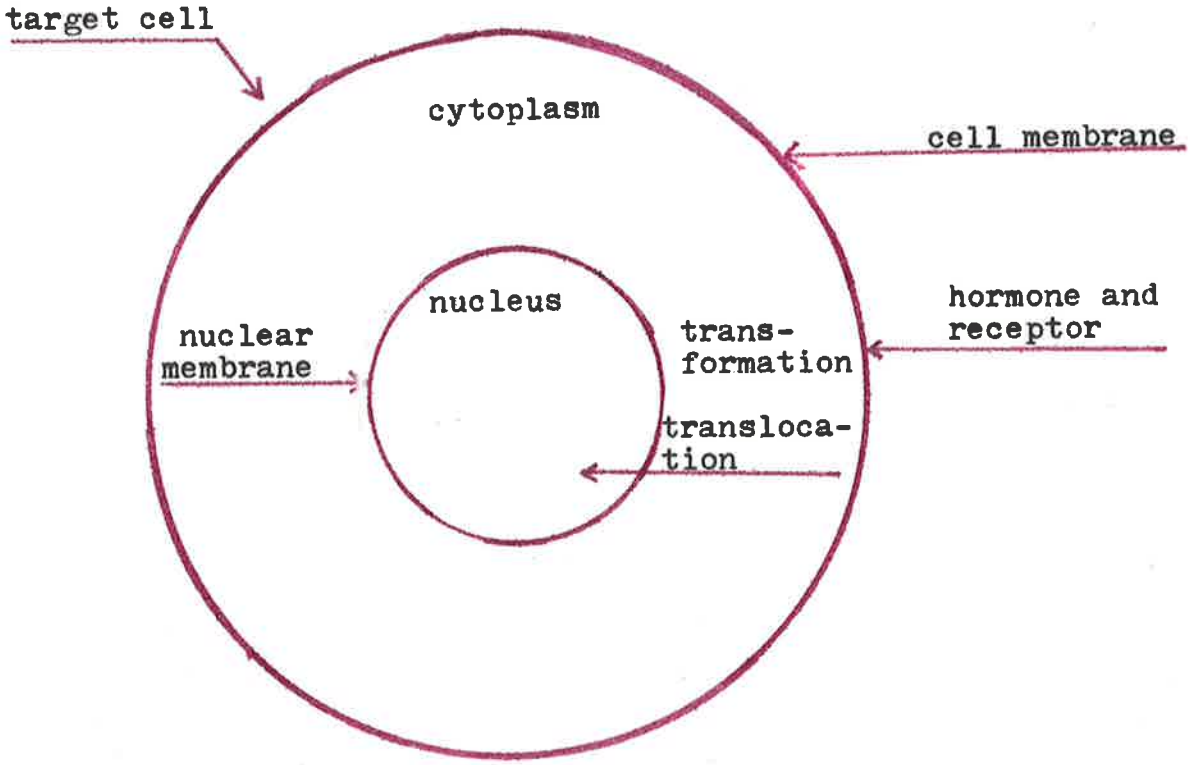
Non-specific binding sites- low affinity, high capacity hormone acceptor sites located within the nucleus of the cell.

Specific binding sites- high affinity, low capacity hormone acceptor sites.

Target cell- a cell upon which a hormone exerts its action.

FIGURE-1

THE MECHANISM OF RECEPTOR ACTION



Transformation- The cytoplasmic receptor binds to the hormone and carries it across the cell membrane and into the cytoplasm.

Translocation- The receptor-hormone complex crosses the nuclear membrane and is bound to acceptor sites on nuclear chromatin.

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Student: Cameron Cole

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