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(54) PREPARATION OF DIRECTLY IODINATED STEROID HORMONES AND RELATED DIRECTLY HALOGENATED COMPOUNDS

(71) I, VELAYUDHAN SAHADEVAN of 3825 Golf Road, Evanston, Illinois 60203, United States of America, a citizen of India, do hereby declare the invention, for which I pray that a patent may be granted to me, and the method by which it is to be performed, to be particularly described in and by the following statement:

The present invention relates to a process for the preparation of halogenated steroid hormones and to halogenated steroid hormones prepared by the process.

In order to quantify the hormone present in an assay tube, it is necessary to determine the amount of hormone bound to the antibody. A radioisotopically labelled steroid is used for this purpose and according to Niwender, G.D., Akbar, A.M., and Nett, J.M. in "Methods in Enzymology, Vol. 36, Hormone Action, Part A: Steroid Hormones", Academic Press, 1975, at pp. 16-33, it is not possible to radioiodinate the cyclopentanophenanthrene nucleus or its substituents directly. Furthermore, these authors report that, although phenolic steroids can be radioiodinated directly at the 2 and 4 positions, this seems to alter the configuration of the molecule and affects the binding to the antibody because the physical dimensions of an iodine atom approximate to those of the complete phenolic A ring in the molecule.

By conjugating the steroid to a tyrosine-containing protein, for example to bovine serum albumin (BSA), it is possible to radioiodinate the protein without affecting the ability of the steroid to bind to an antibody. However, there is a distinct difference between the tyrosine-containing protein and the procedure of this invention in that herein the steroid is directly halogenated, e.g. iodinated. Furthermore, the use of tyrosine containing protein does not ensure that only one steroid molecule is attached to each albumin molecule and, in fact, the steroid-to-protein ratio is at least 20:1.

To prepare one steroid per radioiodinated protein, Niwender *et al* uses the methyl ester of tyrosine (TME) conjugated steroid protein derivative. The phenolic tyrosine ring of this derivative is easily iodinated with $Na^{125}I$ by Chloramine-T procedures of iodination of proteins. The steroid-protein-TME-^{125}I thus prepared is shown to possess antigenic specificity to steroid antiserum and could be used for the radioimmunoassay procedures of estrogen. Chloramine-T has the systemic name (N-chloro-p-toluenesulphonamido)sodium.

According to G.E. Abraham and W.D. Odell, "Solid Phase Radioimmunoassay of Serum Estradiol-17 beta" in "Immunologic Methods in Steroid Determinations". Peron and Caldwell (1970), estrogen (E_2) may be labeled with radioisotopes emitting beta- or gamma-radiations using radioactive halogens such as ^{82}Br, ^{125}I and ^{131}I at positions C-2 and C-4 in the phenolic ring. However, the short half-life of the halogens limits its use to a few days only. Another factor, these authors report, that almost rules out the radio-active halogens as a possible marker on the E_2 molecule is inherent in their physical properties, namely their highly electronegative characteristics which affect the net charge of the molecules and the radioactive halogens with the longest half-life are relatively large atoms that would interfere with the steric fitness of the antibody active sites. According to these authors, estradiol 17β which is directly iodinated at positions 2 and 4 is unable to bind the anti-serum because of the interference induced by the relatively large atoms of iodine with the steric fitness of these active sites of the antibody.

It is known that the steroid synthesizing organs such as the gonads, the fetoplacental unit and the adrenal cortex utilize the same precursor substances for the synthesis of steroid hormones. Also, steroid specific receptor proteins are present in various normal tissues and

in certain malignant tumors. Receptor sites for both estrogen and progesterone are usually found in hormone dependent tumors such as human breast cancer.

When tritiated estradiol is injected into patients with breast cancer, those patients responding to endocrine treatment usually concentrate more tritiated hormones in their tumor tissue than those patients who do not respond to hormonal treatment. The uptake of tritiated hormone by the tumor tissue is facilitated by the presence of specific estrogen receptor sites in such tumors. Other tumors such as endometrial carcinoma and prostate cancer are known to possess specific receptor sites for steroid hormones like estrogen, progesterone and testosterone.

Accordingly, the present invention provides in one aspect a halogenated steroid hormone prepared by reacting a parent steroid hormone (as herein defined) wth an alkali metal halide in the presence of an agent selected from hydrogen peroxide and chloramine-T at ambient temperature.

In a preferred embodiment the present invention provides a radioactive halogenated steroid hormone prepared by reacting a parent steroid hormone (as herein defined) with an akali metal halide containing radioactive halogen in the presence of an agent selected from hydrogen peroxide and chloramine-T at ambient temperature. Preferably the radioactive halogen is selected from iodine-123, iodine-125, iodine-130 and iodine-131.

In another aspect the present invention provides a process for the preparation of a halogenated steroid hormone which comprises reacting a parent steroid hormone (as herein defined) with an alkali metal halide in the presence of an agent selected from hydrogen peroxide and chloramine-T at ambient temperature.

The term "parent steroid hormone" as used herein and throughout this specification includes compounds which are steroid hormones secreted by the ductless or endocrine glands, compounds of the same structure produced synthetically, physiologically active analogues thereof and also the steroid hormone precursors having a cyclopentanophenanthrene nucleus such as cholesterol but does not include conjugates of such compounds. All of these compounds are chemically very similar, though a comparatively slight structural change produces in many instances physiologically disimilar effects which often act on entirely different physiologic systems. In many cases, small structural changes will result in the mere accentuation of certain effects. Thus the parent steroid hormones (as herein defined), when classified by the predominant pharmacologic effects that can be used as starting materials for the process of the present invention include the adrenal corticosteroid, known as anti-inflammatory, antiallergic and antirheumatic agents; the androgens and anobolic agents; the estrogens; the progestogens and progestins; and the diuretic and antidiuretic agents as well as the acetate, succinate, sodium succinate, diacetate, phenylacetate, propionate, benzoate, dipropionate and caproate derivatives thereof.

By the term "halogenated steroid hormone" as used herein and throughout the specification is meant a parent steroid hormone (as herein defined) which has been halogenated (with one or more halogen atoms) directly on the cyclopentanophenanthrene nucleus and/or directly on the substituents of said nucleus.

Normally the halogenated steroid hormones of the present invention have the same antigenicity and specific binding characteristics and/or receptor site specificity of the parent steroid hormone. For example, radioactive iodine labelled estradiol-17β prepared by the process of the present invention retains both the antigenic and receptor sites specificity of the parent estradiol-17β.

The halogenated compounds of this invention are in general prepared by dissolving the parent steroid hormone in an appropriate solvent such as methanol, ethanol or toluene. Methanol is the preferred solvent. To the dissolved steroid hormone, the radioactive or non-radioactive halogen, e.g. iodine in the form of an alkali metal halide is added in an alkaline solution and the halogenation reaction e.g. iodination reaction is allowed to take place at room temperature. The halogenated e.g. iodinated steroid hormone is removed from the solution, for instance by centrifugation, and by subsequent repeated washing, generally with either 0.1 N HCl or water. When the amount of steroid hormone used in halogenation e.g. iodination experiments is only a few micrograms, chromatographic procedures are normally used for the separation of halogenated steroid hormones from the unreacted halide e.g. iodide. The ratio of the reactants can be varied; by increasing the ratio of for example iodide to the steroid hormone concentration, radio-labelled hormone with high specific activity can be prepared.

The purity of the compound thus prepared is tested by precipitation, thin layer chromatography and by the binding affinity of the halogenated compound, e.g. labelled estradiol 17β, to its specific anti-serum and to the naturally occurring estrogen receptor sites.

In order to establish the effectiveness of the halogenated steroid hormones of this invention, prepared samples were subjected to tests whereby the results are shown

graphically and wherein:

Figure 1 is a graphical representation of the thin layer chromatogram wherein the abscissa is the distance of migration; and the ordinate is the count per minute per one cm. of chromatogram;

Figure 2 is a graphical representation of the serial dilutions of an antiserum against estradiol-17β wherein the abscissa is the dilution and the ordinate is the % binding; and

Figure 3 is a graphical representation of a radioimmunoassay of ^{125}I-estradiol with unlabelled estradiol wherein the abscissa is the PGM of non-radioactive estradiol and the ordinate is % bound radioactivity with two curves being shown at different dilutions.

Example I - Iodination of Estradiol-17β by Hydrogen Peroxide:

Estradiol-17β at varying concentration ranging from 0.2724-2.724 mg was dissolved in methanol at a volume of 10 - 100 lambda and allowed to react with radioactive iodide (^{125}I) as sodium iodide at a concentration varying from 10 microCurie to 1mCi in 10 lambda of 0.0005 N KOH or NaOH and 100 lambda of a 3% solution of hydrogen peroxide for about 12 hours. When milligram amounts of steroid are used for the reaction, a visible spontaneous precipitate is formed as soon as the radio-active iodide in alkaline solution and hydrogen proxide is added. At the end of this reaction time, the precipitate was removed by centrifugation. The trace of non-reacted iodide was washed away from the sediment with water or 0.1 N HCl. The washed precipitate was then dissolved in methanol. Diluted aliquot fractions of the supernatant and sediment were counted for their radioactivity, the percent labelling and the specific activity per millimole is calculated. About 50 - 76 percent of added Na-^{125}I is labelled to estradiol-17β by this procedure. Depending on the concentrations of estradiol and ^{125}I used in the reaction, the specific activity of the labelled estradiol varied from 0.075 - 0.8 Ci/mM.

Example II:

To obtain higher specific activity, the concentration of estradiol-17β was decreased to 2.724 microgram (0.01 micromole) and ^{125}I was increased to 1 mCi and the reaction was run as before in Example I. At the end of the reaction time, the unreacted ^{125}I-iodide was removed by transferring the reaction mixture to a Bio-Rad Ag 1×8, 200-400 and the labelled steroid was eluted with methanol. The radioactivity of the collected fractions were determined and the specific activity so obtained was calculated. The percentage labelling so obtained was about 70 and the specific activity of this ^{125}I labelled estradiol was 70 Ci/mM.

Example III - Iodination of Steroid Hormones by Chloramine-T Reaction:

As in previous experiments, estradiol-17β dissolved in methanol was added to ^{125}I as sodium iodide in 0.0005 N sodium or potassium hydroxide in 10 lambda volume. A freshly prepared solution of chloramine-T in distilled water (1mg) was added and spontaneous precipitation of estradiol from the solution takes place. After 5 minutes reaction time, 0.2 ml, 0.1 N HCl or 0.2 ml distilled water was added to precipitate all the steroid from the solution. The sediment was removed by centrifugation and washed with water or 0.1 N HCl to remove the traces of unreacted iodide. The labelled estradiol was dissolved in methanol and aliquot fractions of both supernatant and dissolved sediment are taken for measurement of radioactivity from which the percent labelling is calculated. The percent ^{125}I labelling of estradiol by chloramine-T procedure was 80-90.

Example IV - Iodination of Other Steroids and Steroid Hormones:

A variety of other steroid hormones were labelled both by the hydrogen peroxide and the chloramine-T procedures as described for the iodine labelling of estradiol-17β. The percentage labelling of each of these steroid molecules by both these procedures are summarized in Table I.

^{125}I LABELLING OF STEROID HORMONES

No.	Steroid	Percent Labelling Hydrogen Peroxide	Chloramine-T
1	Cholesterol	20	6.2
2	Δ^5-Pregnenolone	18	14.9
3	Progesterone	39-65	39.0
4	17α Hydroxypregnenolone	11	36.31
5	17α Hydroxyprogesterone	22	11.5
6	Dehydroepiandrosterone	10	21.0
7	Androstenedione	18	52.0
8	Testosterone	18	-
9	Estrone	47.5	85.5
10	Estradiol 17β	46-80	95.0
11	Estriol	47	48.0
12	16-Epiestriol	32	59.5
13	20β-Hydroxypregn-4-en-3-one	19	21.0
14	5β-Pregnane-3α,20α-diol	31.3	21.0
15	Androsterone	24.2	26.0
16	Etiocholanolone	10.3	12.0
17	Adrenosterone	38.9	62.0
18	Corticosterone	10.8	16.0
19	Cortisone	20.5	16.0
20	Deoxycorticosterone	68.8	50.9
21	Dexamethasone	19.3	46.0
22	Hydrocortisone	29.7	7.0

It is to be observed from the results shown in Table I that the steroids numbered 3, 4, 7, 9, 10, 11, 12, 15, 20 and 21 were labelled with ^{125}I to the extent of more than 30% by the use of either hydrogen peroxide or chloramine-T during the reaction.

Example V (Comparison)

Attempts to react Na ^{125}I directly with a steroid were unsuccessful. As described before, 100 mM estradiol-17β dissolved in 100 lambda methanol was added to 10 microcurie ^{125}I as sodium iodide in 0.0005N potassium hydroxide in 10 lambda volume. No hydrogen peroxide or Chloramine-T was added to the reaction mixture. It was allowed to react at room temperature for 12 hours. At the end of the reaction time, the precipitate was removed by centrifugation and washed with water. The percent labelling is calculated as described before, and in the absence of either H_2O_2 or Chloramine-T, it is found to be 9.5; whereas, in the presence of H_2O_2 or Chloramine-T the percent labelling is about 70-90.

Additional Tests on the Purity of the Iodinated Steroid Hormones:

The labelled steroid hormones are separated from the non-reacted iodide either by precipitation and repeated washings or by chromatographic procedures as described before. In addition, they are subjected to the following tests:

1. Thin layer Chromatogram (TLC) - Thin layer chromatogram of the iodinated steroid is run on silica gel using the solvents ethanol, methanol, and ethyl acetate in a 94:5:1 respective ratio and after 1 hour and 40 minutes run, the distribution of radio-activity observed for ^{125}I labelled estrogen as shown in Figure 1.

2. Immunological Specificity of Directly Iodinated Estradiol-17β:

Radioimmunoassay procedures are used for the testing of the antigenic specificity of the ^{125}I labelled estradiol-17β.

A. The effect of serial dilutions of estradiol antiserum in its binding of fixed amount of ^{125}I labelled estradiol-17β (specific activity 0.8 Ci/mM).

Serial dilutions of an antiserum against estradiol-17β starting from 1:100 up to 1.51,200 were made and the percentage binding of about 20 ng ^{125}I labelled estradiol-17β was determined. The unbound ^{125}I estradiol was removed by charcoal adsorption. As shown in Figure 2, at a lower antiserum dilution of 1:100, the percent bound ^{125}I estradiol-17β is 75.3. And, as the concentration of antiserum is decreased (dilution increased), a proportionate decrease in antiserum-bound ^{125}I estradiol is readily observed.

B. The Radioimmunoassay.

The procedures of this assay are identical to those described by the instant inventor and authors C.P. Perlia, S.G. Economou, and H. Sky-Peck in *Journal of Surgical Onocology*, 7:467-477 (1975), except for the following modification. To 0.1 ml anti-serum 1:10,000 or

1:20,000 dilution and ^{125}I estradiol-17β (70 Ci/mM), increments of non radioactive estradiol were added. After incubation, the unbound hormone was removed by charcoal adsorption. As shown in Figure 3, as the concentration of unlabelled estradiol increases, the percent bound ^{125}I estradiol decreases because of the competition for the available binding sites at the antiserum. In comparison with the standard curves obtained for radioimmunoassay of estrogen using ^{3}H labelled estradiol, this ^{125}I labelled estradiol gave higher sensitivity. This indicates the higher specific activity obtained by the procedure described here for ^{125}I labelling of estrogen.

Binding of Directly Iodinated Estradiol-17β to Estrogen Receptor of Human Breast Cancer:
Estrogen receptor assay of human breast cancer was carried out by incubating the receptor-containing tumor cytosol with radioactive estrogen alone and in parallel experiments with both radioactive and non-radioactive estrogen. The non-radioactive estrogen acts as a competitive inhibitor of the binding of radioactive estrogen to the estrogen receptor sites. The receptor-bound radioactive estrogen is identified by sucrose gradient analysis by which the receptor-bound radioactivity appears at 8S or 5S regions which is competitively inhibited by a higher dose of non-radioactive estrogen.

Tumor cytosols prepared from human breast cancer containing estrogen receptor were assayed by sucrose gradient analysis using tritium labelled estradiol. They are compared with the receptor binding of ^{125}I labelled estradiol. It is found that the binding of ^{125}I labelled estradiol to estrogen receptor sites are identical, or even superior as compared to the assay results with ^{3}H labelled estradiol-17β.

Administration of high energy gamma emitting radioiodine labelled steroid hormones or their precursor substances to patients may greatly improve the ability to visualize the steroid synthesizing organs and/or tumor sites that may contain specific steroid hormone receptor sites by radioisotopic scanning procedures. In addition, such radiolabelled steroid hormones may be trapped at the receptor sites of the tumor tissue and the high energy gamma ray emitted by the receptor-bound radiolabelled hormones may induce specific cytotoxicity at the tumor sites. Organs without specific receptor sites and therefore without any specific concentration of the radioactive hormones may not be affected by such treatment. This approach may allow a systematic treatment with radiolabelled hormones and therefore destruction of tumor by radiation induced cytotoxicity. It could be mediated by the interaction of radiolabelled hormone and the receptor sites of the tumor tissue. By conventional radiotherapy, only localized treatment is possible.

Since the halogenated, e.g. iodinated steroids produced by the process of this invention generally retain both the antigenicity and the specific binding characteristics which are identical to the naturally occurring parent steroid hormones, they are physiologically active substances as hormones and they may be used for example as estrogenic or progestational agents or as glucocorticoid and as anti-inflammatory substances depending upon the physiological role of the parent steroid hormone used for the halogenation e.g. iodination process.

The radioactive halogenated steroid hormones within the scope of this invention can be formed by direct reaction with a radioactive alkali metal halide such as sodium iodide-125, potassium iodide-125, sodium iodide-123, sodium iodide-131 and potassium iodide-131. The same procedure can be used to prepare the isotopes ^{123}I and ^{130}I as may be required in tracing receptor sites in tumor tissue where both antigenic and receptor site specificity is required. For these and other purposes other alkali metal halides wherein the halogen is a radioactive isotope having the prerequisite lifetime, mode of decay, decay energy, particle energy and intensity for proper detection and use in such studies can be used. Since the selection of the particular isotope is a matter well known in the art, no further explanation is deemed necessary for purposes of this invention. The process of the invention can also be applied to the use of non-radioactive alkali metal halides to prepare halogenated steroid hormones for selected uses.

WHAT I CLAIM IS:

1. A halogenated steroid hormone (as herein defined) prepared by reacting a parent steroid hormone (as herein defined) with an alkali metal halide in the presence of an agent selected from hydrogen peroxide and chloramine-T at ambient temperature.
2. A halogenated steroid as claimed in claim 1 wherein the agent is hydrogen peroxide.
3. A halogenated steroid as claimed in claim 1 wherein the agent is chloramine-T.
4. A halogenated steroid hormone as claimed in any of claims 1 to 3 having the same antigenicity and specific binding characteristics as the parent steroid hormone.
5. A halogenated steroid hormone as claimed in any of claims 1 to 3 having the same antigenicity and receptor site specificity as the parent steroid hormone.
6. A radioactive halogenated steroid homone (as herein defined) prepared by reacting a parent steroid hormone (as herein defined) with an alkali metal halide containing

radioactive halogen in the presence of an agent selected from hydrogen peroxide and chloramine-T at ambient temperature.

7. A radioactive halogenated steroid hormone as claimed in claim 6 wherein the agent is hydrogen peroxide.

8. A radioactive halogenated steroid hormone as claimed in claim 6 wherein the agent is chloramine-T.

9. A radioactive halogenated steroid hormone as claimed in any of claims 6 to 8 having the same antigenicity and specific binding characteristics as the parent steroid hormone.

10. A radioactive halogenated steroid hormone as claimed in any of claims 6 to 8 having the same antigenicity and receptor site specificity as the parent steroid hormone.

11. A radioactive halogenated steroid hormone as claimed in any of claims 6 to 10 wherein the radioactive halogen is selected from iodine-123, iodine-125, iodine-130 and iodine-131.

12. A halogenated steroid hormone as claimed in any of claims 1 to 11 wherein the parent steroid hormone is estradiol-17β.

13. A halogenated steroid hormone as claimed in any of claims 1 to 11 wherein the parent steroid hormone is selected from cholesterol, pregnenolone, progesterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone, estrone, estriol, 16-epiestriol, 20β-hydroxypregn-4-en-3-one, 5β-pregnane-3α, 20α-diol, androsterone, etiocholanolone, adrenosterone, corticosterone, cortisone, deoxycorticosterone, dexamethasone and hydrocortisone.

14. A halogenated steroid hormone (as herein defined) substantially as herein described with reference to Examples I to IV.

15. A process for the preparation of a halogenated steroid hormone (as herein defined) which comprises reacting a parent steroid hormone (as herein defined) with an alkali metal halide in the presence of an agent selected from hydrogen peroxide and chloramine-T at ambient temperature.

16. A process as claimed in claim 15 wherein the agent is hydrogen peroxide.

17. A process as claimed in claim 15 wherein the agent is chloramine-T.

18. A process as claimed in any of claims 15 to 17 wherein the alkali metal halide contains radioactive halogen.

19. A process as claimed in claim 18 wherein the radioactive halogen is selected from iodine-123, iodine-125, iodine-130 and iodine-131.

20. A process as claimed in any of claims 15 to 19 wherein the parent steroid hormone is dissolved in a solvent.

21. A process as claimed in claim 20 wherein the solvent is methanol, ethanol or toluene.

22. A process as claimed in any of claims 15 to 21 wherein the alkali metal halide is in alkaline solution.

23. A process as claimed in claim 21 wherein the alkaline solution is NaOH or KOH solution.

24. A process as claimed in any of claims 15 to 23 wherein the parent steroid hormone is estradiol-17β.

25. A process as claimed in any of claims 15 to 23 wherein the parent steroid hormone is selected from cholesterol, pregnenolone, progesterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone, estrone, estriol, 16-epiestriol, 20β-hydroxypregn-4-en-3-one, 5β-pregnane-3α, 20α-diol, androsterone, etiocholanolone, adrenosterone, corticosterone, cortisone, deoxycorticosterone, dexamethasone and hydrocortisone.

26. A process as claimed in any of claims 18 to 23 wherein the parent steroid hormone is estradial-17β and the specific activity of the labelled radio-active product is at least 70 Ci/mM.

27. A process as claimed in any of claims 15 to 26 wherein milligram quantities of the parent steroid hormone are used whereby a spontaneous precipitate is formed.

28. A process as claimed in any of claims 15 to 27 which further comprises the step of separating the halogenated steroid hormone from the reaction mixture.

29. A process for the preparation of a halogenated steroid hormone (as herein defined) substantially as herein described with reference to Examples I to IV.

ERIC POTTER & CLARKSON
Chartered Patent Agents
Agents for the Applicants
14 Oxford Street,
Nottingham NG1 5BP.

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1591357 COMPLETE SPECIFICATION

1 SHEET *This drawing is a reproduction of the Original on a reduced scale*

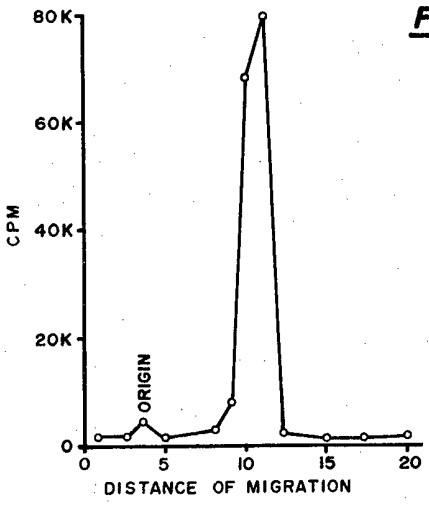

FIG. 1

TLC OF ^{125}I-E_2 FRACTION NO. 5

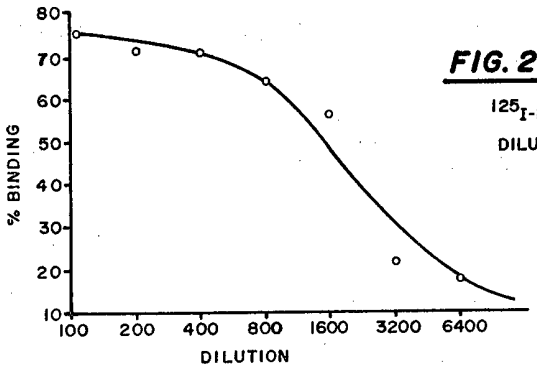

FIG. 2

^{125}I-E_2 BINDING TO SERIAL DILUTION OF E_2-AS

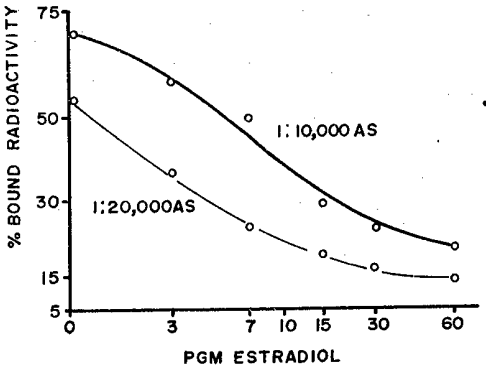

FIG. 3

^{125}I-ESTRADIOL