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ABSTRACT

The paper illustrates some aspects and problems of steroids RIA findings, obtained during the studies on the problem of weak estrous in swamp buffaloes. The work described herein which stem from three aspects of steroids RIA; 1) chromatographic technique of steroids separation 2) immunoreactivity of labelled ^{125}I steroids and displacement efficiency; 3) technique of the separation of free and bound fractions. The non-affinity and low displacement efficiency are also discussed.

INTRODUCTION

The advantage of RIA is that it brings us close to an ideal detection method because of its specificity and sensitivity. This is very much true for proteohormone as it can be performed directly because of its high specific antisera. But the antibody obtained against steroid hormones are not completely specific.

Steroids have low molecular weight and are not immunogenic. The critical point in steroids radioimmunoassay is the raising and availability of the specific antisera and immunoreactivity with the radioligand. Generally, a steroid determination is characterized

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by a three steps in analytical procedure; extraction, chromatographic purification and assay. Most investigators require a simple but accurate assay method rather which would require no chromatographic purification but a direct assay in the crude extract; although an advanced technique for raising highly specific antisera would help to achieve this ideal situation. The direct assay in crude extracts require a careful consideration of all possible interfering effects. The interferences which are coextracted with the steroids can be removed only by chromatography. The chromatographic purification step is, therefore, still needed for determination of some steroids. However, the major problem in steroid RIA is the specificity rather than the sensitivity. The complexity of steroid RIA, and the problems related to its optimization, are therefore, intimately connected with the specificity of the available antisera.

This paper is an attempt to describe some aspects of steroids RIA findings, obtained during our studies on the problem of weak estrous which is common among buffaloes in all parts of Asia. The buffaloes in the Region are characterized by a marked seasonal pattern of calving, with a peak calving season from August to end October; females are sexually inactive from March to end June; and during this period only 3 percent come into heat. On the basis of accumulation information are this subject, it has been clear for long time that research in this field require to be intensified and hence a series of investigations carried by the author, in recent year, in solving some of the reproductive physiological problems of the buffaloes.

STUDIES AND OBSERVATIONS

The work described herein covers three aspects of RIA.

(1) Chromatographic Technique of Steroids Separation.

(2) Immunoreactivity of Labelled ^{125} I Steroids and

Displacement Efficiencey.

(3) Technique of the Separation of Free and Bound Fractions.

1. Chromatographic Technique of Steroids Separation.

Many chromatographic systems are used to purify the steroid hormones prior to RIA. We are concerned with three steroids: progesterone (P), 17-hydroxyprogesterone (17-OHP) and 17-B estradiol (E_2). A number of investigators had found several chromatographic techniques to purify these steroids, which are as:

1. Celite microcolumns (Abraham, 1971).
2. Sephadex LH-20 columns (Mikhai, 1970)
3. Silica gel chromatography (Hotchkiss et al, 1971)

We are using a method which has been modified from what has been used in medical research method for currently measuring P, 17-OHP and E_2 levels simultaneously in the same aliquot of serum (Abraham, 1971). The antiserum which used in the assay reacted completely with P, 17-OHP and deoxycorticosterone (Kamonpatana et al, 1976). The chromatographic purification by celite microcolumn are therefore needed before RIA. The celite microcolumns are preferred because of its suitable qualities, for instance, high capacity and high power of resolution; being an inert support for the stationary phase and nonspecific interferences in the assay. So far, the low cost due to the non-significant interference of reused celite microcolumn, is the most interesting quality (Table I).

The solvent system used for this technique, is the more polar stationary phase of ethylene glycol. It can separate progesterone from other progestins, 17-OHP and 20 a-hydroxy-4-pregnon-3 one and also estradiol 17 B from estrous and estriol (Abraham, 1971). The mobile phases, is a mixture, consisting of ethyl acetate-isooctane.

As regards the level of reliability of this method, it is during the operation of the assay a high degree of accuracy showed be exercised because of the complexity of the method. We are now facing some problems in steroids RIA. They concern the variability of chromatographic steps in the method used and the expenses of the method.

The major factor influencing the reliability is the speed of the elution under nitrogen pressur 'uring celite chromatographic purification. The most important role in this step, in order to achieve a homogenous resolution system for all of the same assayed samples, is the speed control of elution. We found that the flow rate of elution should not exceed 4-8 drops/min, as otherwise it would decrease the efficiency of resolution (Table II). This factor causing the variability is mainly due to the distinguishable density of packing celite microcolumn. The used columns are therefore have to be selected. It accounts for the high expense of this procedure.

Furthermore, Abraham (1974) insisted that the temperature has an effect on the mobility of the mobile phases. It should be between $18^{o}C$-$25^{o}C$. A higher temperature increases the mobility and a lower one decreases the mobility. It may even cause the overlapping of steroids. A hot climate also increases the expense of keeping temperature ranges constant.

The limitations mentioned above make operations more difficult. Skill is the chief factor which ought to be considered strictly. We have now realized that ^{3}H-labelled steroids RIA cost ten times higher than the industrialised countries. There is a general agreement among our investigators that the high expense of the technique is an economic problem faced by the poorer countries.

Obviously, this acts as a constraint to proceed with the assay in such an area. So, attempt to solve this problem must depend

selecting of a suitable method. Although low cost is a major factor in this regard but the reliability and practicalbility are also equally important qualities essential to obtain an acceptable method.

Our objectives for steroids RIA are, therefore, aimed at

(a) Avoiding chromatography in ^{3}H-labelled steroids RIA.

(b) Examining the using ^{125}I-labelled steroids RIA.

In the first objective, we would like to avoid chromatography even if the nonchromatographic system is more time-consuming. The longer time might be involved in the investigation of more specific antisera rise. We have tried to perform a direct assay of serum progesterone, without chromatographic purification. The antiserum code "L", a 11-a conjugated progesterone raised antiserum obtained from Dr. G.E. Abraham was used in the assay. The specificity of the antiserum was carried out with various steroids; it showed no significant cross reaction(Fig.1). The levels of serum progesterone in swamp buffalo during one estrous cycle were determined to compare with the levels measured by the previously described method(Fig.2). It was obvious that the method without chromatography is an acceptable one. Obtaining the closely parallel progesterone pattern indicates the suitability of this method. Although we could avoid the complexity of the method, yet we have approached a new aspect of the more specific antisera raising technique, and a more reliable method achieved. So far, it had tended to be general major problem of steroids RIA.

It is a general knowledge among investigators that antisteroid antibodies are most specific for the portion of the steroid projecting out of the carrier protein and least specific for the portion of steroid linking to the protein. Niswender and collaborators (1970) studied the effect of site of conjugation on the specificity of antiprogesterone antibodies. Three conjugates were prepared,

with attachment to the protein carrier at C-3, C-11 and C-20 respectively. The C-11 conjugates gave the most specific antibodies. Such antibodies have been used in RIA procedures to measure plasma progesterone without chromatography. (Bodley, 1973).

The second objective, the possibility of using ^{125}I-labelled steroids RIA, we think that this investigation might be of great help to lower the cost of steroid RIA. Several factors control the choice between ^{3}H-labelled and ^{125}I-labelled steroids RIA. The advantages and disadvantage must be considered in the choice of what to use.

The advantages of using ^{125}I-labelled steroids compare with ^{3}H- labelled steroids are as follows:

1. higher precision.
2. easier operation.
3. cheaper cost.

The disadvantages are:

1. Its shorter half life causes the necessity to prepare the tracer and to concurrently operate the assay within certain time limited (not more than 2 months) in order to avoid adverse effects on reliability.

2. Purification of the prepared tracer must be done from time to time during that certain time limited to eliminate free iodine and degraded products.

3. Lower sensitivity.

The certain active site of steroids is really hard to know. Some informations can be derived from experiments aimed at assessing the condition to raise a specific anti-steroids antibodies and to identify the site of attachment of steroids. Even though a

number of investigators have produced very specific antisera, the possibility of RIA must depend on the relationship between affinity and displacement. The high affinity but low displacement make RIA impossible.

The following example illustrate the validity and limitations of the view that the site of attachment of steroid is significantly affected by the steric factor of labelled ^{125}I conjugated molecule.

2. Immunoreactivity of ^{125}I-Labelled Steroids and Displacement Efficiency.

The experiment using ^{125}I-labelled steroid derivative as ligands for steroid RIA was developed for estradiol measurement. The procedure used, followed the description by Cameron of the Tenovus Institute for Cancer Research Cardiff, U.K., which was based on those described by Nars & Hunter (1973) and Hunter, Nars & Rutherford (1974). Activation of estradiol at position 6 and 17 in a form of oxime and hemisuccinate were performed respectively. Coupling reaction took place with activated steroids and Iodo-histamine in dioxan-aqueous solution. The ^{125}I-labelled estradiols were purified by TLC on silica gel prior to use. Usually, the upper band showed effective binding with ligand. The successful use of the E_2-6-^{125}I ligand for estradiol RIA (Hunter et al, 1974) showed a limitation to one particular antiserum (Exley and Johnson, 1971). Immunoreactivity was performed in two systems; a homologous system and a heterologous system. These are definded as position of attachment of ^{125}I and the original antigenic protein identical and different respectively. The antibodies Ab-R_1, Ab-R_3 are obtained from Tenovus Institute for Cancer Research.

In homologous system, the reactions of a) anti E_2-6-BSA vs E_2-6-oxime Iodo histamine and b) anti E_2-17-BSA vs E_2-17-hemisuccinate-Iodohistamine were carried out. As a result in a) a dilution

curve for Ab-R_1 and Ab-R_3 was performed with and without inhibition of ligand binding by 1,000 pg non-radioactive estradiol (Fig. 3). Presence of binding but low displacement with non-radioactive estradiols appeared. The affinity of Ab-R_3 for the ligand greatly exceeded one of estradiol so practical assay system could not proceed. Furthermore, a high immunoreactivity could be achieved from Ab-R_3 more than Ab-R_1, indicating that homologous systems are not generally useful. The results in b) were tried in two comparative tracer dilution. It showed the presence of binding but low inhibition of non radioactive estradiol (Fig. 4). The reaction in heterologous system is completely inexplicable (Table III). Absence of binding seems to be the most interesting and a challenge for further investigation.

The results indicated that the anti-E_2-6-BSA/E_2-6-oxime-Iodohistamine and anti-E_2-17-BSA/E_2-17-hemisuccinate-Iodohistamine, the homologous systems cannot compete with the corresponding ones using a ^{3}H-labelled ligand in terms of specificity. The low displacement efficiency between the non-radioactive ligand and radioligand antibody complex might be due to the steric effect of ^{125}I-conjugated molecule. The reason for this opinion seems to be due chiefly to the free rotation of a single bond at C-6 conjugate between estradiol and ^{125}Iodo-histamine-oxime. Furthermore, due to the stereochemistry of ring A and B of the ligand, the -H and -OH at the 5 and 6 positions are cis to each other, but trans to the 3-OH and to the angular methyl at the 10 position. Fusion of the rings to each other can be cis or trans, thus increasing the complications of the stereochemistry. Finally, in any rigid cyclic system like this, conformational effects are marked and often completely control the course of reaction (Fig. 5).

According to the heterologous systems, there is no appearence of affinity, the explanation to this phenomenon may be the effect of ^{125}Iodine on electrophilic aromatic substitution. So far,

the chemical structure of estradiol is composed of aromatic properties in ring A. Moreever, the position 3-OH of ring A increase Iodide substitutions. Because of high reactivity of phenolic ring A, treatment with aqueous-buffer solutions of Iodide results in replacement of every hydrogen ortho- to the -OH group and may even cause displacement of certain other groups. If iodination is carried out in a solvent of low polarity, reaction may be limited to monoiodination. The substitutions of Iodides in their large size at position 2 and 4 might screen the active site of attachment. It, therefore, shows no affinity. As a result, assay conditions have to be re-optimized to take full advantage of this.

3. The Separation of Free and Bound Fractions.

Various techniques are used to separate free and bound fractions in steroids RIA.

1. Differential migration of free and bound fractions. This technique include paper chromatoelectrophoresis, electrophoresi on starch gel, cellulose acetate or polyacrylamide and gel filtration (Haber et al, 1966).

2. Fractional precipitation of bound fraction. Many organic solvents, inorganic salt and acids had been used by a number of investigators for instance; ethanol-NaCl, ethanol, dioxane and polyethylene glycol (Desbuquois and Aurbach, 1971). Besides, salts and acids were also used i.e.: sodium sulfate, ammonium sulfate (Ismail, 1972) and trichloroacetic acid (Mitchell and Byron, 1967).

3. Double antibody methods. These techniques were widely used (Mahajan et al, 1972) but the expense of obtaining the second antibody often prevent many workers from applying the technique.

4. Solid-phase antobodies offer advantages as separation systems, particularly in their suitability for automation. However, in some instances such systems have tended to limit assay sensitivity (Mikhai et al, 1970 และ Abraham, 1969).

5. Adsorption methods. These methods were commonly employed using many solid adsorbents such as anion exchange resin (Frenkil et al, 1966) silicate (Rosselin et al, 1966) and coated char-coal.

We used the dextran-coated charcoal suspension to absorb and precipitate the free steroid. There is an agreement that this separated technique gave many advantages, for example, it has low non-specific in an assay. It is also simple and fast to perform.

We found that 0.6% charcoal was the lowest possible concentration to give the acceptable blank value,the steepest curve as well as the high initial binding (Fig. 6). Although higher the charcoal concentration, the more it would adsorb the free steroids, the excess charcoal concentration does not pack well at the bottom of the tube and decrease the efficiency of the separation. It is also know that not only the charcoal concentration affects the dissociation of the bound but also the other factors do;for instance, polarity of steroids, time and temperature of incubation. Furthermore, we have observed that different centrifugation significantly affects the efficiency of separating free from bound fractions.

It would be valuable if one separation method were to prove suitable for general reference use. No such method yet appear to quality for this purpose.

CONCLUSIONS

Several limitations still influence application of the RIA of some steroids; such as the ^{125}I-labelled steroids and assay

technique. New problems are now appearing. A readily available source of ^{125}I-labelled radioligands for steroid immunoassay may well be the most difficult analytical challenge for the investigators working with steroids.

The studies on reproductive physiology of female buffalo and Artificial Insemination (A.I.) technique in buffalo are needed in order to achieve, as rapidly as possible, the required emphasis on quality of buffalo productivity. The hormonal parameter as a guide line in connection with breeding programme will be a great help to successful A.I. Since swamp buffalo has an weak estrous phenomena, Prostaglandin F_{2a} by means of induction and synchronization the estrous, would be helpful for A.I. in the future.

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Table I Comparative resolution of new and reused celite microcolumns in the seperation of progesterone (P), 17 - hydroxyprogesterone (17 -OHP) and 17 B - estradiol (E_2) added to $1cm^3$ of pooled free hormone male buffalo serum.

Steroids added		Found value ± SD in 5 determinations (pg/cm^3)		% Deviation (from the expected value)	
Name	pg/cm^3	new celite (1)	reused celite (2)	1	2
P	0	below sensitivity	below sensitivity	-	-
	250	245.8 ± 17.9	263.8 ± 29.4	- 1.7	+ 5.5
	500	511.5 ± 69.7	522.1 ± 68.5	+ 2.3	+ 4.4
	2,500	1,951.7 ± 104.8	2,391.5 ± 371.7	-21.9	- 4.3
				$\bar{x}$ = -7.1	$\bar{x}$ = +1.9
17-OHP	0	below sensitivity	below sensitivity	-	-
	250	241.7 ± 36.4	247.2 ± 43.3	- 3.3	- 1.1
	500	534.4 ± 33.6	525.5 ± 58.3	+ 6.9	+ 5.1
	2,500	2,441.6 ± 172.9	2,385.3 ± 121.4	- 2.3	- 4.6
				$\bar{x}$ = +0.4	$\bar{x}$ = -0.2
E_2	0	0	0	-	-
	100	101.3 ± 9.3	109.4 ± 16.4	+ 1.3	+ 9.4
	500	427.4 ± 27.4	521.3 ± 40.4	- 1.5	+ 4.3
	1,000	902.0 ± 77.4	1,248.2 ± 200.5	- 9.8	+ 24.8
				$\bar{x}$ = -3.3	$\bar{x}$ =+12.8

Table II The effect of eluted speed on the resolution efficiency by celite microcolumns in the separation of progesterone (P), 17 - hydroxyprogesterone (17 - OHP) and 17B -estradiol (E_2) as the relative values.

Flow rate drops / min	Steroids	Relative values between % CPM bound of each steroid VS the combination of P, 17 - OHP and E_2.		
		Isooctane	15 % Ethyl Acetate in Isooctane	40 % Ethyl Acetate in Isooctane
4 - 8	P	1.026 1.152	0.040 0.043	0.014 0.009
	17 - OHP	0.046 0.056	1.001 0.997	0.036 0.053
	E_2	0.038 0.052	0.041 0.045	0.828 0.944
30 - 40	P	0.841 0.876	0.026 0.082	0.013 0.027
	17 - OHP	0.236 0.049	0.818 0.588	0.043 0.042
	E_2	0.010 0.043	0.050 0.065	0.699 0.626

TABLE III BINDING OF RADIOLIGANDS WITH VARIOUS ANTISERA

REACTED SYSTEM	ANTISERUM	RADIOLIGAND	BINDING
HOMOLOGOUS	E_2-6-BSA	E_2-6-^{125}I	REACT
HOMOLOGOUS	E_2-17-BSA	E_2-17-^{125}I	REACT
HETEROLOGOUS	E_2-6-BSA	E_2-17-^{125}I	NONE
HETEROLOGOUS	E_2-17-BSA	E_2-6-^{125}I	NONE

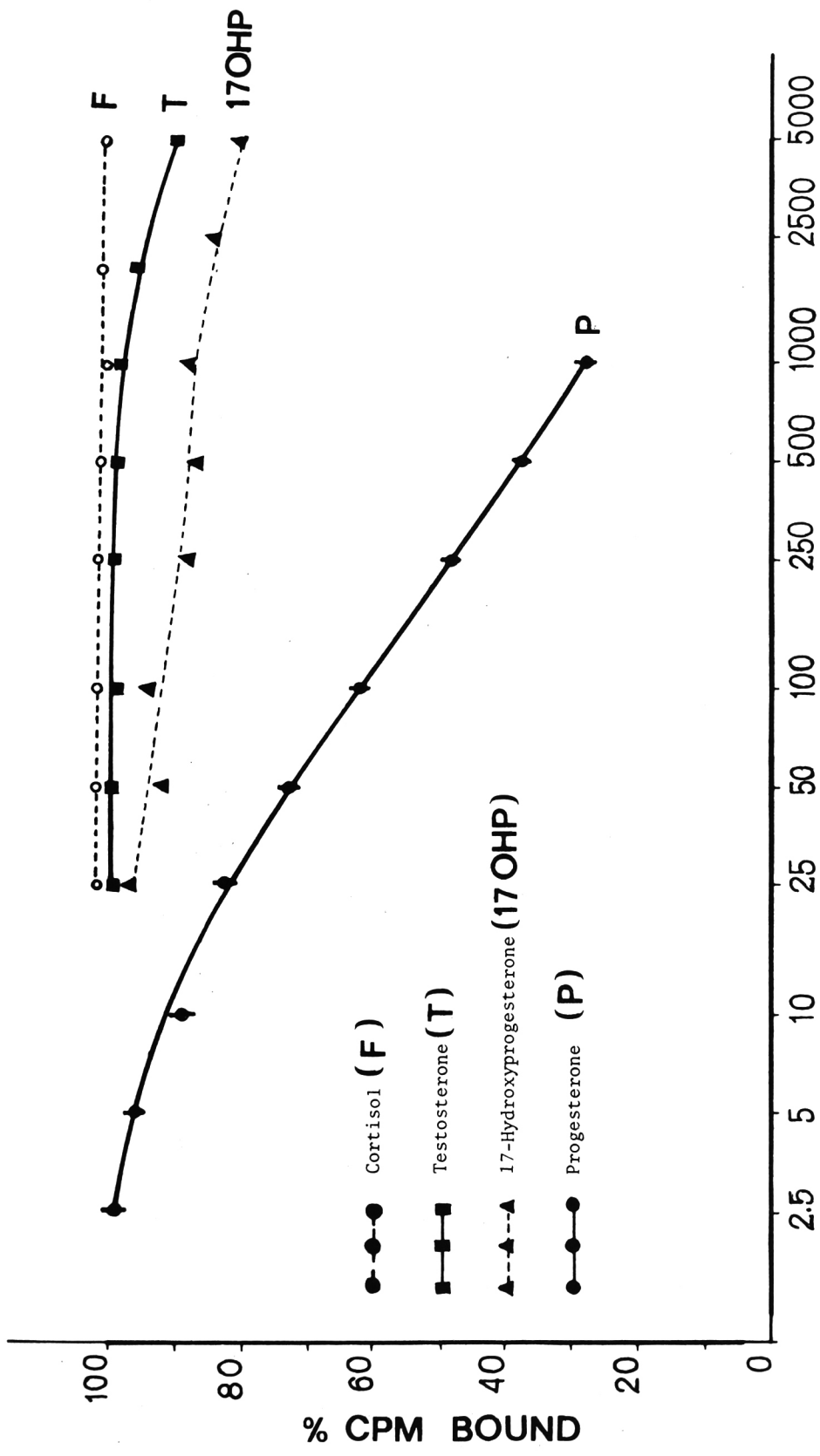

Fig. 1 The Specificity of antiserum "L" dilution 1/35,000, 0.1 cm^3 with various steroids.

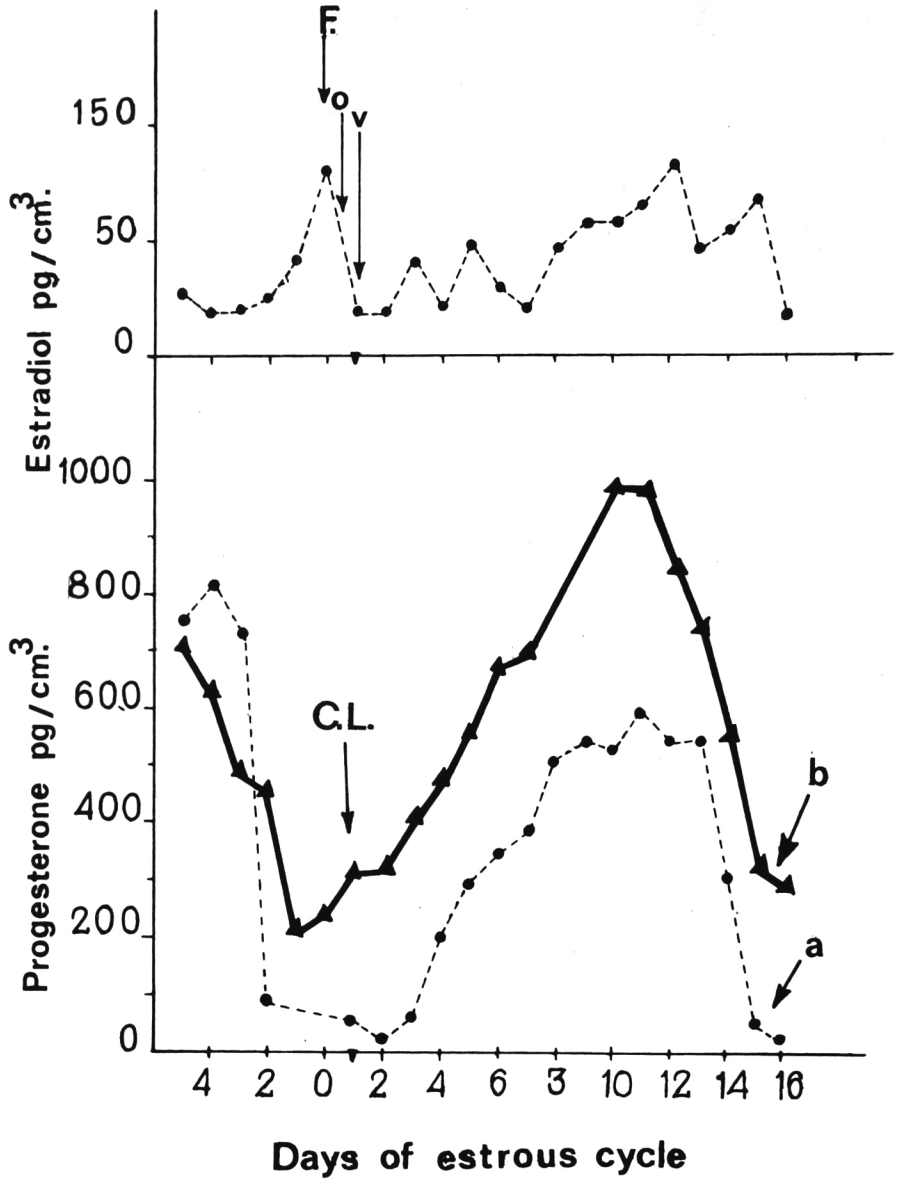

Fig.2 The comparative serum levels of progesterone with celite microcolumn vs antiserum S - 257 # 2 (a) and without celite microcolumn vs antiserum "L" (b).

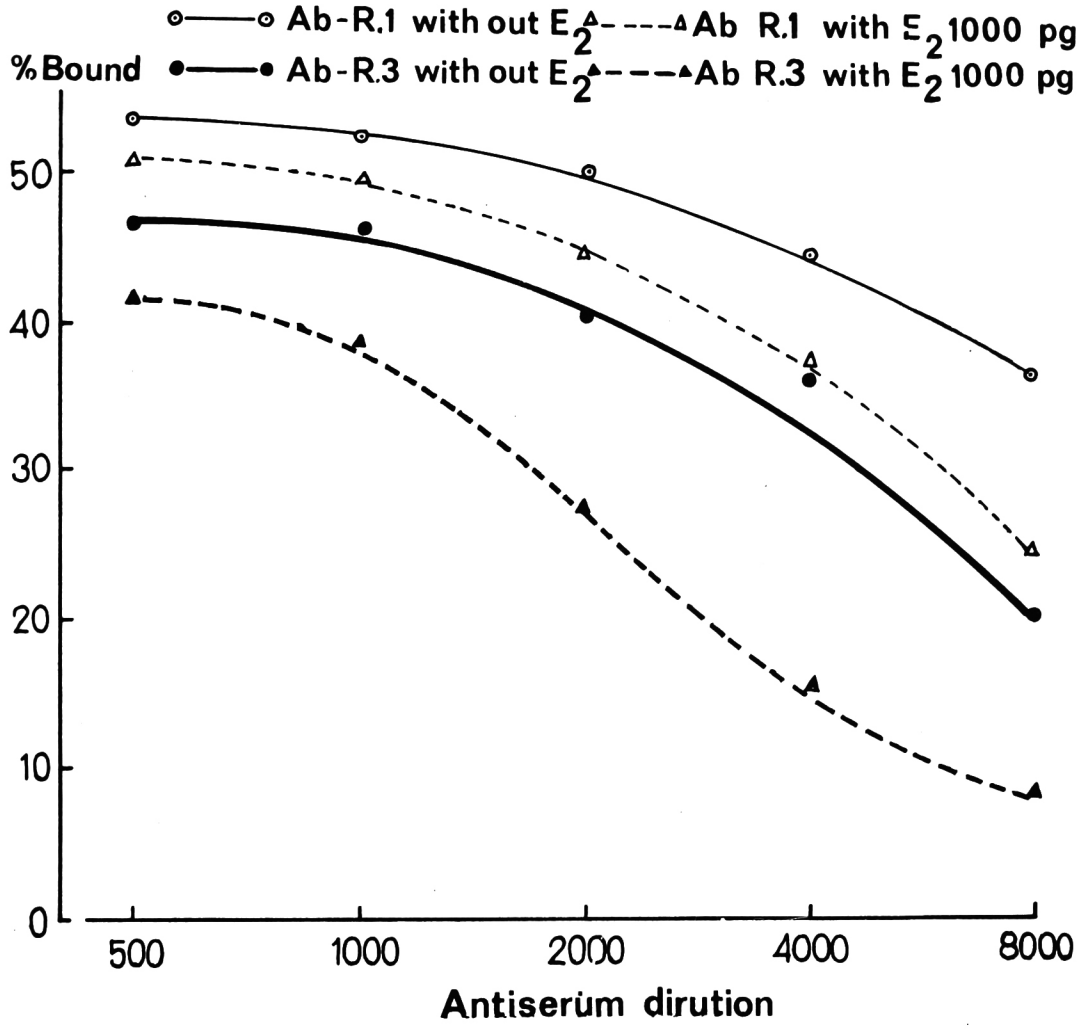

Fig 3. Dilution curves for anti-E_2-6 BSA Serum (Ab-R_1, Ab-R_3) using the E_2-6-^{125}I radioligand and performed with and without inhibition of ligand binding by 1.000 pg non-radioactive estradiol.

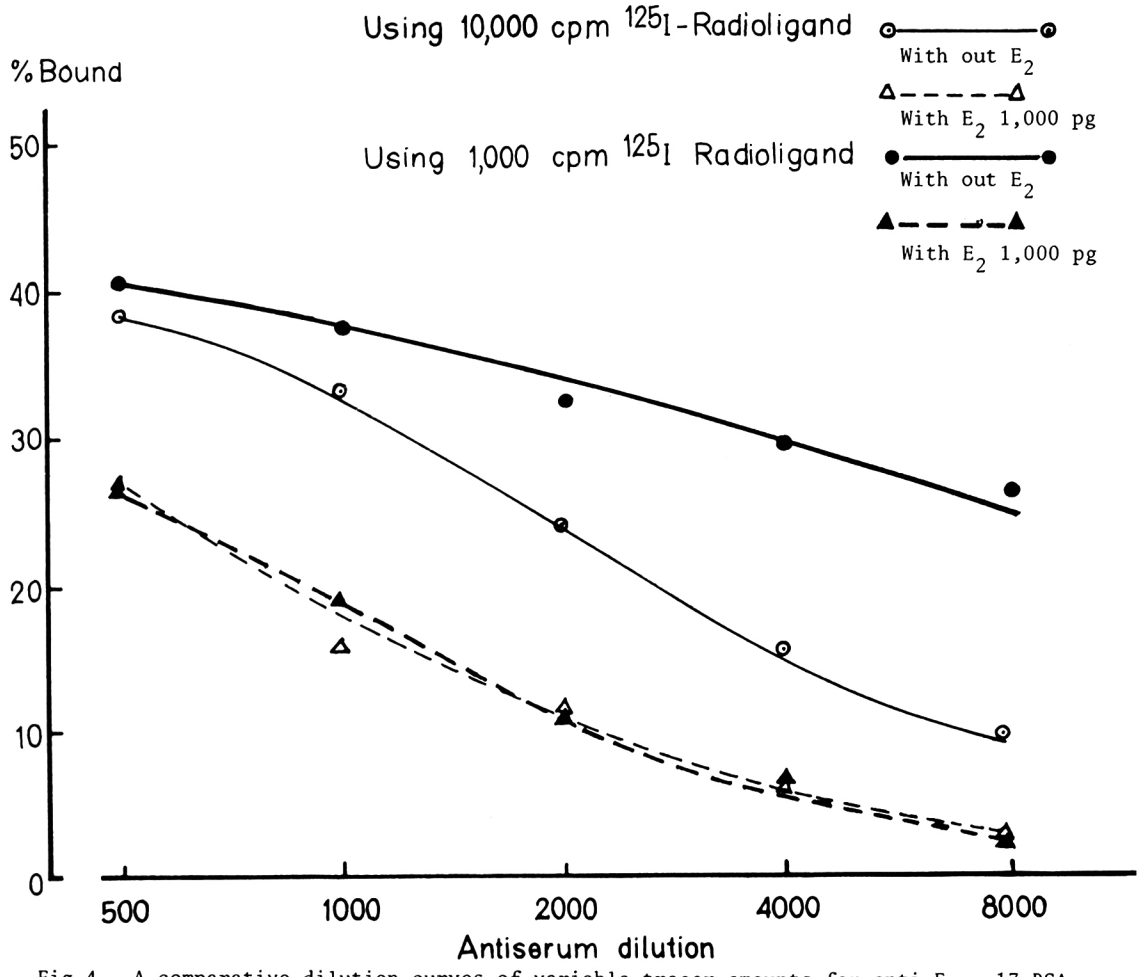

Fig 4. A comparative dilution curves of variable tracer amounts for anti-E_2 - 17-BSA serum using the E_2-17-^{125}I radioligand and performed with and without inhibition of ligand binding by 1,000 pg non-radioactive esteradiol.

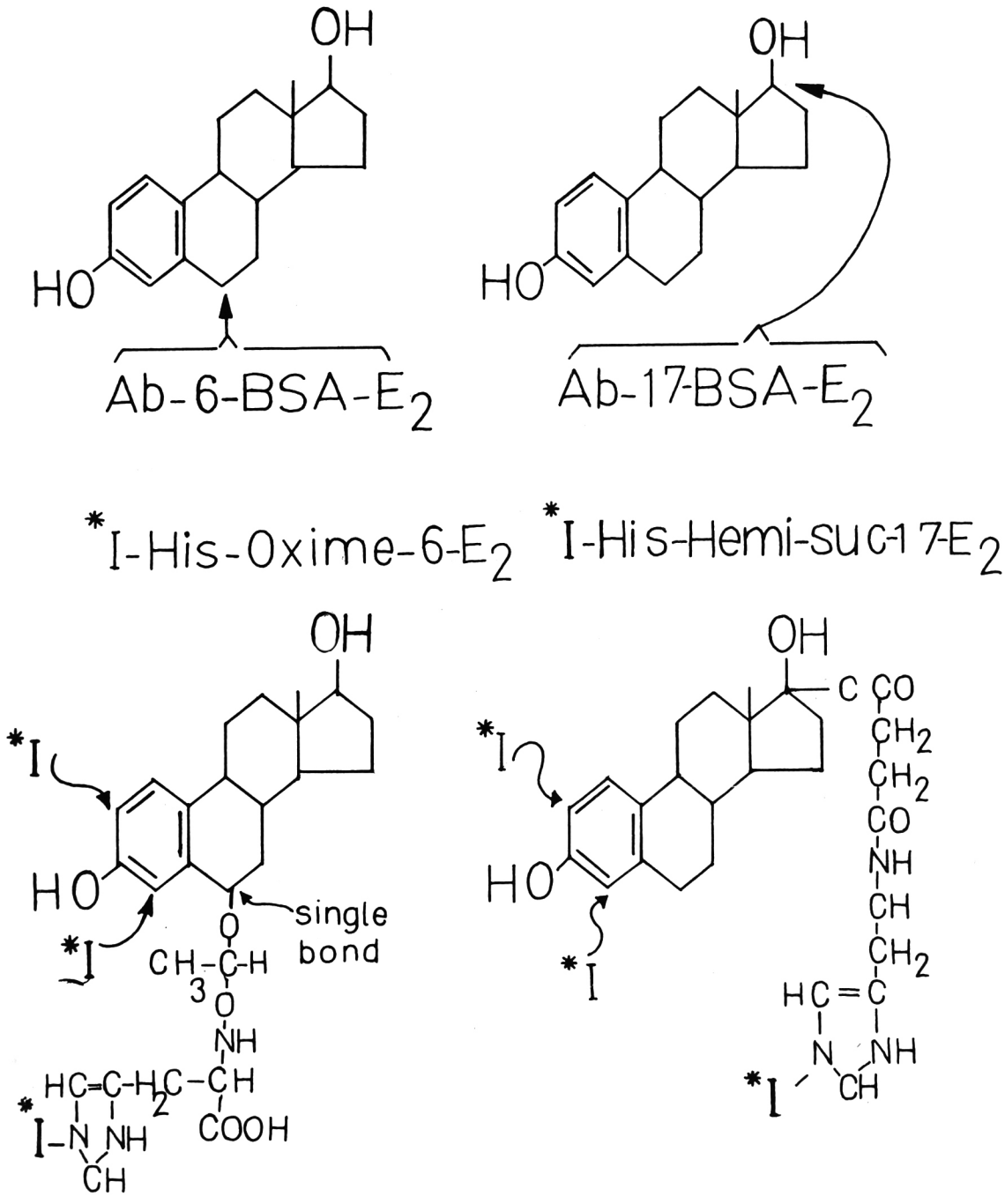

Fig. 5 The views of possible steric factor and interaction between ^{125}I labelled estradiol and antibodies at 6 & 10 positions.

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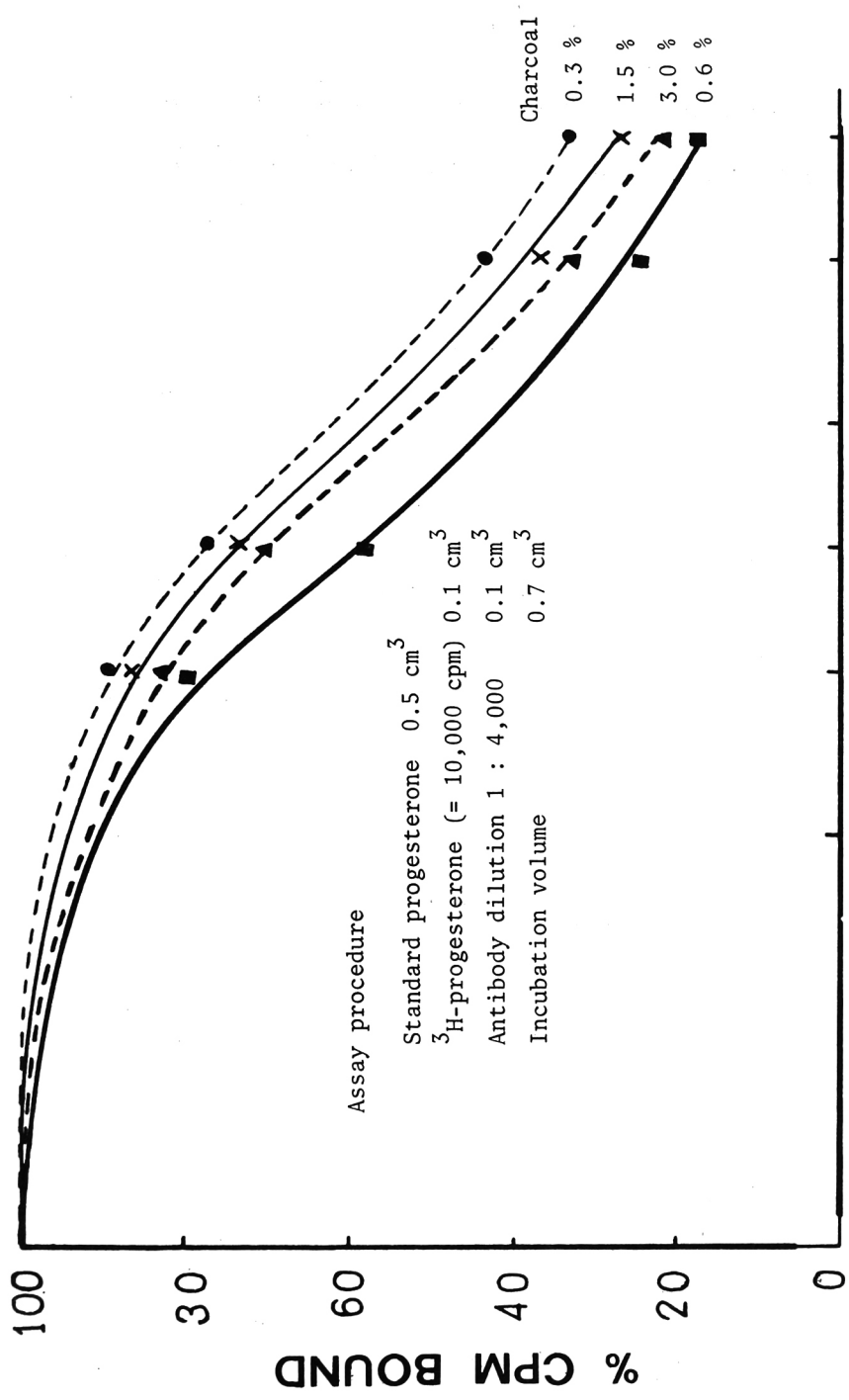

Fig. 6 Effect of Different Concentrations of Charcoal on The Progesterone Standard Curve.

บทย่อ

ปัญหาการวัดปริมาณสเตอรอยด์ฮอร์โมน โดยวิธี เรดิโอ-อิมมิโน-เอสเซ ซึ่งเกี่ยวกับการศึกษาปรากฏการณ์การเป็นสัดในควายปลักไทย

มณีวรรณ กมลพัฒนะ

ผลการวิจัยนี้ได้รวบรวมปัญหาบางประการ ในขบวนการ เรดิโอ-อิมมิโน-เอสเซ ที่ค้นพบระหว่างการศึกษา ปรากฏการณ์การเป็นสัดในควายปลักไทยเพื่อการผสมเทียม งานนี้ได้อธิบายถึงปัญหาและข้อเสนอแนะในการแก้ปัญหาเหล่านั้น โดยขึ้นกับขั้นตอน 3 ประการดังต่อไปนี้

1) เทคนิคการแยกสเตอรอยด์ โดยวิธีโครมาโตกราฟฟี

2) ปฏิกริยาและการแทนที่ที่เกิดขึ้นระหว่าง แอนตีเจน และ แอนตีบอดี ของสเตอรอยด์ โดยติดสารกัมมันตรังสี ไอโอดีน 125

3) เทคนิคการแยกกัมมันตรังสีอิสระออกจากส่วนที่แอนตีเจน และ แอนตีบอดีเกาะเกี่ยวกัน
