รายงานผลการวิจัย

ทุนวิจัยรัชดาภิเษกสมโภช

เรื่อง

การศึกษาระดับโปรเจสเตอโรน และเอสตราไดออล ในชีรัมกระบือไทย

โดย

มณีวรรณ กมลพัฒนะ

ปีงบประมาณ 2518

The Study of Serum Progesterone 17 Hydroxyprogesterone and 17 β Estradiol by radioimmunoassay in Thai swamp buffalo

by

Maneewan Kamonpatana, B.Sc., M.Sc.,
Dip. in Animal Physiology and
Biochemistry (Copenhagen).

Department of Physiology
Faculty of Veterinary Sciences
Chulalongkorn University
Bangkok, Thailand.

1975

Acknowledgements.

The authors wish to thank Assistant Professor Vichai Poshyachinda and Dr. B.N. Saxena, WHO Consultants for the advisory during the course of work. The project was supported by the Royal Silver Juilee Aniversary Grant of Chulalongkorn University. And being supported the facilitation and in part of chemicals by the WHO Research Team Chulalongkorn Hospital and Medical School Bangkok, Thailand.

Abstract A radioimmunoassay for the measurement of serum progesterone (P), 17 hydroxyprogesterone (17P)and 17B estradiol (E_2) were described. The antisera obtained from Dr. G.E. Abraham, Department of Obstetrics and Gynecology, University of California. Male buffaloes free hormone serum (FHS) was prepared for pooled steroids preparation. Separation of free from bound hormone was achieved by dextran coated charcoal suspension. One ml of pooled serum with tritiated these steroids added for recovery, was extracted with ether, then chromatographed on a celite microcolumn. The specificity of antisera has been show significantly specific cross reaction. After purification step by celite chromatography, the contaminants could be all removed. Recovery of the labelled P, 17P and E_2 after extraction and chromatography were 71.1 - 94.5 %, 70.2 - 94.1 % and 61.0 - 80.7 % respectively. The percentage recovery of standard P, 17P and E_2 added in FHS varied in the ranges of 72.9 - 89.6, 77.0 - 97.0 and 85.5 - 101.3 respectively. The presision of within and between assay variance was evaluated by duplicated measuraments of the same sample in the same assay and in 5 different assays. The coefficient of variation (CV) were 8.2 % (P and 17P), 8.3 % (E_2) and 13.2 % (P), 11.2 % (17P), 14.6 % (E_2) respectively. The sensitivity varied between 3.5 - 25.0 pg of P, 4.0 - 25.0 pg of 17P and 2.5 - 10.0 pg of E_2

บทคัดย่อ

การศึกษาการเปลี่ยนแปลงทางสรีรวิทยาของฮอร์โมนเพศ ระหว่างวงจรการเป็นสัดในกระบือไทย นับว่าจะเป็นประโยชน์อย่างยิ่งต่อการผสมเทียมแล้ว ยังเป็นประโยชน์ต่อการศึกษาระบบการสืบพันธ์ของเพศเมียอีกด้วย เนื่องจากลักษณะการเป็นสัดของกระบือที่สังเกตุจากภายนอก มีอาการไม่ชัดเจน และมีระยะสั้น ถ้าเทียบกับกระบือนม ซึ่งมีระยะการเป็นสัด 24 – 36 ชั่วโมง Kaleff (1942), Hafez (1954), และ Ivanov and Sachriev (1960). ผลเด่นชัดที่จะได้จากการศึกษานี้ ก็คือ สามารถตรวจหาระยะตกไข่ที่แน่นอน ตลอดจนการเปลี่ยนแปลงของระดับ ฮอร์โมนโปรเจสเตอโรน (P), 17 ไฮดรอกซี่โปรเจสเตอโรน (17P) และ 17 เบตา เอสตราไดออล (E_2) ระหว่างวงจรการเป็นสัด.

ในการศึกษานี้ได้ศึกษาวิธีวัดฮอร์โมน ทั้ง 3 ตัวดังกล่าว โดยถือวิธี, radio-immunoassay ของ Abraham et al ,(1971) เป็นหลักเพื่อทดสอบหามาตราฐานที่เชื่อถือได้ในการวัดระดับของ ฮอร์โมนเหล่านี้ในซีรัมวงจรสัดของกระบือไทย ได้เตรียมซีรัมกระบือผู้ที่กำจัดฮอร์โมนออกแล้ว เติมฮอร์โมนมาตราฐานที่ทราบจำนวนแน่นอน เพื่อใช้ทดสอบหามาตราฐานของวิธีวัดโดยใช้ 1cc ของซีรัมที่ผ่านขบวนการวัดตามวิธีที่กำหนดแล้ว ผลการทดลองพบว่า specificity ของ Antiserum มี cross reaction กับเสตียรอยด์ที่ต้องการวัดอย่างมีนัยสำคัญทางสถิติ การผ่าน celite chromatograply สามารถจะกำจัดสิ่งเจือปนที่รบกวนปฏิกริยาได้หมด ทำให้วิธีนี้สามารถวัดสเตียรอยด์ได้ทั้ง 3 ตัวในซีรัม 1cc .เปอร์เซ็นต์ recovery จากการเติม H^3P, $H^3 17P$ และ H^3E_2 ได้ผล 71.1 –94.5 %, 70.2 – 94.1 % และ 61.0 – 80.7 % ตามลำดับส่วนเปอร์เซ็นต์ recovery ของ P, 17 P และ E_2 มาตราฐานได้ผล 72.9 – 89.6 %, 77.0 –97.0 % และ 54.5 – 101.3 % ตามลำดับ Precision ของผลการทดลองวัดตัวอย่างเดียวกัน 5 ครั้ง ในการทดลองครั้งเดียวกันได้ Coefficient of variation(CV)8.2 % สำหรับ P และ 17 P กับ 8.3 % สำหรับ E_2 ส่วน Precision ของผลการทดลองวัดตัวอย่างเดียวกันซ้ำกัน 5 ครั้ง ในการทดลองต่างกัน 5 ครั้ง ได้ CV 13.2 % สำหรับ

P 11.2 % สำหรับ 17 P และ14.6 % สำหรับ E_2 ความไว (sensitivity)ของวิธีวัดพบว่าใน P มีความไววัดได้ละเอียดระหว่าง 3.5 – 25.0 พิโครกรัม, ใน 17P ได้ 4.0 – 25.0 พิโครกรัม และใน E_2วัดได้ 2.5 – 10.0 พิโครกรัม.

สรุปได้ว่าเราสามารถที่จะใช้วิธีนี้ สำหรับวัดระดับของฮอร์โมนทั้ง 3 ในซีรัมกระบือไทยได้.

Table of Contents

List of Tables

List of Figures

Introduction Animal protein consumption is one of the serious problems in the world. The relative rate of increasing human population militats against the food animal production. The demand for animal protein is ever increasing. Artificial insemintion (A.I.) may be one of the answer to solve this problem. The development and application of A.I. to increase animal production must be attended greatly. Buffaloes should be the most interesting animal production in particular in Asia.

In South East Asia, which swamp buffalo population being concentrated faces the local problems, and needs further investigation. Calving interval of swamp type in Malaysia averages 639 days. (Fadzil 1969) that is usually longer than river type. This may be due to lack of fertilization while the females passed through several estrous without being mated. The farmers in Indonesia who own only one to two buffaloes, mostly females (Toelihere, 1974) faced the same problem of lacking male buffaloes. The same problem occours in Thailand but deals mainly with the vasectomized male animals. Lack of male buffaloes or insufficient ones is one of the main factors causing reproductive failure in these countries. A.I. may be of great help for the farmers to get their female buffaloes bred.

The developing of A.I. in buffaloes must be based on the reproductive physiology of this species. Publication on reproductive physiology and A.I. in buffaloes is indeed extremely rare, a lot of it comes from India.

These papers concern mostly the river or dairy buffaloes. Very lilttle is known on physiology of reproduction of swamp buffaloes and so far there is no application of A.I. in this type. This paper is mainly based on the study of reproductive physiology in swamp female buffaloes for A.I. because the female buffalo shows weak estrous phenomena. The attendance has been carried out to standardize the method by combined radioimmunoassay (Abraham et al 1971) for measurement the levels of serum progesterone 17 hydroxy-progesterone and 17β estradiol during the estrous cycle. The purposes are : finding out the normal length of estrous cycle, the normal sex female steroid pattern of estrous cycle and the proper time for insemination.

Materials and Methods

Progesterone, 17 - hydroxyprogesterone and 17 β - estradiol values were determined by the combined radioimmuno assay of Abraham et al (1971). The materials and methods have been described in the following steps.

1. Solvents and reagents.

Ether, anhydrons A.R. Mallinckrodt Chemicals.

Ethylacetate A.R. Mallinckrodt Chemicals.

Isooctane A.R. Mallinckrodt Chemicals.

Ethylene glycol, Chromatoquality

Toluene Matheson, Coleman and Bell.

Norit A Charcoal Matheson, Coleman and Bell

Dextran T-70 Mann Research Laboratories

Benzene nanograde Mallinckrodt Chemicals

Ethanol Absolute A.R. Mallinckrodt Chemicals

Dioxane Baker Analyzed J.T. Baker

Liquidfluor NEW New England Nuclear Corporation.

Sodium phosphate dibasic heptahydrate A.R. Mallinckrodt.

Sodium phosphate monobasic monohydrate A.R. Mallinckrodt.

Sodium azide A.R. E. Merck.

Sodium chloride A.R. Mallinckrodt.

Gelatin Difco laboratories, Detriot, Michigan

Decon 90^{R} concentrate Decon labs. limited, Eleen street, Brington.

Celite Johns - Manville, analytical filter aid.

2. Steriods

Nonradioactive steriods were obtained from Mann Research Laboratories.

C21 Steroids.

Cortisol

11-Deoxycorticosterone

Deoxycorticosterone

Progesterone

17 - Hydroxyprogesterone

C19 Steroids

Testosterone

Androsterone

Androstenediol

C18 Steroids

Estradiol - 17 β

Estriol

Estrone

16 Epiestrone

3 - Deoxyestrone

Radioactive steroids were supplied by New England Nuclear Corporation

1, 2 - ^{3}H - progesterone 0.25 mci/0.25 ml (P^{*})

1, 2 - ^{3}H - 17 - hydroxyprogesterone 0.25 mci/ 0.25 ml ($17\ P^{*}$)

6, 7 - ^{3}H - 17 - β estradiol 0.25 mci/0.25 ml (E_2^{*})

The tracers were diluted to a concentration of 20 - 25 μci/ml of benzene : ethanol (9 : 1) and stored at 4°C for up to 6 months.

3. Instruments

3.1 Packard liquid scintillation spectrometer 3390

3.2 Centriguge MSE Mistral 4L

4. Materials.

All glass wares and pipettes were reused through out the assay by cleaning with Decon : water (15 : 100 V/V), and rinsed with ether prior to use.

5. Preparation of reagents.

5.1 Assay Buffer

To a 2 lit. volumetric flask add 32.7 gms of sodium phosphate dibasic hepatahydrate (M.W. 268), 10.8 gms of sodium phosphate monobasic monohydrate (M.W. 138), 2.0 gms of sodium azide (M.W. 65) and 18.0 gms of sodium chloride (M.W. 58). Then add distilled water to a total volume of two liters. The pH of buffer should be adjusted to 7.0 $\pm$ 0.1. The assay buffer consists of a 0.1 % Gelatin solution and should be stored at 4°C. This buffer is good as long as no evidence of mold or bacterial growth is present.

5.2 Charcoal Suspension

To a 200 ml flask add 1.25 gms Norit A and 0.125 gms of Dextran T-70 and 200 ml of assay buffer. Stopper the flask and shake vigorously for 1 min. It should be stored at 4°C and is stable for up to one month. The charcoal suspension must be shake vigorously 15 mins before use.

5.3 Counting Solution

Add 640 ml of liquifluor and 3,000 ml of dioxane in 4 gallons of toluene, mix well and transfer into an automatic jet pipetter (Beckman instruments). Use 10 ml of this counting fluid per vial. This is a two phase counting system. The dioxane dissociates the labelled steroid from the antibody and allows the labelled steroid to move into the upper phase (counting fluid) where it is "seen" by the liquid scintillation counter. Without dioxane this two phase system must be equilibrated for 16 to 20 hours prior to counting.

5.4 Radioactive steroids.

A 0.25 mci/0.25 ml of tritiated steroid in a 5 ml screwcapped vial, complete the volume to 5 ml with benzene : ethanol (9 : 1). This stock solution store at 4°C can be used for up to six months.

To prepare tritiated steroid for use in the assay, pipet 5 μci ($\cong$ 100 μl) of stock solution in a clean 100 ml Q/Q conical flask and dry under nitrogen gas. Add 50 ml of assay buffer and mix well. Let stand at room temperature for one hour. Check the total dpm in 0.1 ml of solution, should be 20,000 $\pm$ 1,000 dpm (10,000 cpm). If not adjust by adding more buffer or labelled steroid. This solution can be used up to 2 months if stored at 4°C.

5.5 Antisera.

The antisera obtained from G.E. Abraham, Division of Reproductive Biology, Department of Obstertrics and Gynecology University of California.

Dissovlved the lyophilized antisera in 2 ml of distilled water. Transfer 0.1 ml aliquots to screw capped glass tube and frozen at - 20° to - 40°c. Each time use tube to add 49.9 ml of assay buffer. Mix well and stored at 4°C. It is stable for 2 months under this conditions.

A volume of 0.1 ml diluted antisera will bind 40-50 % of about 50 pg of ^{3}H labelled steroid when a total incubating volume of 0.7 ml is used in the assay.

5.6 Standard Steroids.

Stock solutions were prepared in a concentration of 1 µg/ml with absolute ethanol and stored in freezer at - 20°C to - 40°C.

Solution A	contained standard steroid	1,000 pg/0.5 ml
Solution B	contained standard steroid	500 " "
Solution C	contained standard steroid	250 " "
Solution D	contained standard steroid	100 " "
Solution E	contained standard steroid	50 " "
Solution F	contained standard steroid	25 " "
Solution G	contained standard steroid	10 " "
Solution H	contained standard steroid	5 " "
Solution I	contained standard steroid	2.5 " "
Solution J	contained standard steroid	0.0 " "

6. Preparation of Celite Microcolumns

Microcolumns of Celits were prepared by weigh out 20 gms of celite (keeping in an oven at 540°C overnight) and mix thoroughly with 10 ml of ethylene glycol while celite

is still warm for 10 min. Mix in a clean glass beaker and glass rod. Do not use plastic bags because they cause high blanks. Place a small bead (3 mm diametes) in each pipette. Then filled the well mixed celite about 0.5 g/columm into microcolumm. Load fluffy celite in the microcolumn. Elute the microcolumn with 3.5 ml isooctane under nitrogen gas pressure 2 times before use.

7. Procedure

7.1 Preparation of Free Hormones Serum (FHS)

7.1.1 The activated charcoal was performed by washing charcoal Norit A through the suction flask ten times with redis-tilled water. And filtered with whatmann no 1 filtered paper. Then dried in the oven at 110°C overnight.

7.1.2 Add 2.0 gms of sodium azide and 40.0 gms of activated charcoal (from above preparation) into 2.0 liters of male buffalo serum. Let vigorously stir at room temperature for 24 hrs. Then centrifuge the mixture for several times until the free hormones serum looked clear. Filtered through whatman no. 42 several times. Finally the clear solutiom would be accepted as Free Hormones Serum (FHS)

7.2 Plasma extraction

One ml aliquots of serum samples were pipetted into 18 x 150 mm glass test tube and mixed with 0.1 ml of assay buffer containing 2,000 dpm (1,000 cpm) each of P^*, $17P^*$ and E_2^*. This tracer used as internal standard for recovery estimations. Equilibrated the mixture at room temperature for 30 mins. Extraction was carried out by Rotamixer mixing for 30 sec. With 10 volume of cold ether. Let stand for 5 min to set clear separation of 2 phases, the lower phase (serum) was quick - frozen by dipping in 95 % ethanol containing chips of dry ice. The ether was decanted in a 20 ml vial and evaporated to dryness under filtered air.

7.3 Chromatography

Chromatography of the dried residue on Celite microcolumns was carried out by adding 1.0 ml isooctane to ether residue in vial. Transfer sample from vial to column once using the pasteur pipette and rinsed the vial with 0.5 ml isooctane. The microcolum then elute with 3.5 ml of issoctane, 15 % ethylactate in isooctane and 40% ethylactate in isooctane respectively. The zero fraction contains P, the 15% fraction contains 17 P and 40% fraction contains E_2. Dry collected fractions under filtered air.

7.4 Radioimmunoassay

Pre-warm assay buffer at room temperature. Add 1.7 ml of buffer to dried residue and mix well with rota-mixer. Let stand for 1-2 hrs. At room temperature. Pipet one aliquot 0.5 ml in counting vial for recovery estimation

and duplicate aliquot 0.5 ml in 10 x 75 mm test tube and mixed with 0.1 ml of diluted antiserum in assay buffer and 0.1 ml of 20,000 dpm (10,000 cpm) radioactive steroid. Then incubate at 4°C overnight. The standard curve was performed by a set of standard steroids in triplicates is run together with the unknown samples. With each determination, the eluates of 4 columns containing no ether extract residue are used as "blanks" in the assay. Together with quadruplicate of three pools serum containing the standard steroids as in the following table are used as "control" in the assay

Pool	Standard steroids added in pg/ml		
	P	17 P	E_2
A	0	0	0
B	500	500	100
C	2,500	2,500	500

After incubation at 4°C overnight, 0.2 ml of a suspension containing 0.625 % Norit A and 0.0625 % dextran in assay buffer was added to the incubation media, mixed and incubated for 20 min in an ice bath 4°C. The samples were centrifuged for 20 min 4°C at 2,500 rpm. The supernatant was decanted into counting vials, 10 ml of counting solution added, and mixed. The vials were allowed to the scintillation counter for counting

8. **Calculations.**

Let : U = unknown value read on standard curve (pg/ml of serum)

B = mean blank value (pg/ml)

R = fraction recovered

$$= \frac{\text{cpm recovered}}{\text{total cpm added for recovery}}$$

X = pg steroid/ml of serum

Then $$X = \frac{U - B}{R}$$

Results.

1. Effect of temperature of incubation.

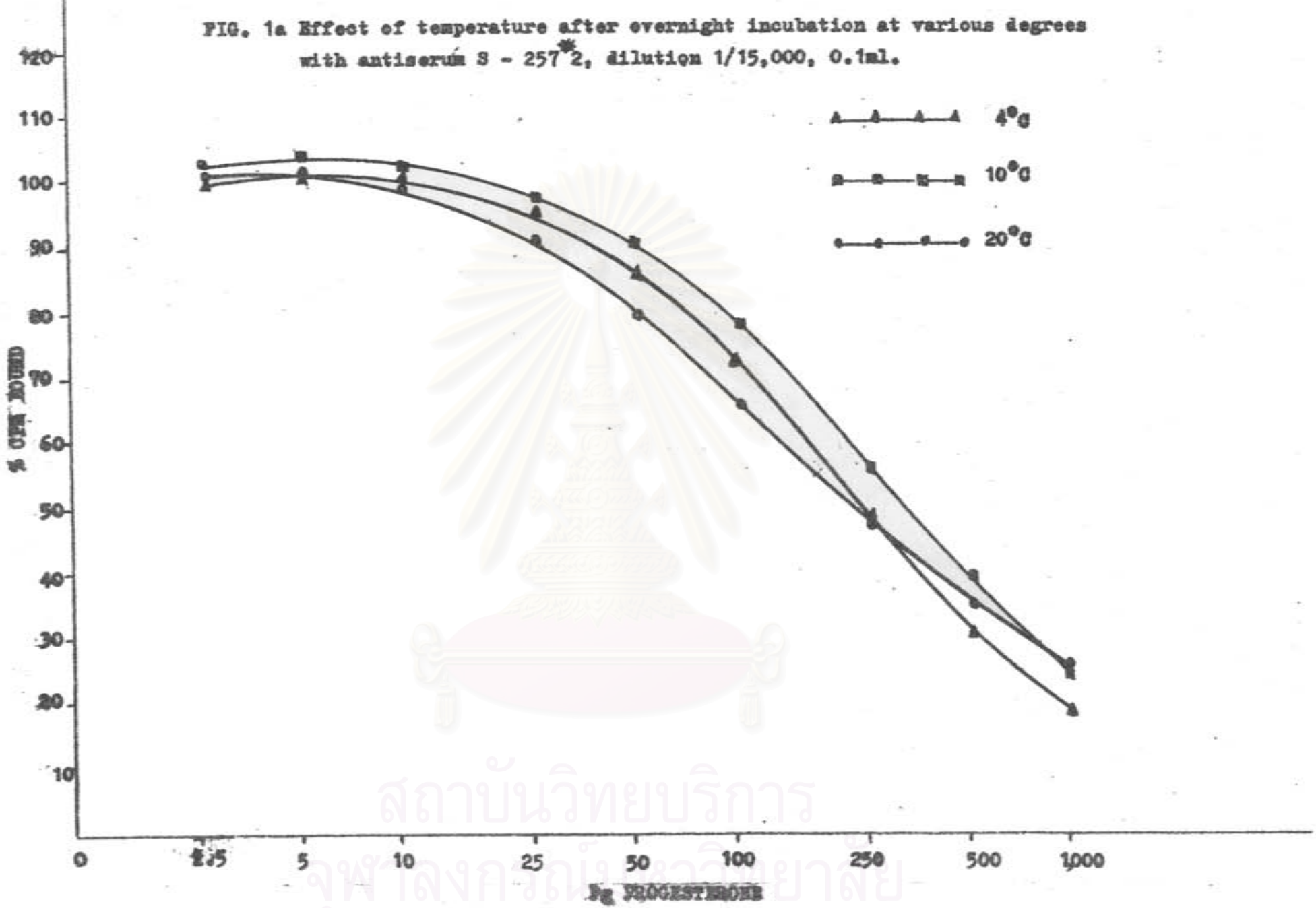
A set of standard steroids (P, 17P and E_2) were carried out for radioimmunoassay by vareing incubated temperature at 4°C, 10°C and 20°C respectively. No significant difference in binding was obtained after 4°C and 10°C incubation. But at 20°C the binding was decrease and get more narrow range of accurated amount in the standard curve. The results have been show in Fig 1a, 1b and 1c.

The temperature of ice bath also was oberved for 1 hr of setting. Inorder to indicate the constantcy and variation of temperature in ice bath during incubation time. The ice bath should be used after an half hour of setting and the outer area shows no significant change of temperature from the inner area. As the results in Table 1.

2. Sensitivity

The standard curve plotted from the per cent cpm bound versus the logarithm of pg of steroids, varied from 2.5 - 1,000 pg. The cpm bound when no presence of steroids was defined as 100 % confidence limit, 25 pg was significantly different from zero pg and the coefficient of variation at each point of the standard curve was less than 5 % (Fig 2a, 2b, and 2c). The sensitivity of progesterone, 17 hydroxy-progesterone and 17β - estradiol varies from 3.5 to 25.0 pg, 4.0 to 25.0 ph and 2.5 to 10.0 pg respectively (Table 2).

FIG. 1a Effect of temperature after overnight incubation at various degrees with antiserum S - 257*2, dilution 1/15,000, 0.1ml.



with antiserum S - 49#6, dilution 1/15,000, 0.1ml.

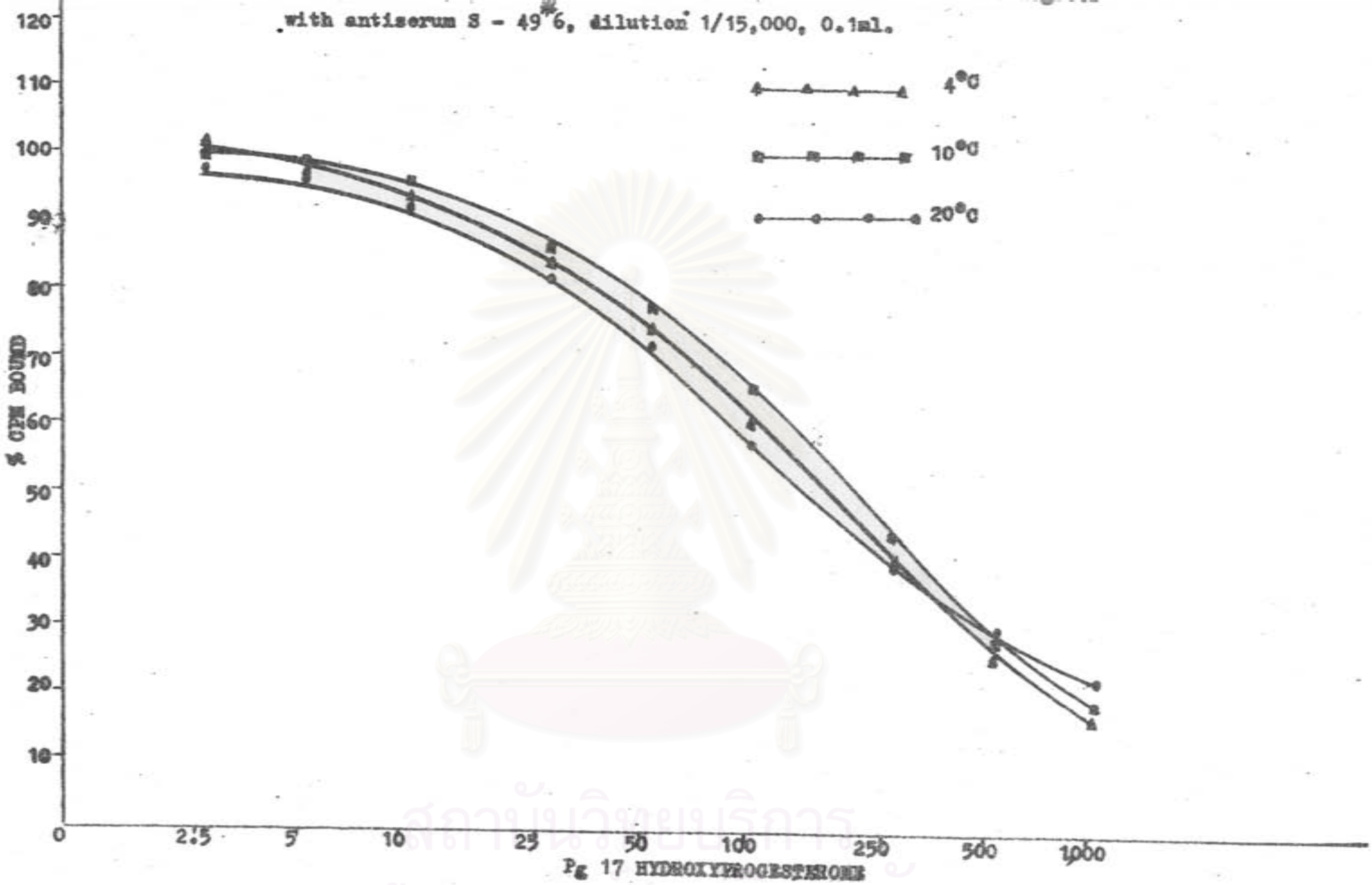
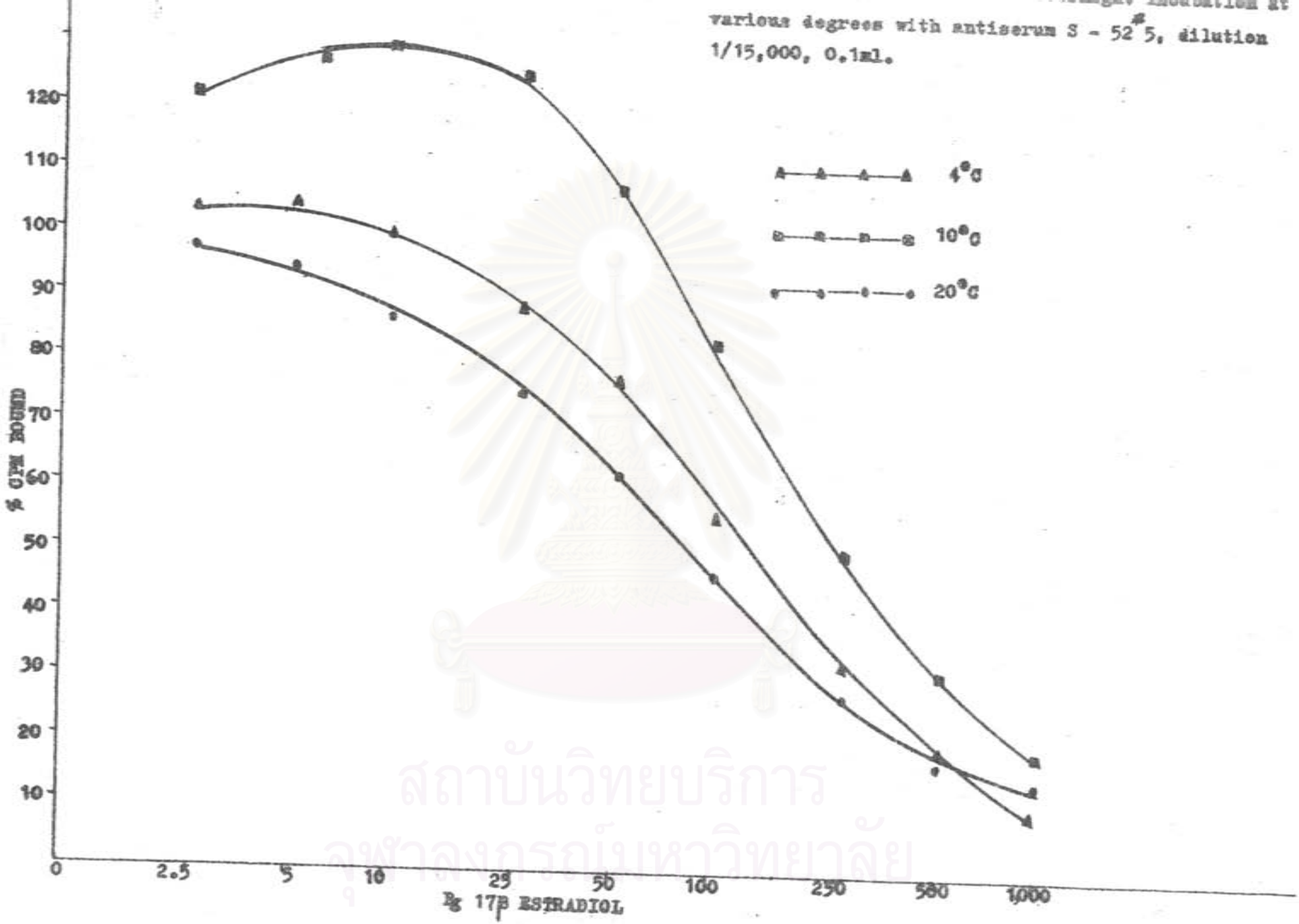


Fig 17 HYDROXYPROGESTERONE



various degrees with antiserum S - 52 #5, dilution 1/15,000, 0.1ml.

Table 1. Tho distribution of temperature in ice bath during 1 hour observation.

Time after setting mins	Temperature in the ice bath								
	1st Observation			2nd Observation			3rd Observation		
	Ia	Oa	ΔT	Ia	Oa	ΔT	Ia	Oa	ΔT
0	7.0	6.5	0.5	7.9	7.5	0.4	7.8	7.2	0.6
5	6.8	6.2	0.6	7.8	7.4	0.4	7.7	7.1	0.6
10	6.7	6.0	0.7	7.7	7.3	0.4	7.6	7.0	0.6
15	6.6	5.9	0.7	7.6	7.1	0.5	7.5	6.9	0.6
20	6.5	5.8	0.7	7.5	7.0	0.5	7.4	6.8	0.6
25	6.5	5.8	0.7	7.4	6.9	0.5	7.3	6.7	0.6
30	6.4	5.7	0.7	7.3	6.8	0.5	7.2	6.6	0.6
35	6.3	5.5	0.8	7.2	6.7	0.5	7.1	6.5	0.6
40	6.2	5.4	0.8	7.1	6.6	0.5	7.0	6.4	0.6
45	6.1	5.3	0.8	7.0	6.5	0.5	6.9	6.3	0.6
50	6.0	5.2	0.8	6.9	6.4	0.5	6.8	6.2	0.6
55	5.9	5.1	0.8	6.8	6.3	0.5	6.7	6.1	0.6
60	5.8	5.0	0.8	6.7	6.2	0.5	6.6	6.0	0.6

Ia = Inner area in the ice bath

Oa = Outer area in the ice bath

ΔT = The difference temperature between Ia & Oa

FIG. 2a Standard curve with antiserum S-257 *2, dilution 1/15,000, overnight incubation at 4°C. Each point represents the mean of triplicate determinations ± SD.

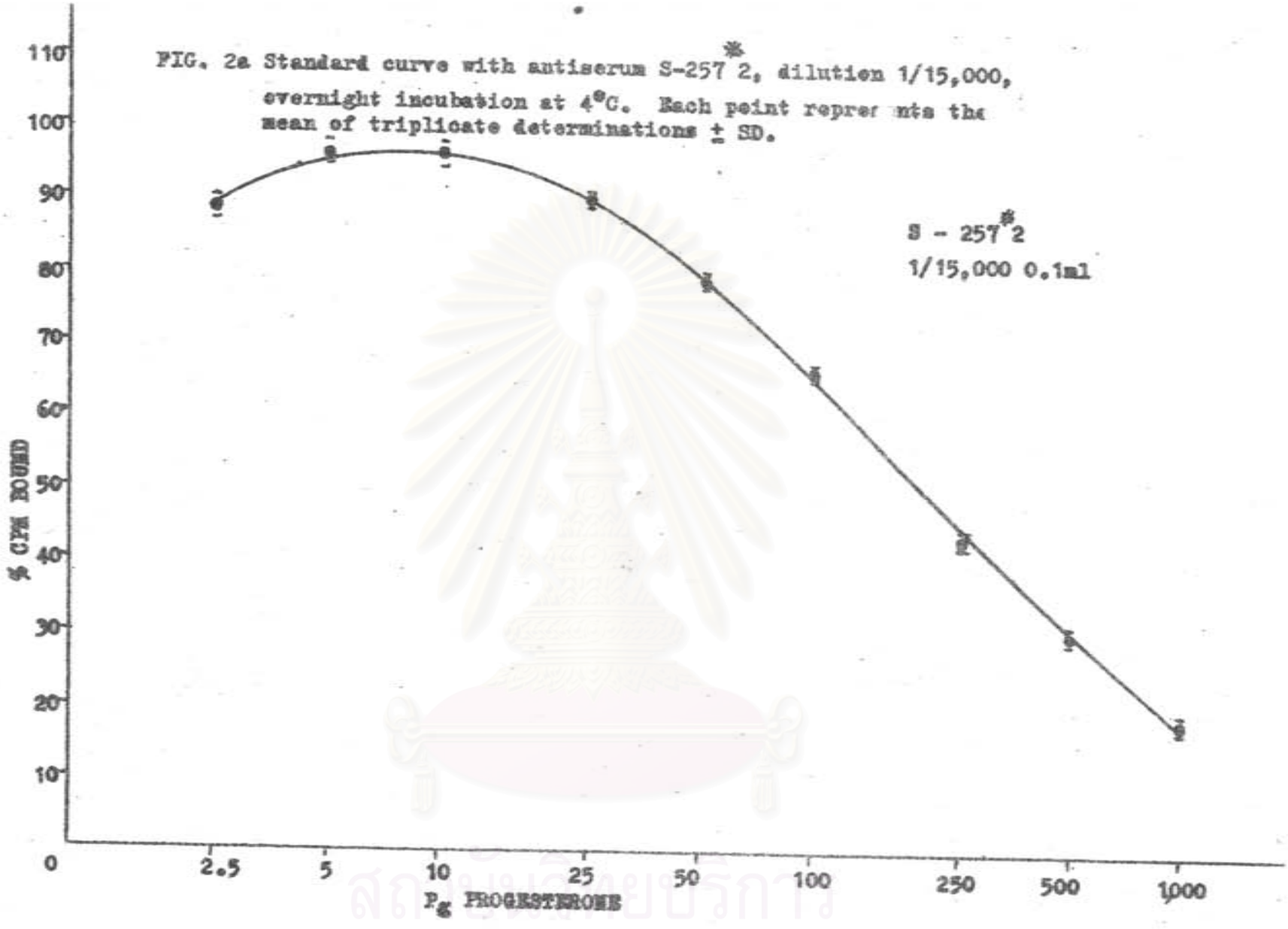


FIG. 2b Standard curve with antiserum S-49*6, dilution 1/15,000, overnight incubation at 4°C. Each point represents the mean of triplicate determinations ± SD.

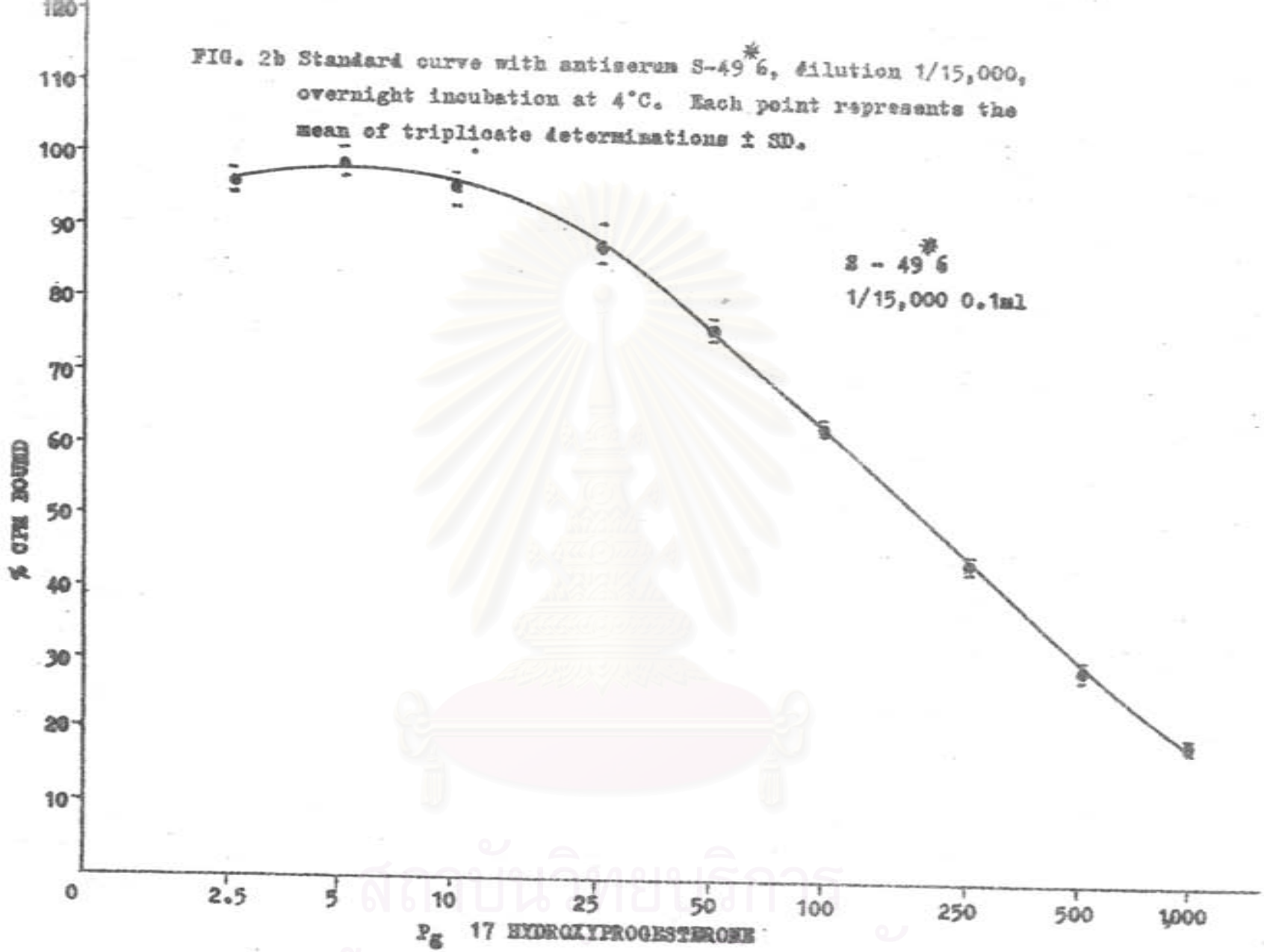


FIG.2c Standard curve with antiserum S-52*5, dilution 1/15,000, overnight incubation at 4°C. Each point represents the mean of triplicate determinations ± SD.

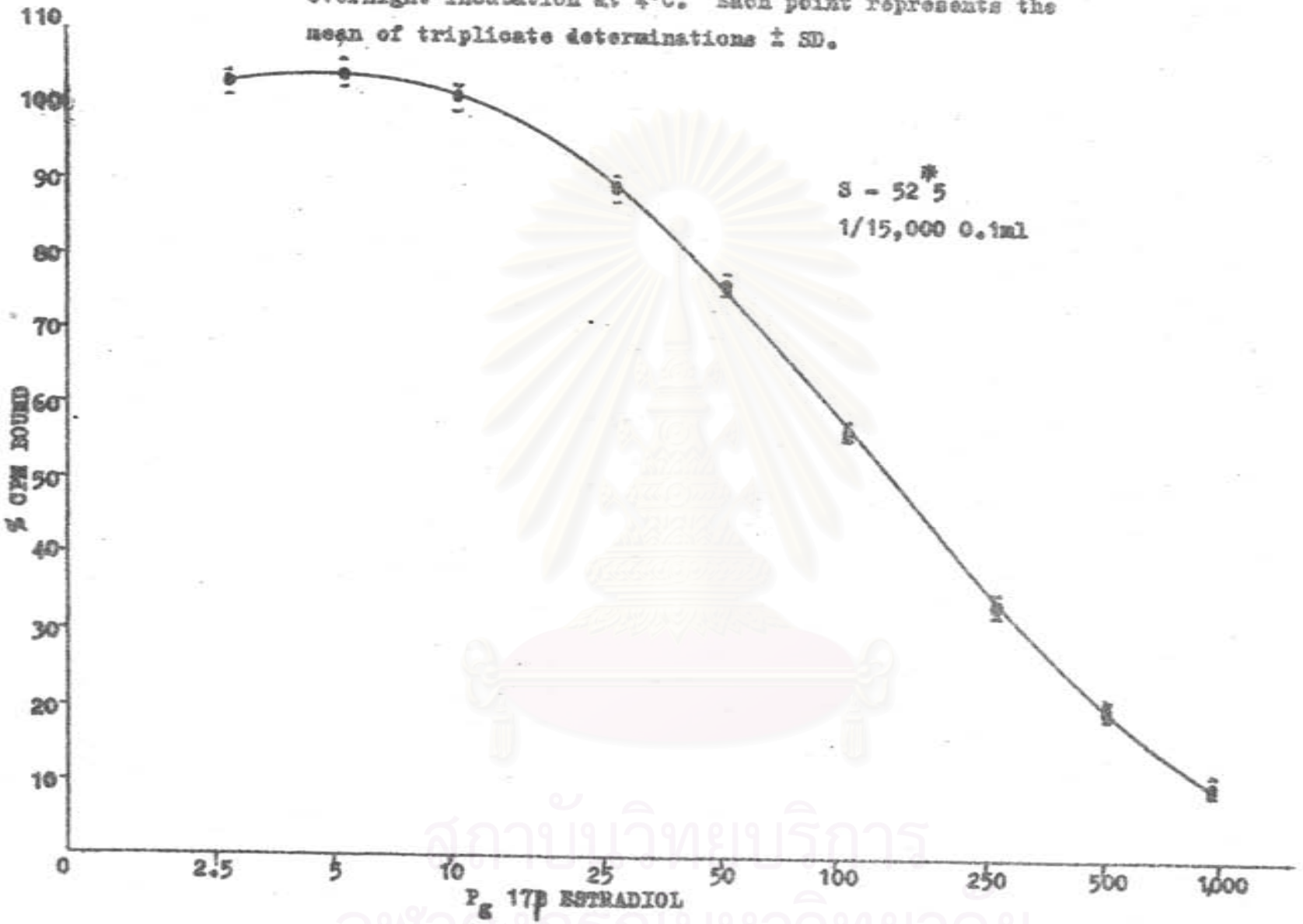


Table 2 Sensitivity of progesterone, 17 hydroxyprogesterone and 17-β -estradiol in various days of determination.

Days of Determination	Steroids Measured	Antiserum used	Sensitivity (Bo - 2S.D. / Bo x 100)	
			%Cross Reaction	pg / ml
1	P	S -257* 2	92.84	25.0
2			87.62	25.0
3			94.01	12.0
4			98.64	3.5
5			94.25	13.0
6			88.18	25.0
7			95.44	8.5
8			93.46	9.0
9			87.90	11.5
10			89.08	21.0
1	17P	S - 49* 6	88.83	4.5
2			91.58	11.0
3			80.45	16.5
4			93.96	4.0
5			89.46	25.0
6			92.09	10.0
7			93.46	8.0
8			95.58	12.0
9			93.20	19.0
10			96.04	13.0
1	E_2	S - 52* 5	98.03	5.0
2			85.56	6.0
3			94.54	8.0
4			96.35	2.5
5			97.15	4.0
6			88.75	6.0
7			94.67	10.0
8			96.62	5.0
9			96.18	7.5
10			95.03	2.5

3. Specificity

Specificity of an assay system refers to the ability to respond only to the compound the assay is intended to quantify. Thus,a completely specific assay for progesterone, 17 hydroxyprogesterone and 17 - β - estradiol would quantify only these steroids. Absolute specificty is difficult to demonstrate. If a partially nonspecific detection method is used, adequate specificity can be expected if the purification steps performed prior to detection remove all the know contaminants that interfere. The specificity of the antiserum S - 257* 2, S - 49* 6 and S - 52* 5 were tested by cross-reaction studies with various steroids (Fig 3a, 3b, 3c Fig. 4a, 4b, 4c and Fig 5a, 5b, 5c.) The cross reaction of progesterone with antiserum S - 257* 2 was investigated as 100%. The steroid deoxycorticosterone showed significantly cross reaction with 20.83 %. The other steroids tested showed little or no detectable cross reaction.(Table 3a). The same series of steroids were carried out through the cross reaction with antiserum S - 49* 6. The cross reaction of 17 hydroxyprogesterone was taken as 100 %. Two steroids showed complete cross reaction namely deoxycorticosterone (80.0 and progesterone (100%). The other steroids show no signifcantly cross reaction. (Table 3b). The specificity of antiserum S - 52* 5 was performed with various steroids. The result showed that 17 β estradial taking 100 % cross reaction.

FIG. 3a The specificity of antiserum # - 257 2, dilution 1/15,00⁻, 0.1ml with various steroids.

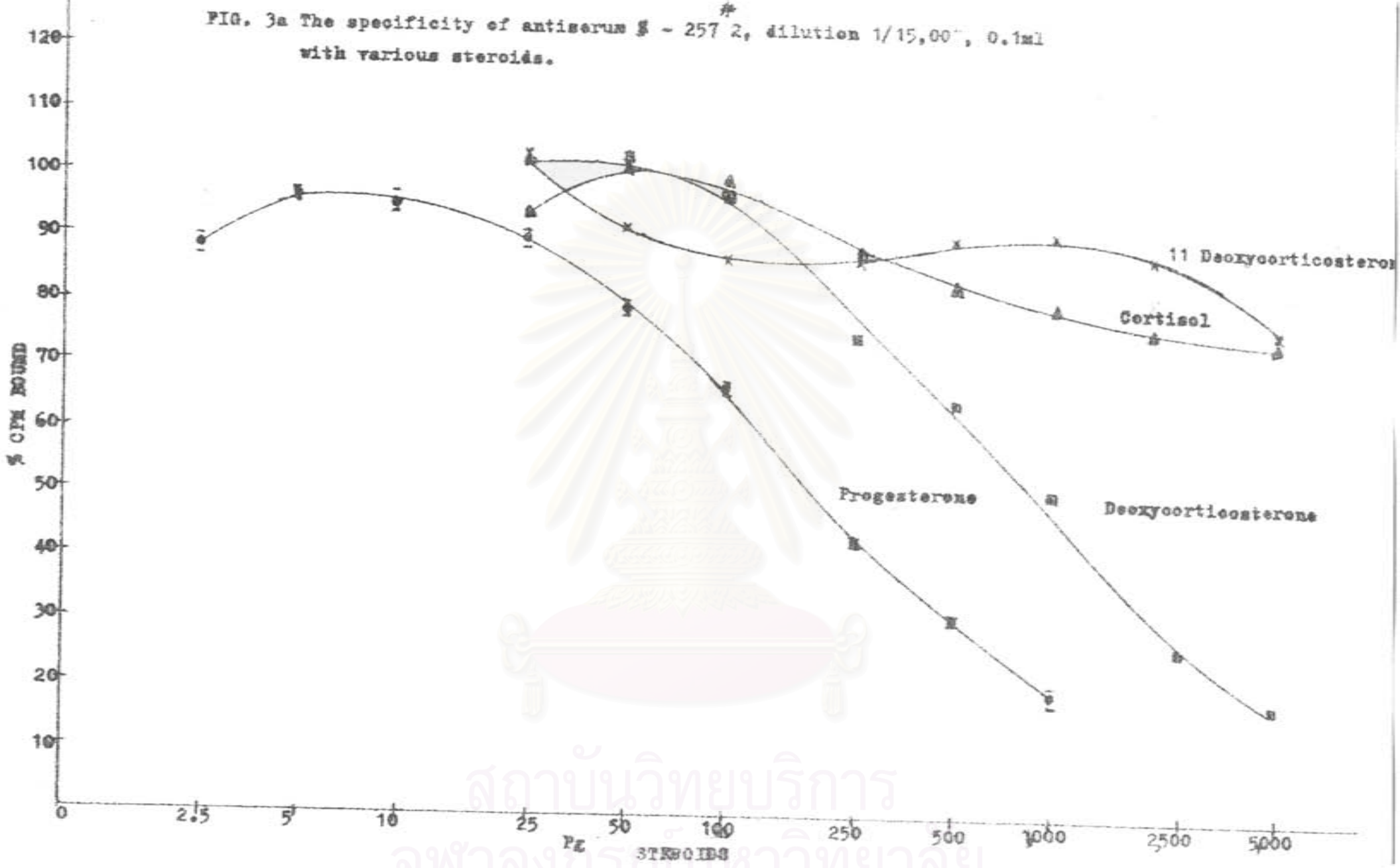


FIG. 3b The specificity of antiserum S - 257 2, dilution 1/15,000, 0.1ml with various steroids.

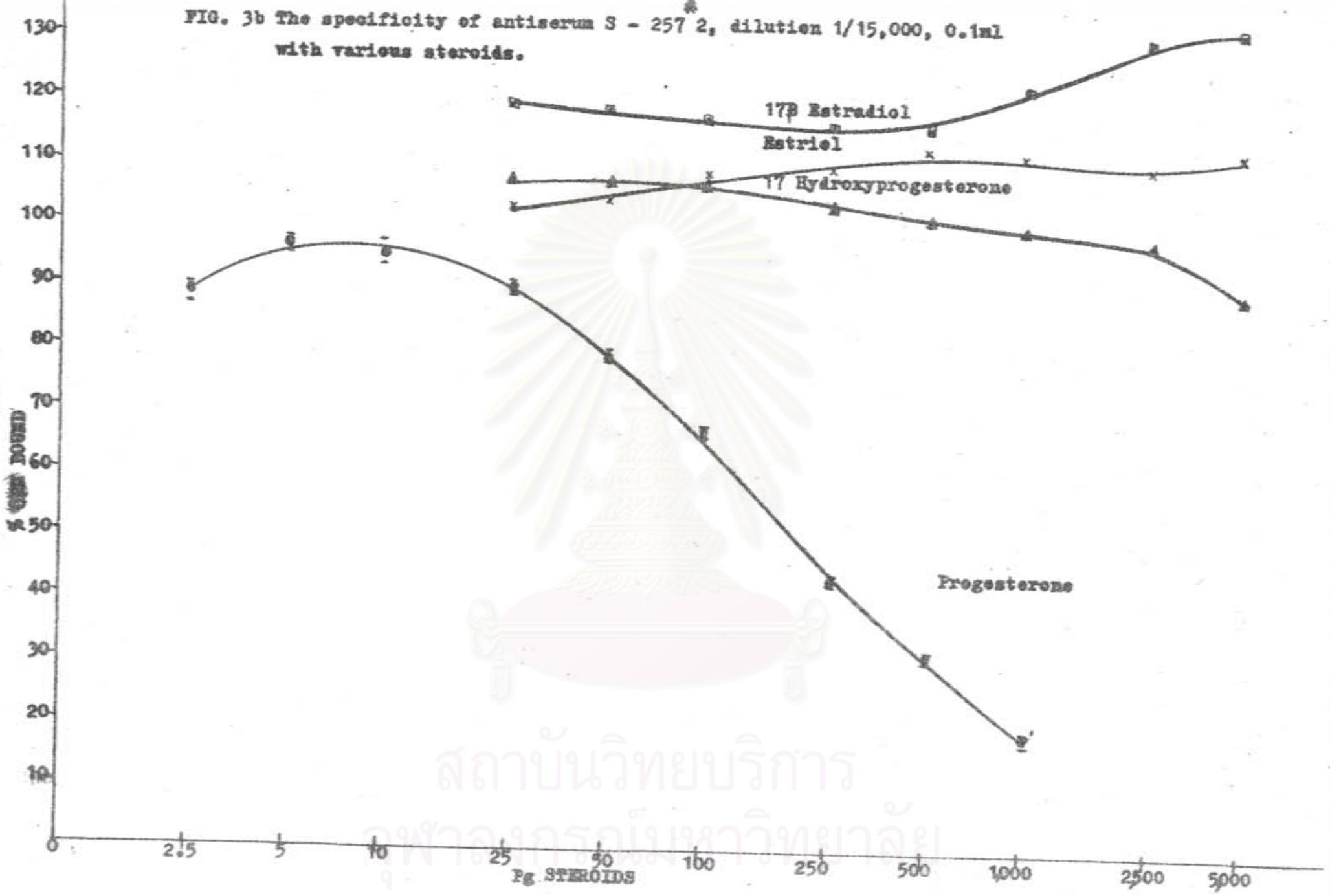


FIG. 3c The specificity of antiserum S - 257*2, dilution 1/15,000, 0.1ml with various steroids.

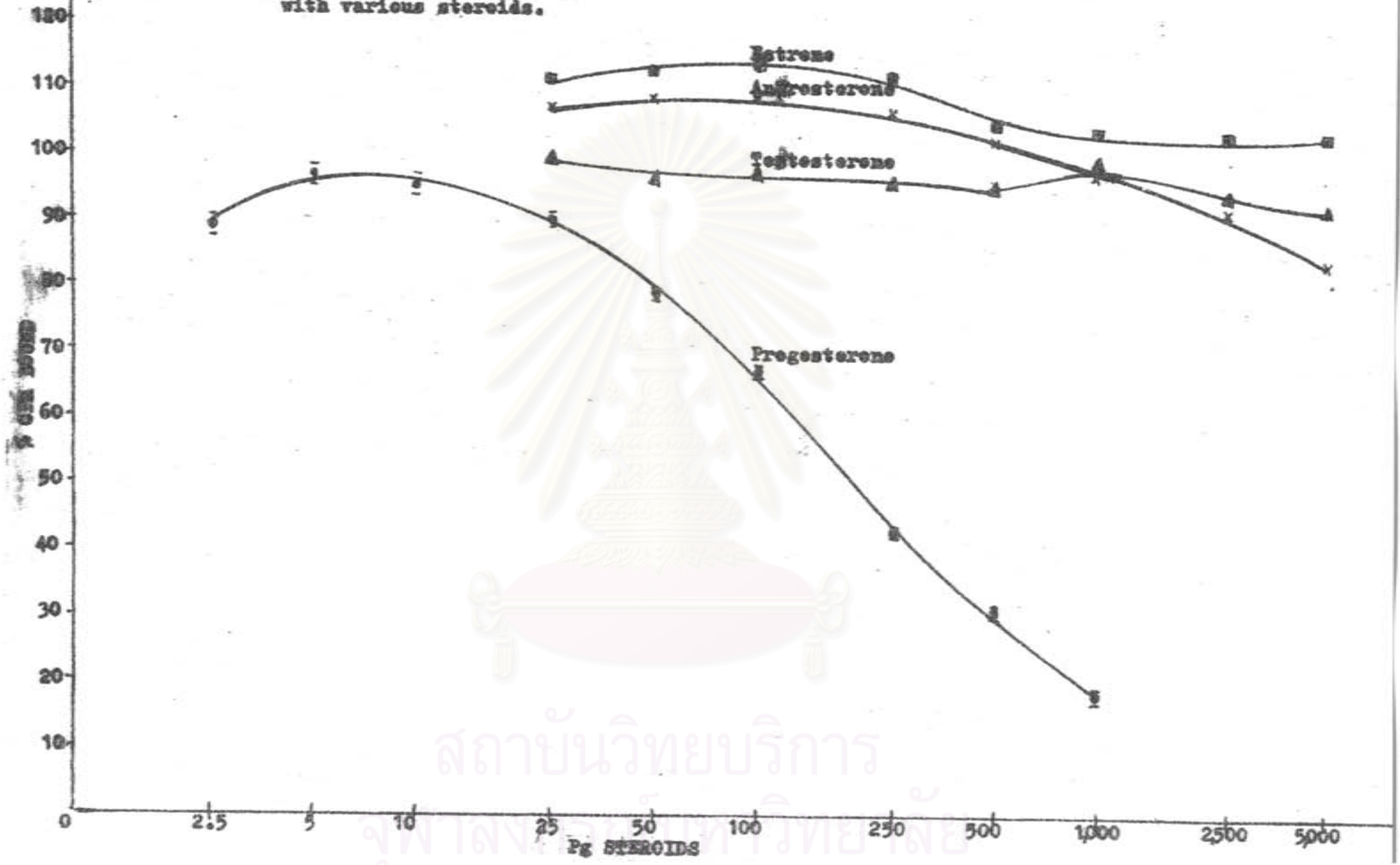


FIG. 4a The specificity of antiserum S - 49 #6, dilution 1/15,000, 0.1ml with various steroids.

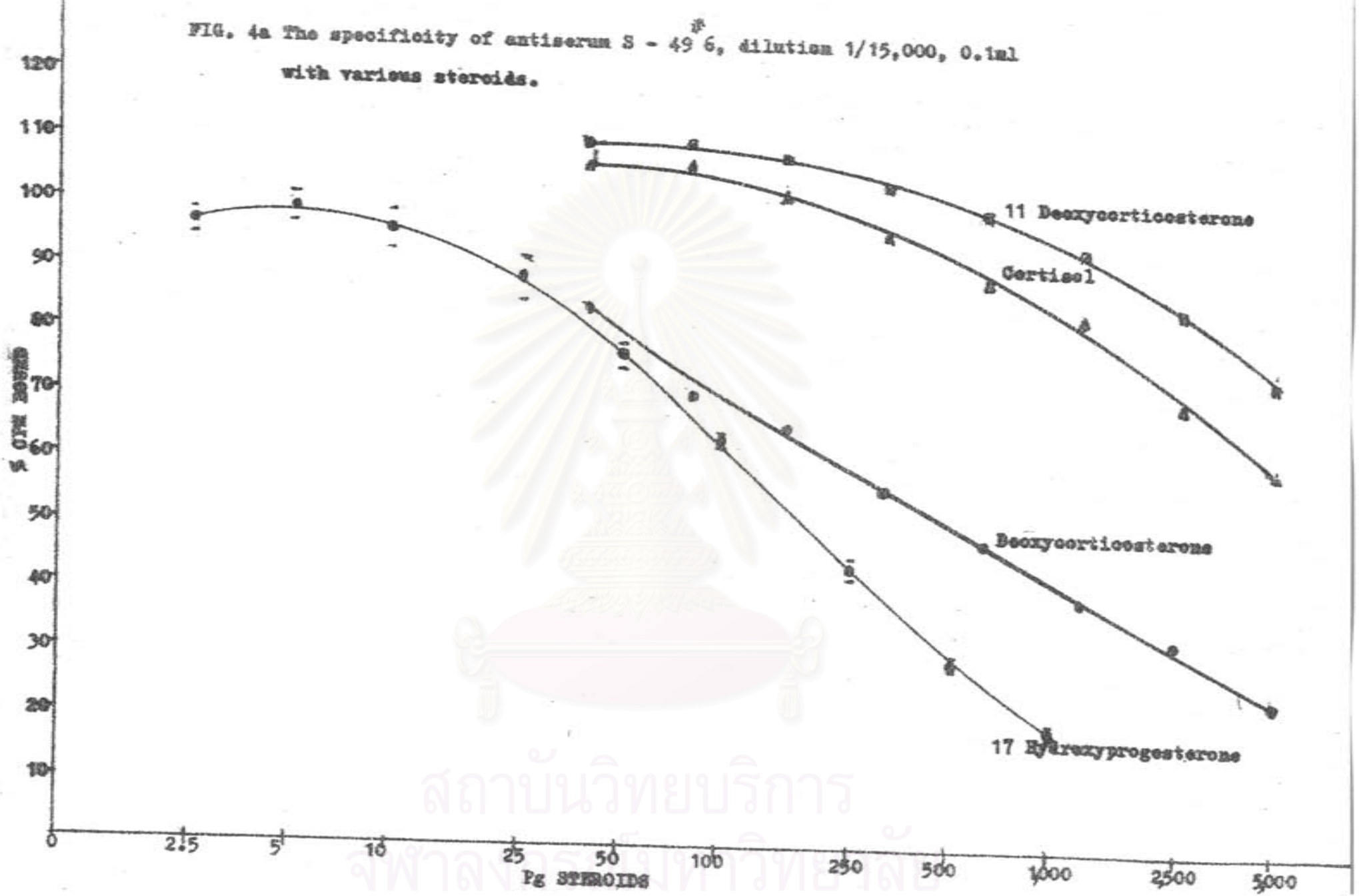


FIG. 4b The specificity of antiserum S - 49 6, dilution 1/15,000, 0.1ml with various steroids.

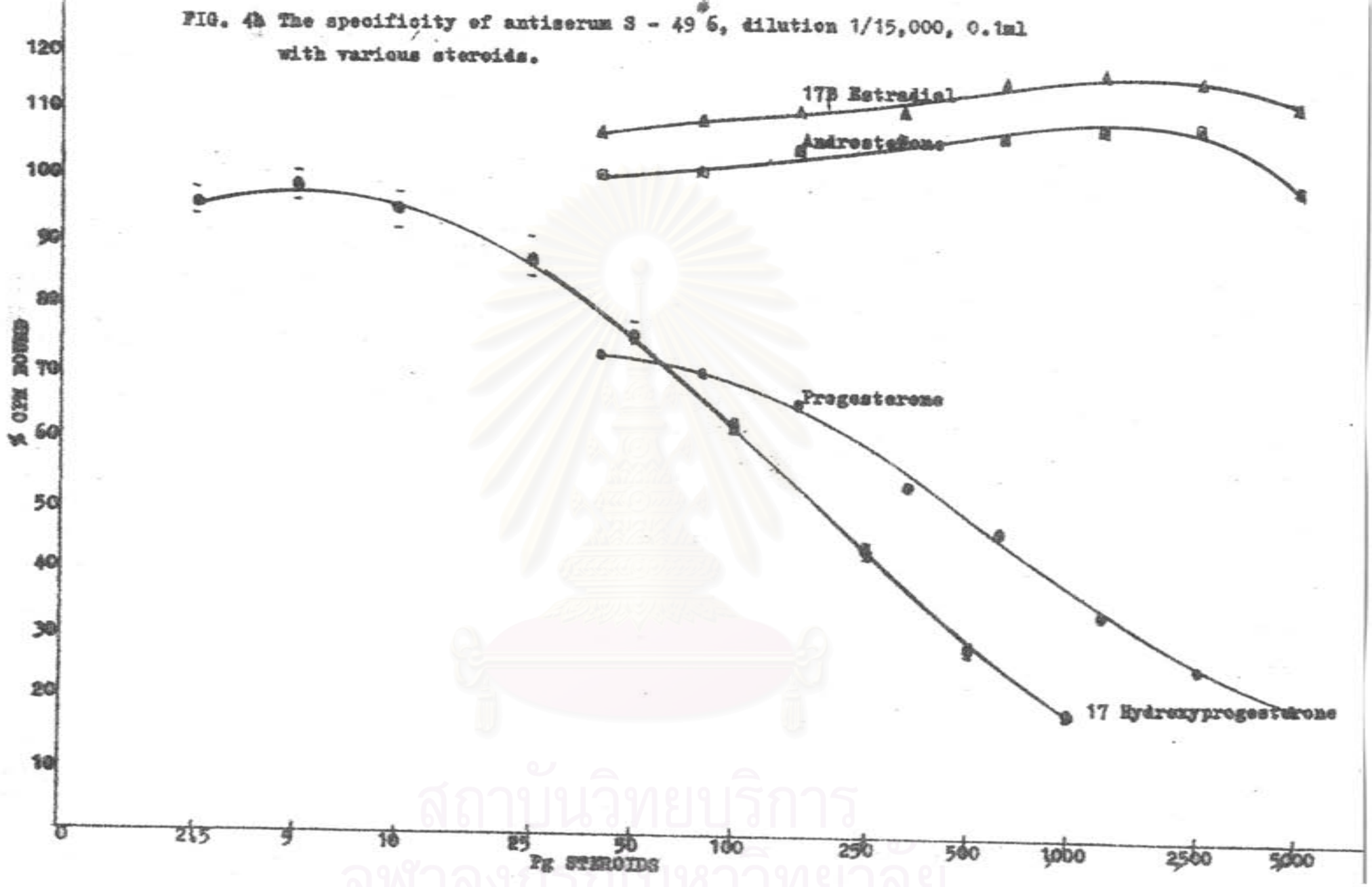


FIG. 4c The specificity of antiserum S - 49* 6, dilution 1/15,000, 0.1ml with various steroids.

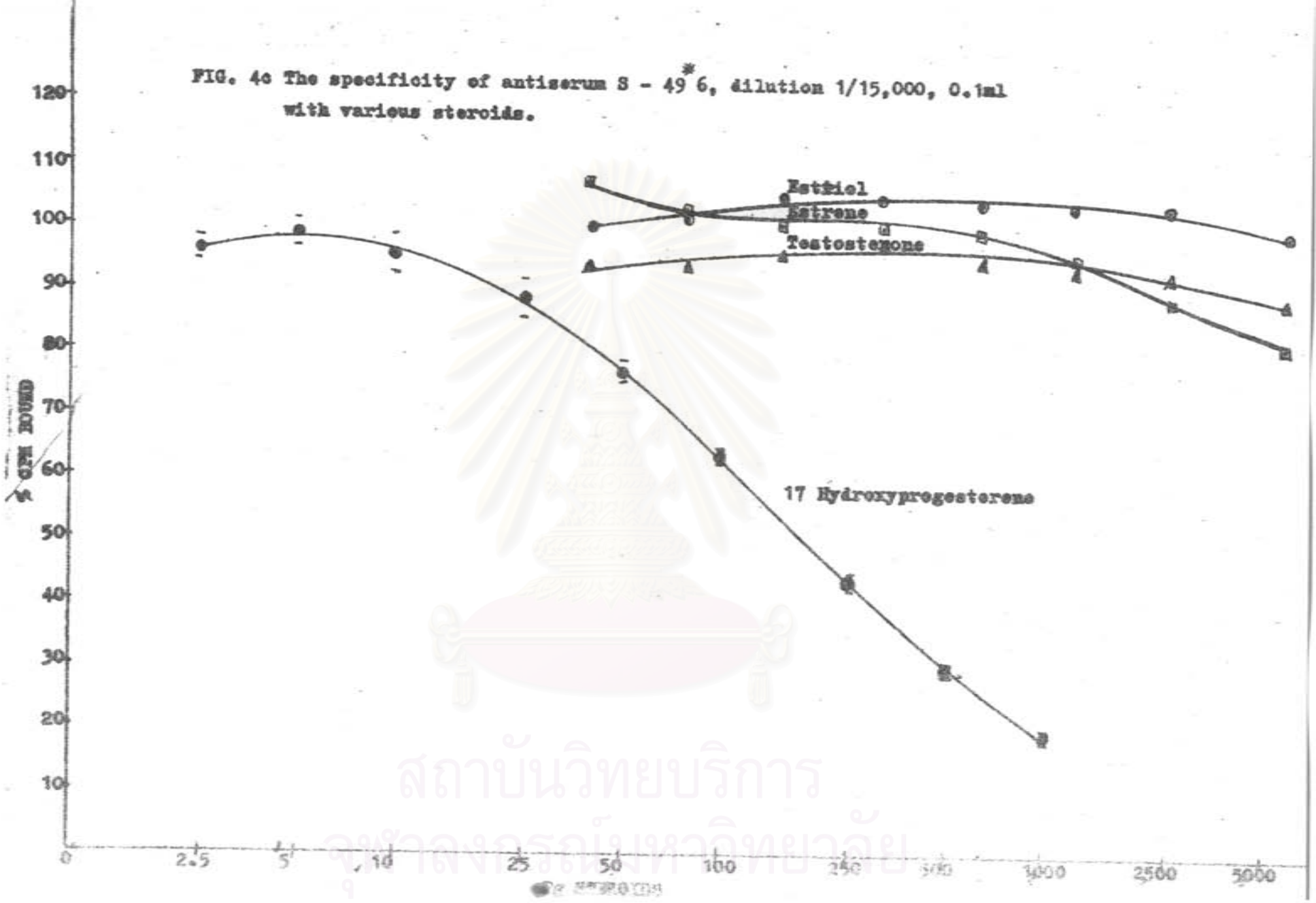


FIG. 5a The specificity of antiserum S - 52 #5, dilution 1/15,000, 0.1ml with various steroids.

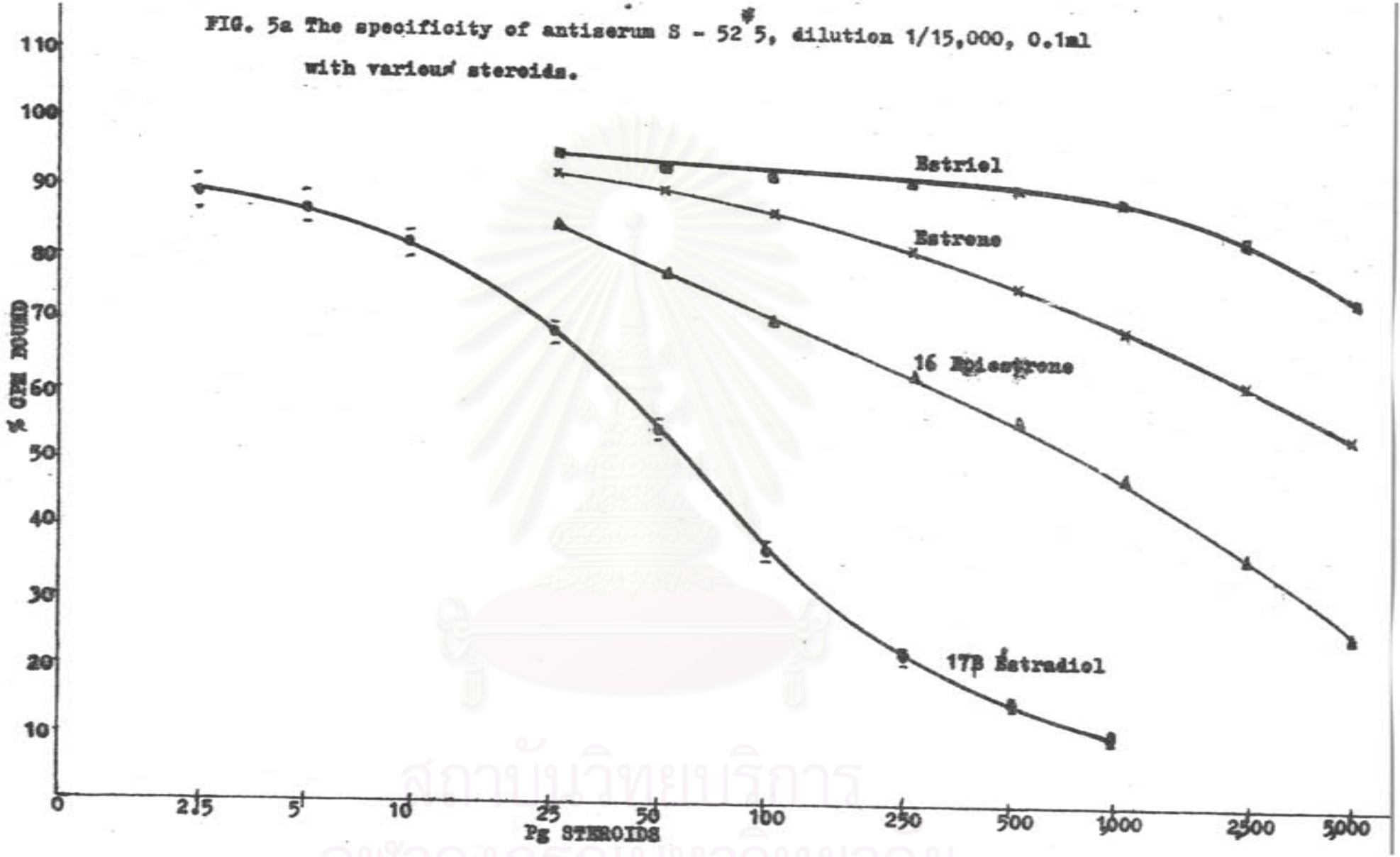


FIG. 5b The specificity of antiserum S 52*5, dilution 1/15,000, 0.1ml with various steroids.

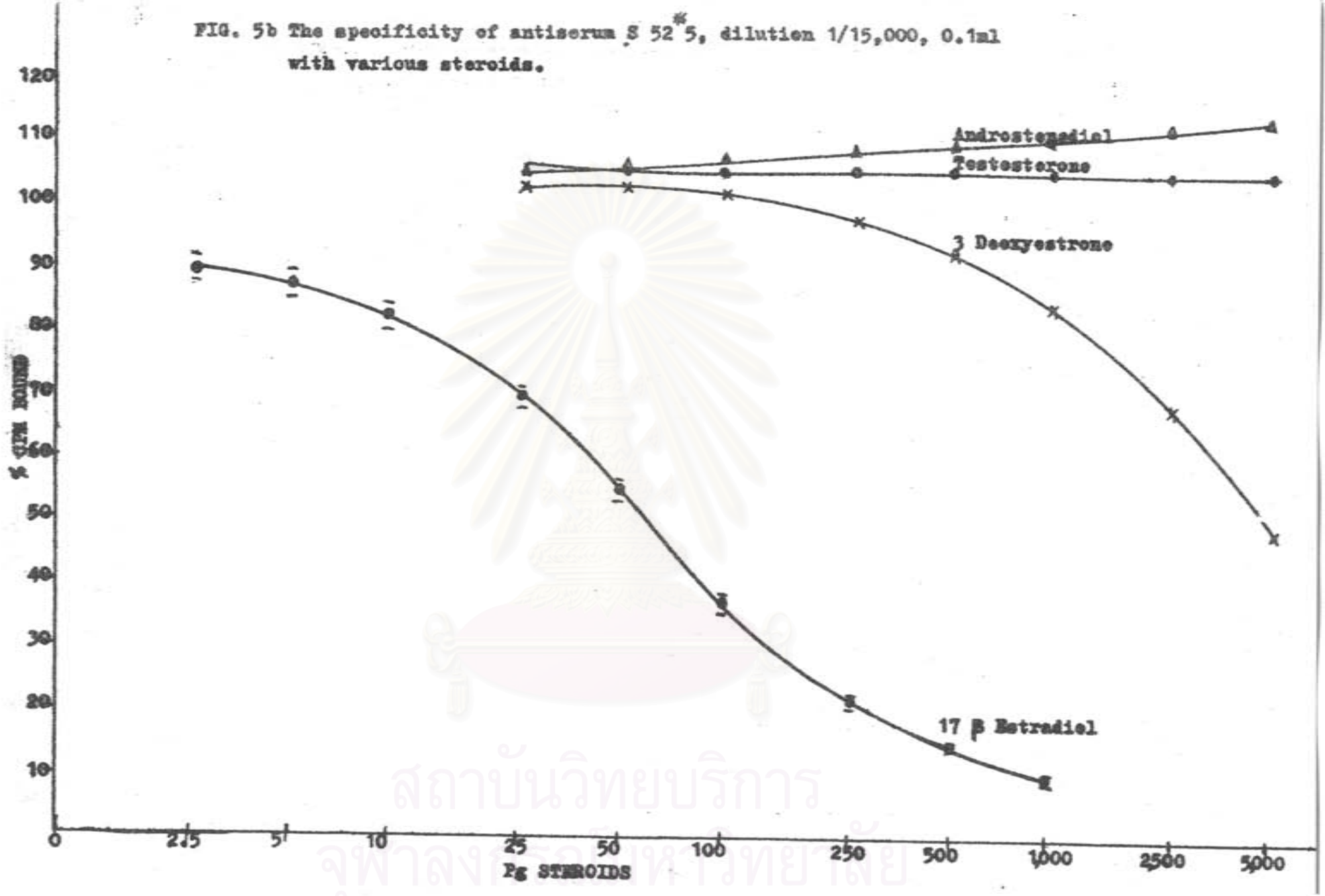


FIG. 5c The specificity of antiserum S 52 5, dilution 1/15,000, 0.1ml with various steroids.

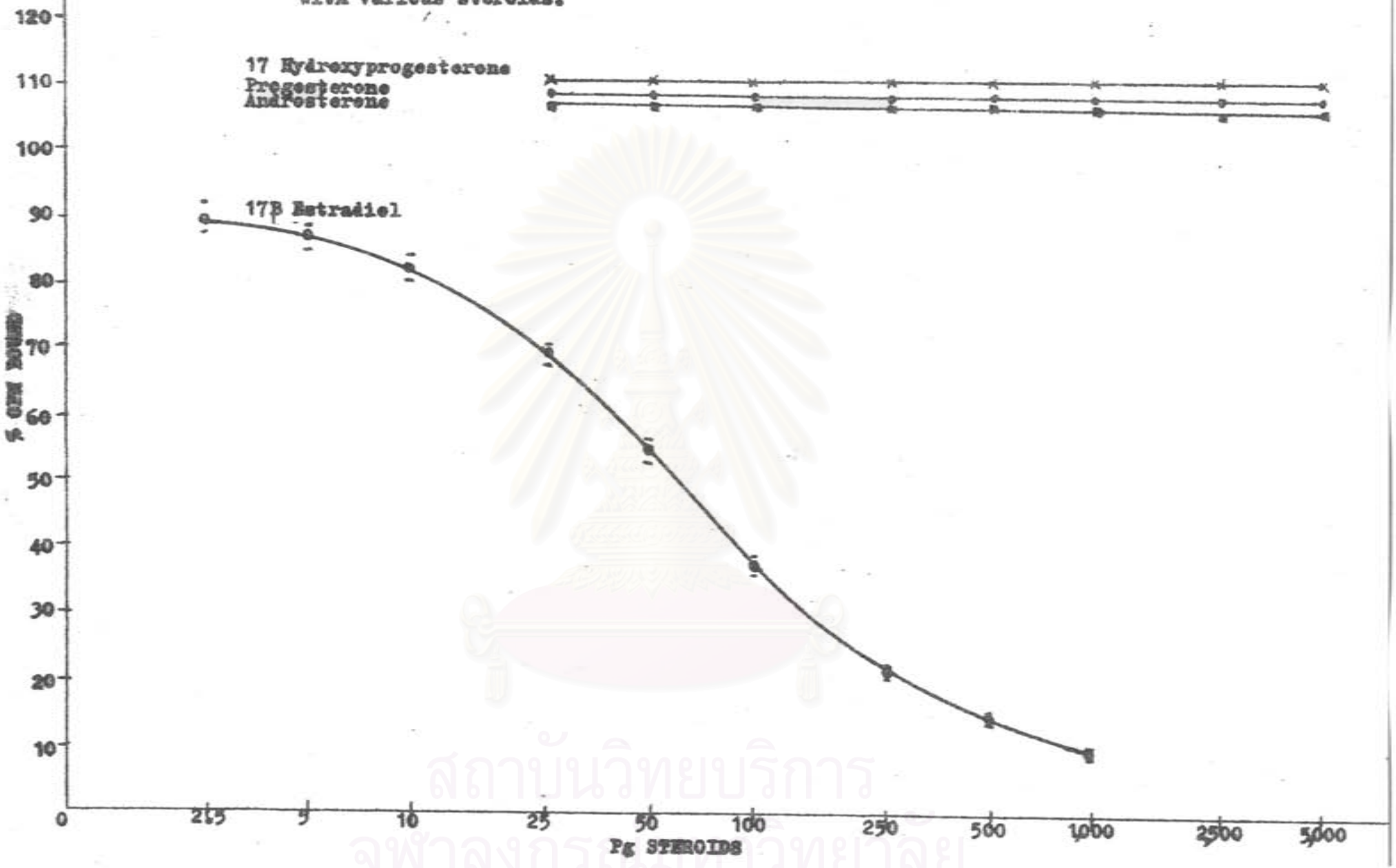


Table 3a Cross Reaction of Various steroids with Antiserum

S - 257 #2

Steroids	Cross Reaction (in %)
C_{21} Steroids	
Cortisol	5.55
11- Deoxy corticosterone	2.00
Deoxycorticosterone	20.83
Progestcrone	100.0
17α -hydroxy progesterone	0.50
C_{19} Steroids	
Testosterone	1.00
Androsterone	0.40
C_{18} Steroids	
Estradiol - 17β	0.00
Estriol	0.00
Estrone	0.00

Table 3b Cross Reaction of Various Steroids with Antiserum

S - 49#6

Steroids	Cross Reaction (in %)
C_{21} Steroids	
Cortisol	3.07
11 - Deoxycorticosterone	1.29
Deoxycorticosterone	80.00
Progesterone	100
17α - hydroxyprogesterone	100
C_{19} Steroids	
Testosterone	0.80
Androsterone	0.00
C_{18} Steroids	
Estradiol - 17β	0.00
Estriol	0.11
Estrone	0.80

And the steroid 16 epiestrone having significantly cross reacted in 25.0 %. But the others showed a little or no detectable cross reaction (Table 3c).

The purification step is very important for simultanously measurement of these three steroids. The celite chromatographic separation was investigated for purification. Celite microcolumn utilizes ethlene glycol as statioenary phase. While the mobile phase was varied in various percentage of ethylactate in isooctane. The steroid deoxycorticosterone (showing significant cfoss reaction chromatographic separation) suddenly decreased the % cross reaction up to undetectable level. The result indicated that best eluants for P, 17β and E_2 were 0, 15, 40 % of ethylene acetate in isooctane respectively (Table 4).

4. Precision

4.1 Preparation of pooled serum

Adding standard P, 17P and E_2 into 100 ml FHS (from 7.1) and stired vigorously at room temperature for 24 hrs. The amounts of steroids added as in the following table.

Table 3c Cross Reaction of Various Steroids with Antiserum

S - 52#5

Steroids	Cross Reaction (in %)
C_{21} Steroids	
Progesterone	0.00
17 - α hydroxyprogesterone	0.00
C_{19} Steroids	
Testosterone	0.00
Androstenediol	0.00
Androsterone	0.00
C_{18} Steroids	
Estradiol -17β	100
Estriol	0.33
Estrone	2.50
16 Epiestrone	25.00
3 -Deoxyestrone	1.13

Table 4 Chromatographic Separation of Various Steroids on Celite Microcolumns (utilizes ethylene glycol as stationary phase) and the Cross Reaction of these Steroids with Antisera S 257#2, S 49#6 and S 52#5

Mobile Phase	Steroids tested in the eluated fraction	Cross Reaction (in %) with S 257#2	S 49#6	S 52#5
Isooctane	Progesterone	100.0	100.0	0.0
	17α-hydroxyprogesterone	0.0	0.0	0.0
	Cortisol	0.0	0.0	0.0
	Deoxycorticosterone	0.0	0.0	0.0
	Estradiol- 17β	0.0	0.0	0.0
15% Ethyl acetate in isooctane	Progesterone	0.0	0.0	0.0
	17-α hydroxyprogesterone	1.0	90.0	0.0
	Cortisol	0.0	0.0	0.0
	Deoxyeorticosterone	0.0	0.0	0.0
	Estradiol - 17β	0.0	0.0	0.0
40% Ethyl acetate in isooctone	Progesterone	0.0	0.0	0.0
	17-α hydroxyprogesterone	0.0	0.0	0.0
	Estradiol - 17β	0.0	< 0.1	100
	Cortisol	0.0	< 0.1	0.0
	Deoxycorticosterone	0.0	5.3	0.0

Table 4 (Continue)

Mobile Phase	Steroids tested in the eluated fraction	Croos Reaction (in %) with S 257 2	S 49 6	S 52 5
60% Ethyl acetate in isooctane	Progesterone	0.0	0.0	0.0
	17-∝hydroxyprogesterone	0.0	0.0	0.0
	Estradiol - 17β	0.0	0.0	0.0
	Cortisol	<0.01	0.0	0.0
	Deoxycorticosterone	0.0	6.2	0.0
70% Ethyl acetate in isooctane	Progesterone	0.0	0.0	0.0
	17-∝hydroxyprogesterone	0.0	0.0	0.0
	Estradiol -17β	0.0	0.0	0.0
	Cortisol	0.0	0.0	0.0
	Deoxycorticosterone	0.0	<0.1	0.0
80% Ethyl acetate in isooctane	Progesterone	0.0	0.0	0.0
	17-∝ hydroxy progesterone	0.0	0.0	0.0
	Estradiol -17β	0.0	0.0	0.0
	Cortisol	0.0	0.0	0.0
	Deoxycorticosterone	0.0	0.0	0.0

Pool Serum.	Steroids added. in FHS (pg/ml)		
	P	17P	E_2
I	62.5	62.5	25
II	125	125	50
III	250	250	100
IV	500	500	500
V	2,500	2,500	1,000

4.2 Reproducibility

The within assay variance was evaluated by 5 duplicated determinations of each pool (I,II,III,IV,and V) in the same assay. And varied in 5 different days of assay (Table 5a, 5b, 5c, 5d, 5e, 6a, 6b, 6c, 6d, 6e, and 7a, 7b, 7c, 7d, 7e). The coefficient of variations (CV) with values ranging from 250 - 2,500 pg/ml were 8.2 % for both progesterone and 17 hydroxyprogesterone. While the CV in ranging from 62.5 - 125 pg/ml were 17.1 % and 19.0 % respectively. The CV in within assay of 17 β estradiol with values ranging between 50 - 1,000 pg/ml was 8.3 % but the level of 25 pg/ml was 18.4 %

The between assay variance was investigated by 5 duplicate determinations in 5 different assays (Table 8a, 8b, 8c,). The CV of progesterone, 17 hydroxyprogesterone and 17 β

Table 5a **Roproducibility** of 62.5 pg progesterone added in 1 ml FHS.

Days of determination	No. of determination in the same sample	Found (pg/ml)	$\bar{X} \pm$ SD	CV	SE
1	1	92.3			
	2	87.5			
	3	117.3			
	4	127.9			
	5	123.4	109.7 ±18.5	16.9	8.3
2	1	92.2			
	2	81.6			
	3	110.3			
	4	108.2			
	5	134.0	105.2 ±19.9	18.9	8.9
3	1	59.8			
	2	43.2			
	3	47.7			
	4	43.5			
	5	43.2	47.5 ± 7.1	15.1	3.2
4	1				
	2				
	3	-	-		-
	4				
	5				
5	1				
	2				
	3	-	-	-	-
	4				
	5				

Table 5b Reproducibility of 125 pg progesterone added in 1 ml PHS

Days of determination	No.of determination in the same sample	Found (pg/ml)	$\bar{X} \pm SD$	CV	SE
1	1	184.5			
	2	154.5			
	3	179.4			
	4	156.5	184.0		
	5	245.3	±36.8	19.9	6.4
2	1	152.7			
	2	125.4			
	3	148.0			
	4	100.1	133.4		
	5	140.5	±21.3	15.9	9.5
3	1	176.9			
	2	153.8			
	3	130.3			
	4	126.1	142.8		
	5	126.7	±22.2	15.6	9.9
4	1				
	2				
	3	-	-	-	-
	4				
	5				
5	1				
	2				
	3	-	-	-	-
	4				
	5				

Table 5c Reproducibility of 250 pg progesterone added in 1 ml FHS

Days of determination	No.of determination in the same sample	Found (pg/ml)	$\bar{X} \pm SD$	CV	SE
1	1	248.9			
	2	232.5			
	3	220.4			
	4	221.5	231.6		
	5	234.6	±11.6	5.0	5.2
2	1	233.1			
	2	312.9			
	3	238.1			
	4	284.1	259.9		
	5	231.6	±36.7	14.1	16.4
3	1	251.4			
	2	261.4			
	3	220.7			
	4	233.9	245.8		
	5	261.4	±18.0	7.3	8.0
4	1	263.4			
	2	262.6			
	3	314.1			
	4	256.9	273.4		
	5	250.0	±25.6	9.4	9.1
5	1	272.9			
	2	339.6			
	3	305.8			
	4	281.9	308.3		
	5	341.5	±31.8	10.3	14.2

Table 5d Reproducibility of 500 pg progesterone added in 1ml FHS

Days of determination	No.of determination in the same sample	Found (pg/ml)	$\bar{X} \pm SD$	CV	SE
1	1	464.7			
	2	472.2			
	3	429.1			
	4	4 5.8	450.6		
	5	451.3	±18.4	4.1	8.2
2	1	440.5			
	2	474.2			
	3	488.8			
	4	470.6	463.0		
	5	441.1	±21.4	4.6	9.6
3	1	429.0			
	2	464.9			
	3	497.8			
	4	577.6	511.5		
	5	588.3	±69.7	13.6	31.2
4	1	509.9			
	2	494.0			
	3	690.6			
	4	630.7	598.8		
	5	668.9	±91.2	15.2	40.8
5	1	462.2			
	2	589.5			
	3	576.0			
	4	628.2	586.7		
	5	677.5	±79.9	13.6	35.8

Table 5e Reproducibility of 2500 pg progesterone added in 1 ml FHS

Days of determination	No.of determin the same sample	Found (pg/ml)	$\bar{X} \pm$ SD	CV	SE
1	1	3,093.4			
	2	2,747.6			
	3	2,956.8			
	4	2,849.4	2,948.8		
	5	3,097.0	± 152.7	5.2	68.3
2	1	2,329.6			
	2	2,588.6			
	3	2,622.7			
	4	2,581.4	2,510.2		
	5	2,428.8	± 125.6	5.0	56.2
3	1	1,938.2			
	2	1,820.6			
	3	2,087.3			
	4	1,892.4	1,951.7		
	5	2,020.2	± 104.8	5.4	46.9
4	1	2,309.9			
	2	2,172.9			
	3	2,185.2			
	4	2,209.5	2,215.7		
	5	2,200.3	± 45.6	2.5	24.4
5	1	2,531.7			
	2	2,088.6			
	3	2,447.6			
	4	2,325.6	2,331.3		
	5	2,262.8	± 171.3	7.3	76.5

Table 6a Reproducibility of 62.5 pg 17 hydroxyprogesterone added in 1 ml FHS

Days of determination	No.of determination in the same sample	Found (pg/ml)	$\bar{X} \pm SD$	CV	SE
1	1	75.2			
	2	48.7			
	3	62.7			
	4	53.1	58.3		
	5	52.1	±10.8	18.4	4.8
2	1	53.4			
	2	86.7			
	3	75.1			
	4	71.3	75.1		
	5	88.9	±14.2	18.9	6.4
3	1	29.7			
	2	27.4			
	3	21.7			
	4	53.4	32.1		
	5	28.4	±12.3	38.2	5.5
4	1				
	2				
	3	-	-	-	-
	4				
	5				
5	1				
	2				
	3	-	-	-	-
	4				
	5				

Table 6b Reproducibility of 125 pg 17 hydroxyprogesterone added in 1ml FHS

Days of determination	No.of determination in the same sample	Bound (pg/ml)	$\bar{X} \pm SD$	CV	SE
1 1	1	82.5			
	2	93.2			
	3	128.4			
	4	107.0	106.6		
	5	122.9	±19.4	18.2	8.7
2	1	125.7			
	2	154.7			
	3	156.9			
	4	126 6	133.6		
	5	104.2	±22.2	16.6	9.9
3	1	77.5			
	2	83.8			
	3	78.1			
	4	82.3	81.0		
	5	83.4	±3.0	3.7	1.3
4	1				
	2				
	3	-	-	-	-
	4				
	5				
5	1				
	2				
	3	-	-	-	-
	4				
	5				

Table 6c Reproducibility of 250 pg 17 hydroxyprogesterone added in 1 ml FHS

Days of determination	No.of determination in the same sample	Found (pg/ml)	$\bar{X} \pm$ SD	CV	SE
1	1	244.5			
	2	247.8			
	3	242 9			
	4	236.7	244.		
	5	249.3	± 4.9	2.0	2.2
2	1	251.9			
	2	281.4			
	3	221.2			
	4	280.1	267.2		
	5	301.5	±31.2	11.7	13.9
3	1	166.6			
	2	157.1			
	3	180.0			
	4	208.0	182.3		
	5	200.0	±21.6	11.8	9.6
4	1	224.5			
	2	206.2			
	3	267.0			
	4	218.6	241.7		
	5	292.3	±36.4	15.0	16.3
5	1	291.8			
	2	277.1			
	3	327.3			
	4	279.5	300.6		
	5	327.3	±25.0	8.3	11.2

Table 6d Reproducibility of 500 pg 17 hydroxyprogesterone added in 1 ml FHS

Days of determination	No. of determination in the same sample	Found (pg/ml)	$\bar{X} \pm SD$	CV	SE
1	1	492.6			
	2	495.6			
	3	480.9			
	4	498.2	485.0		
	5	457.9	±16.5	3.4	7.4
2	1	561.8			
	2	633.1			
	3	601.8			
	4	635.	603.9		
	5	587.0	±31.3	5.2	14.0
3	1	438.5			
	2	404.6			
	3	485.6			
	4	459.6	454.0		
	5	481.8	±33.5	7.4	14.9
4	1	540.9			
	2	489.7			
	3	581.2			
	4	518.7	534.4		
	5	541.3	±33.6	6.3	15.0
5	1	520.2			
	2	596.9			
	3	540.4			
	4	554.3	550.3		
	5	539.7	±28.7	5.2	12.9

Table 6e Reproducibility of 2,500 pg 17 hydroxyprogesterone added in 1ml FHS

Days of determination	No.of determination in the same sample	Found (pg/ml)	$\bar{X} \pm SD$	CV	SE
1	1	2,187.6			
	2	2,342.7			
	3	2,500.0			
	4	2,657.8	2,494.1		
	5	2,728.3	±238.1	9.5	106.5
2	1	2,360.7			
	2	2,245.4			
	3	2,204.4			
	4	2,032.1	2,285.7		
	5	2,586.1	±205.3	8.9	91.8
3	1	2,392.6			
	2	2,201.3			
	3	2,162.9			
	4	2,208.4	2,226.4		
	5	2,166.9	±95.1	4.3	42.5
4	1	2,640.9			
	2	2,454.2			
	3	2,172.3			
	4	2,417.4	2,441.6		
	5	2,523.1	±172.9	15.0	77.3
5	1	2,309.6			
	2	2,699.9			
	3	2,234.3			
	4	2,461.1	2,478.8		
	5	2,689.3	±213.3	8.3	95.4

Table 7a Reproducibility of 25 pg 17β estradiol added in 1 ml FHS

Days of determination	No.of determination in the same sample	Found (pg/ml)	$\bar{X} \pm$ SD	CV	SE
1	1	16.0			
	2	17.0			
	3	20.0			
	4	10.0	16.4		
	5	19.0	±3.9	23.9	1.7
2	1	30.5			
	2	34.2			
	3	31.9			
	4	32.5	33.2		
	5	36.9	±2.4	7.3	1.1
3	1	23.0			
	2	19.0			
	3	19.0			
	4	19.0	18.2		
	5	11.0	±4.4	24.1	2.0
4	1				
	2				
	3	-	-	-	-
	4				
	5				
5	1				
	2				
	3	-	-	-	-
	4				
	5				

Table 7b Reproducibility of 50 pg 17β estradiol added in 1 ml FHS

Days of determination	No.of determination in the same sample	Found (pg/ml)	$\bar{X} \pm SD$	CV	SE
1	1	49.7			
	2	54.7			
	3	53.3			
	4	53.5	50.4		
	5	41.0	±5.6	11.1	2.5
2	1	61.6			
	2	69.9			
	3	59.2			
	4	59.4	62.1		
	5	60.4	±4.5	7.8	2.0
3	1	41.0			
	2	42.0			
	3	43.0			
	4	43.0	42.0		
	5	41.0	±1.0	2.4	0.5
4	1				
	2				
	3	-	-	-	-
	4				
	5				
5	1				
	2				
	3	-	-	-	-
	4				
	5				

Table 7c Reproducibility of 100 pg 17β estradiol added in 1ml FHS

Days of determination	No.of determination in the same sample	Found (pg/ml)	$\bar{X}$ ± SD	CV	SE
1	1	95.2			
	2	97.9			
	3	95.1			
	4	98.9	94.7		
	5	86.4	±4.9	5.2	2.2
2	1	86.3			
	2	106.1			
	3	92.5			
	4	86.2	89.5		
	5	76.2	±11.0	12.3	4.9
3	1	129.3			
	2	129.8			
	3	120.8			
	4	115.9	122.2		
	5	120.5	±5.0	4.1	2.2
4	1	118.9			
	2	123.1			
	3	118.8			
	4	109.3	115.1		
	5	105.7	±7.3	6.4	3.3
5	1	121.2			
	2	118.1			
	3	124.3			
	4	145.4	125.3		
	5	117.3	±11.6	9.3	5.2

Table 7d Reproducibility of 500 pg 17β estradiol added in 1ml FHS

Days of determination	No.of determination in the same sample	Found (pg/ml)	$\bar{X}$ ± SD	CV	SE
1	1	512.5			
	2	487.8			
	3	471.9			
	4	513.3	501 3		
	5	520.8	±20.6	4.1	9.2
2	1	460.8			
	2	481.0			
	3	521.6			
	4	446.1	492.0		
	5	550.7	±43.4	8.8	19.4
3	1	525.6			
	2	535.9			
	3	453.6			
	4	470.4	484.7		
	5	438.4	±43.7	16.1	19.5
4	1	612.7			
	2	531.9			
	3	515.5			
	4	519.8	575.7		
	5	698.7	±79.3	13.8	35.5
5	1	607.2			
	2	432.2			
	3	595.9			
	4	633.5	552.7		
	5	494.0	±85.6	15.5	38.3

Table 7c Reproducibility of 1000 pg 17β estradiol added in 1 ml FHS

Day of determination	No.of determination in the same sample	Found	$\bar{X}$ ± SD	CV	SE
1	1	1,070.3			
	2	991.0			
	3	1,009.2			
	4	1,033.3	1,029.4		
	5	1,043.1	±30.7	3.0	13.7
2	1	1,406.5			
	2	1,154.7			
	3	1,258.3			
	4	1,206.1	1,235.8		
	5	1,153.5	± 140.7	8.8	19.4
3	1	1,703.1			
	2	1,121.4			
	3	1,616.2			
	4	1,735.5	1,561.6		
	5	1,631.9	± 251.0	9.0	112.2
4	1	1,221.5			
	2	1,116.8			
	3	1,053.5			
	4	1,172.4	1,132.6		
	5	1,098.9	± 65.5	5.8	29.3
5	1	1,197.4			
	2	1,222.4			
	3	1,355.2			
	4	1,353.3	1,281.7		
	5	1,278.2	± 73.2	5.7	32.8

Table 8a Reproducibility of progesterone (P) in five different assays

Amounts of added in FHS (pg/ml)	Days of determination	Found (pg/ml)	$\bar{X}$ ± SD	CV	SE
62.5	1	109.7			
	2	105.2			
	3	47.5			
	4		87.5		
	5		±34.7	39.7	20.1
125	1	184.0			
	2	133.4			
	3	142.8			
	4		153.4		
	5		±26.9	17.5	15.5
250	1	231.6			
	2	259.9			
	3	245.8			
	4	273.4	263.8		
	5	308.3	±29.4	11.1	13.1
500	1	450.6			
	2	463.0			
	3	511.5			
	4	598.8	522.1		
	5	586.7	±68.5	13.1	30.6
2500	1	2948.8			
	2	2510.2			
	3	1951.7			
	4	2215.6	2391.5		
	5	2331.3	±371.7	15.5	166.2

Table 8b Reproducibility of 17 hydroxyprogesterone (17P) in five different assays

Amounts of 17P added in FHS (pg/ml)	Days of determination	Found (pg/ml)	$\bar{X} \pm$ SD	CV	SE
62.5	1	58.3			
	2	15.1			
	3	32.1			
	4		55.2		
	5		±21.7	39.3	12.5
125	1	106.6			
	2	133.6			
	3	81.0			
	4		107.1		
	5		±26.3	24.6	15.2
250	1	244.2			
	2	267.2			
	3	182.3			
	4	241.7	247.2		
	5	300.6	±43.3	17.5	19.4
500	1	485.0			
	2	603.9			
	3	454.0			
	4	534.4	525.5		
	5	550.3	±58.3	11.1	26.1
2,500	1	2,494.1			
	2	2,285.7			
	3	2,226.4			
	4	2,441.6	2,385.3		
	5	2,478.8	±121.4	5.1	54.3

Table 8c Reproducibility of 17β estradiol (E_2) in five different assays

Amaunts of E_2 added in FHS(pg/ml)	Days of determination	Found (pg/ml)	X ± SD	CV	SE
25	1	16.4			
	2	33.2			
	3	18.2			
	4		22.6		
	5		±9.2	40.8	5.3
50	1	50.4			
	2	62.1			
	3	42.0			
	4		51.5		
	5		±10.1	19.6	5.8
100	1	94.7			
	2	89.5			
	3	122.2			
	4	115.1	109.4		
	5	125.3	±16.3	14.9	7.3
500	1	501.3			
	2	492.0			
	3	484.7			
	4	575.7	521.3		
	5	552.7	±40.4	7.8	18.1
1,000	1	1,029.4			
	2	1,235.8			
	3	1,561.6			
	4	1,132.6	1248.2		
	5	1,281.7	±200.5	16.1	89.6

estradiol were 13.2 %, 11.2 % and 14.6 % respectively.

5. Recovery Following ether extraction and Celite chromatography, the percentage recovery of progesterone, 17 hydroxyprogesterone and 17 β - estradiol varied in the ranges of 72.9 - 89.6, 77.0 - 97.0 and 85.5 - 101.3 respectively (Table 9)

Following the same procedure, the recovery of ^{3}HP, ^{3}HP, ^{3}H 17P and $^{3}HE_2$ varied in the ranges of 71.1 - 94.5%, 70.2 - 94.1 % and 61.0 - 80.7 % respectively. The mean recovery of 30 samples were 77.5 ± 4.7 %, 79.5 ± 6.1 % and 68.2 ± 4.3 % respectively. The smallest amount of the steroids in an unknown sample that could be measured was 50 pg for both progesterone and 17 hydroxyprogeoterone and 25 pg for 17 β estradiol after correction for recovery.

6. Accuracy Recovery experiments were carried out by measuring the levels of progesterone, 17 hydroxyprogesterone and 17 β estradiol present in 1 ml free hormone male buffalo serum samples which were added increasing amounts of these steroids (Table 10). The value of measured steroids did not differ by more than 20 % deviation from the expected value.

Table 9 Percentage Recovery of progesterone, 17 hydroxyprogesterone and 17β-estradiol added to 1 ml of pooled free hormone male buffalo serum

Steroids added		No. of determination in the same pool	Found value ± SD (pg/ml)	Percentage Recovery
Name	pg/ml			
P	0	5	63.5	-
	250	5	245.8 ± 17.9	72.9
	500	5	511.5 ± 29.7	89.6
	2,500	5	1951.7 ± 104.8	75.5
17P	0	5	49.2	-
	250	5	241.7 ± 36.4	77.0
	500	5	534.4 ± 33.6	97.0
	2,500	5	2441.6 ± 172.9	95.7
E_2	0	5	0	-
	100	5	101.3 ± 9.3	101.3
	500	5	427.4 ± 27.4	85.5
	1,000	5	902.0 ± 77.4	90.2

Table 10 Recovery of progesterone, 17 hydroxyprogesterone and 17β - estradiol added to 1 ml of pooled free hormone male buffalo serum

Steroids added		Found value in duplicate (pg/ml)		Expected value	% Deviation (from the expected value)	
Name	pg/ml	1	2	(pg/ml)	1	2
P	0	0	0	0	-	-
	125	125.4	140.50	125	+ 0.3	+ 12.4
	250	234.6	248.9	250	- 6.2	- 0.4
	500	476.2	509.9	500	- 4.8	+ 2.0
	2,500	2,172.9	2,172.9	2,500	-13.1	- 13.1
					mean 2.6	mean 4.5
17P	0	0	0	0	-	-
	125	128.4	122.8	125	+ 2.7	- 1.8
	250	266 3	249.3	250	+ 6.5	- 0.3
	500	498.2	457.9	500	- 0.4	- 8.4
	2,500	2,187.6	2,657.8	2,500	-12.5	+ 6.3
					mean 7.8	mean 6.88
E_2	0	0	0	0	-	-
	50	53.5	49.7	50	+ 7.0	- 0.6
	100	106.2	93.9	100	+ 6.2	- 6.1
	500	527.4	512.5	500	+ 5.5	+ 2.5
	1,000	1,009.2	1,043.1	1,000	+ 0.9	+ 4.3
					mean 13.4	mean 6.9

Discussion Although the method for currently measuring of human serum P, 17P and E_2 by radioimmunoassay were already established (Abraham et al, 1971). It needs the standard - ization of the method with buffalo serum prior use. The flucuation of temperature of incubation between 4°C and 10°C was investigated that no significantly effect in % cross reaction. However the temperature fluctuation in ice bath below 10°C during experiment can be accepted . Therefore this is not a factor to influence the flucuations in between assay or the stability of the method. It probably comes from the contamination of the reused glasswares.

The antiserum S - 49#6 used in the present assay was obtained from a ewe immunized with 11 - deoxycortisol - 21- monosuccinate - human serum albumin conjugate (Abraham et al , 1971**). It reacted completely with P, 17P and deoxy- corticosterone. Anyhow, the efficiency of the chromatographic system on Celite microcolumns is high enough to separate these three steroids from each other (Table 4). Together with a simple purification of nonspecific antiserum, it was able to use this antiserum as a binding reagent in the radio.- immunoassay of P (Abraham et al, 1971*). and 17P. The advantage of this method was currently measuring the levels of P, 17P and E_2 simultaneously in the same 1 ml aliquot of serum, making use of the chromatographic system on Celte micr.co- lumms to separate these three steroids.

The comparable values between the standardization of human and buffalo serum P, 17P and E_2 were shown in the following table

Standardized values	P		17P		E_2	
	HS*	BS**	HS	BS	HS	S
specificity(in% cross reaction)	100	100	90	100	100	100
Recovery (in%) labelled ster.	75-9	71.1-94.5	66-93	70.2-94.1	-	61.0-80.7
Recovery (in%) Standard ster.	-	72.9-89.6	-	77.0-97.0	-	85.5-101.3
Precision within assay	8.6	8.2	7.7	8.2	-	8.3
Precision between assay	18.0	13.2	14.7	11.2	-	14.6
Sensitivity	10-25	3.3-25.0	10-25	4.0-25.0	10	2.5-10.0

HS = Human Serum (Abraham et al, 1971),

BS** = Buffalo Serum

ster. = Steroids

The indications from this table show that it was able to use this method under current investigation of serum P, 17P and E_2 in buffalo.

Conclusion

1. Specificity of antiserum S-257#2, S-49#6 and S-52#.5 were investigated significant specific to P, 17P and E_2 respectively.

2. The purification step by chromatographic Celite microcolumn is needed for simultanously measurement of these three steroids.

3. The precision under within assay and between assay were 8.2 % (of P and 17P), 8.3 % (of E_2) and 13.2 % (of P), 11.2 % (of 17P), 11.2 % (of E_2) respectively.

4. The smallest amounts of both P and 17P which can be detected accurately were 50 pg or 125 pg/ml, and also being the same with 25 pg or 50 pg/ml of E_2.

5. Precentage recovery of standard P, 17P and E_2 were ranging between 72.9 - 89.6, 77.0-97.0 and 85.5-101.3 respectively. And the mean recovery of this three labelled steroids in 30 samples were 77.5 $\pm$ 4.7 % , 79.5 $\pm$ 6.1 % and 68.2 $\pm$ 4.3 % respectively. While the actual recovery is three times less than this.

6. The sensitivity of P, 17P and E_2 vary from 3.5-25.0 pg, 4.0-25.0 pg and 2.5-10.0 pg respectively.

7. The results indicate that it is valid to use this method for currently measurement of these three steroids in swamp buffalo during estrous cycle.

References

*Abraham, G.E., R. Swerdloff, D. Tulchinsky, and W.D. Odell: Radioimmunoassay of plasma progesterone. J. Clin. Endocrinol. Metab. 32:619, 1971.

**Abraham, G.E. R.S. Swerdloff, D.Tulchinsky, K. Hopper, and W.D. Odell: Radioimmunoassay of plasma 17-hydroxyprogesterone. J. Clin. Endocrinol. Metab. 33:42, 1971.

Abraham, G.E.,K. Hopper, D. Tulchinsky, R.S. Swerdloff, and W.D. Odell: Simultaheous measurement of plasma progesterone, 17-hydroxyprogesterone, and estradiol-17β by radioimmunoassay. Analyt. Letters 4:325, 1971.

Fadzil, M.: The Influence of Age and Season on the Interval between Parturition and Conception in Malayan Swamp buffaloes. Kajian Vet. 2:64, 1969.

Hafez, E.S.E. : Oestrus and Some Related Phenomena in buffalo. J. Agric. Sci. 44:165, 1954.

Ivanov, P. and S.J. Sachariev : Biologische Eigenschaften und Wirtschaftlichkeit der Büffel in Bulgarien. Z. Tierz. Zücht. Biol. 74:340 (A.B.A., 29:400), 1960.

Kaleff, F. : Der Hausbüffel und seine Züchtungcbiologie in Vergleich ZUM Rind. Z. Tierz. Zücht. Biol. 51:131, 1942.

Toelihere, M.R. : Contribution to Biology and Pathology of Reproduction in Buffaloes in Indonesia. Research Report. Bogor Agric. Univ. 1974.