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EVALUATION OF IMMUNOASSAY METHODS FOR PROLACTIN IN BODY FLUIDS

D. A. DILLEY, FIRST LIEUTENANT, USAF

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The experiments reported herein were conducted according to the "Principles of Laboratory Animal Care" established by the National Society for Medical Research.

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FOREWORD

This research was performed at the Toxic Hazards Division, Aerospace Medical Research Laboratories, Wright-Patterson Air Force Base, Ohio. The experiment was in support of project 6302, "Toxic Hazards of Propellants and Materials," tasks 630202, "Pharmacology-Biochemistry" and 630206, "Toxicological Support." D. A. Dilley, First Lieutenant, USAF was the principal investigator. The work was begun 16 August 1965 and was terminated 1 July 1966.

The author wishes to thank A. A. Thomas, MD, Major F. R. Robinson, K. C. Back, PhD, Major D. B. Gisler, Major R. F. Ziegler, Mr. S. F. Brokeshoulder, S. A. London, PhD, and his staff, Staff Sergeant J. G. King, III, Airman Second Class W. A. Knight, and Airman First Class D. B. Jones for their help on various stages of this project.

This technical report has been reviewed and is approved.

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ABSTRACT

A practical immunologic assay to detect the hormone, prolactin, in body fluids was evaluated. Prolactin content was assayed using positive antiprolactin sera prepared in rabbits and test samples of serum, urine, and urinary extract from a borane exposed female monkey in micro-Ouchterlony gel diffusion and agglutination inhibition tests. The gel diffusion test gave qualitative results while the agglutination inhibition test allowed quantitation of prolactin content. Results of the later test indicated a possible difference in pre- and postborane exposure prolactin content of monkey urine. Further work in this area will need to be done to clarify this preliminary evidence and to expand on its applicability to human exposure to boranes.

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SECTION I

INTRODUCTION

Detection and quantitation of specific serological factors in body fluids induced through stress are used in the diagnosis of many clinical syndromes (Dubos, 1965). In this laboratory female monkeys (Macaca mulatta) exposed to borane-containing fuels have lactated following exposure. The hypothesis has been made that the lactation was mediated by increased prolactin levels in the serum. Therefore, an assay to measure increased amounts of prolactin in body fluids (i.e., serum or urine) would be of benefit in determining levels of borane exposure. The feasibility of quantitating prolactin levels in serum and urine following exposure to decaborane by selected immunological assay methods was investigated.

Primates secrete a hormone with growth hormone (HGH) and prolactin (CGP)-like activity that is different from pituitary growth hormone as determined by physiochemical and biologic tests (Cohen, Grumbach, and Kaplan, 1964). Hayashida (1961) cites several investigators who have provided evidence that CGP and HGH are two different molecules. However, CGP and HGH appear to have certain similar antigenic groups as determined by immunologic cross reactions in serological test systems (Cohen, Grumbach, and Kaplan, 1964; Josimovich and Brande, 1964; Grumbach and Kaplan, 1964; Sciarra, Kaplan, and Grumbach, 1963).

Josimovich and Brande (1964) state that bovine CGP and bovine growth hormone (BGH) can be separated chemically and that their physiologic effects are clearly distinct and separate. Primate pituitaries seem to produce peptides with common lactogenic or BGH properties.

Gonadotropin, HGH, and prolactin in the urine or serum of a test subject can be demonstrated by a bioassay method. One method is dependent on the increase in crop (ingluvies) weight in pigeons (Hayashida, 1961). The increase in crop weight is reported to be proportional to the amount of hormone (prolactin or gonadotropin) present in the test sample of either urine or serum.

Another common hormone bioassay method is based on a proportional increase in mouse uterine weights due to prolactin or gonadotropin levels in test samples (Keller and Rosenberg, 1965).

Investigators have detected increased amounts of HGH and CGP in blood and urine of pregnant women (Grumbach and Kaplan, 1964). Recent advances in immunologic techniques have opened new frontiers in testing for hormone and sera factors associated with pregnancy and various other hormone mediated conditions (Islami et al., 1964). Precipitin tests

(Yakulis and Heller, 1959), fluorescent antibody tests (Sciarra, Kaplan, and Grumbach, 1963), agglutination inhibition (Read, 1960), complement fixation (Brody and Carlstrom, 1961), and latex agglutination (Islami et al., 1964) have been successfully adapted for hormonal assays.

In 1960, Read and Bryan modified Boyden's protein coated-tanned erythrocyte agglutination technique and produced a more sensitive means of detecting antigen-antibody reactions. They were able to determine species differences in growth hormones. Read et al. (1961) refined the tanned erythrocyte agglutination test of Boyden (1951) and observed that occasionally during incubation the settling cells would adhere to the sides of the tube. This type of distorted settling pattern can be caused by the effects of auto-agglutination of aged cells, and cellular aging which causes the cells to resuspend poorly (Read et al., 1961). These investigators used formalin treated sheep erythrocytes in this test. This agglutination test was reported to have a sensitivity of 0.004 μgm HGH/ml. At this time some discrepancy was also reported to exist between the results of bioassay techniques and the immunochemical methods.

Hayashida (1961) used the pigeon crop assay method in his assay for prolactin and found that the maximum sensitivity was 0.026 μgm of prolactin.

An important immunologic method which has been used is the Ouchterlony gel diffusion test (precipitation of antigen-antibody in a gel). Grumbach et al. (1960), using this method, produced high antiCGP titers in rabbits with a 10 mgm dose. They analyzed their sera and found 10^{-2}mg/ml by the quantitative precipitin method.

The width of a precipitin band in the Ouchterlony gel diffusion test of Grumbach and Kaplan (1961) varied with the antigen concentration when the central well contained undiluted standard antisera. In this work, the line of precipitate curved towards the antisera well suggesting that the molecular weight of the antibody was greater than that of the antigen.

Growth hormone differences between species of primates have been noted (Tashjian et al., 1965). Certain immunological similarities between three nonprimate growth hormones have also been detected. A microcomplement fixation procedure used by these investigators demonstrated porcine, bovine, and ovine growth hormone reactions with antihuman growth hormone sera. This led to a hypothesis that antigenic determinants of the various growth hormones are similar if not identical. Structural differences in growth hormone from different species have been demonstrated by physical and chemical methods.

Brody and Carlstrom (1961) state that protein hormones are comparatively weak antigens and for this reason the adjuvant technique has been adopted for the preparation of good quality antisera.

Antibodies to purified-growth hormone preparations have been obtained from rabbits by a number of investigators (Wallace and Sobey, 1965). In spite of structural differences, ovine and bovine growth hormone apparently are immunologically identical (Hayashida and Li, 1958). Hayashida and Li (1958) state that the quantities of prolactin or prolactin-like antigen in the growth hormone preparations are sufficient to cause precipitins for prolactin.

Hipkin (1965) has been able to demonstrate that antihuman chorionic gonadotropin (HCG) serum contains factors specific for inert urinary and serum protein as well as the hormone. He compared bioassay and immunoassay techniques and found discrepancies. Immunologic data indicates the apparent absence of positive correlation between immunoassay and bioassay results (Hipkin, 1965 and Brody and Carlstrom, 1961). Hipkin (1965) had false positive results in his immunologic assay for CGP in urine because of interference by inert urinary protein. It was his conclusion that bioactivity and antigen specificity are probably independent of each other. As a result of his work, HCG estimated by immunologic methods during pregnancy is apparently biologically inactive. Hipkin stated that antiHCG sera gives accurate results for serum levels for HCG and CGP hormones but that the urinary assay using the same antisera may not be reliable.

Our experiment was carried out as a pilot study to compare the modified micro-Ouchterlony gel diffusion test and the tanned sheep erythrocyte agglutination inhibition test, and to determine whether the sensitivity demonstrated by these tests could lead to a qualitative analysis and quantitation of prolactin in unknown test sera which would be superior to existing bioassay methods.

SECTION II

MATERIALS AND METHODS

IMMUNIZATION

Twelve young adult male 2-1/2 kg albino rabbits were used. Four were injected according to the method of Grumbach and Kaplan (1961) with a Freund's complete adjuvant-saline-prolactin emulsion containing a total of 2.048 mg of prolactin. Controls consisted of 4 rabbits which were sham injected with equivalent volumes of sterile saline, and 4 rabbits were injected with Freund's adjuvant (FA).

The prolactin used for the injections was lyophilized NIH-P-S 6 (ovine) with a mean potency of 24.8 IU/mg.* The prolactin was reconstituted in Sorensen's PO_4 buffer pH 7.3 and 1/10,000 merthiolate added as a preservative. The final concentration of the stock prolactin was 2.56 mg/ml. Intradermal injections were made into the foot pads of the rabbits with 1.0 ml disposable tuberculin syringes and 5/8 inch 26-gauge needles. The paw was clipped prior to injection, and bottle tops and the injection area prepared with 70% alcohol.

Aliquot samples of unknown urine, urinary extract, and serum were obtained from a borane exposed monkey and a "blind sample" was also tested. This material was obtained from the Toxicology Branch of this laboratory and had been used in bioassay tests.**

SAMPLE COLLECTION AND TREATMENT

Prior to blood collection each rabbit was anesthetized with ether and the chest was clipped and washed with 70% alcohol. At 2-week intervals over a 12-week period, 5 ml blood samples were obtained from all animals by the cardiac puncture technique and sera separated. Disposable 5-ml syringes and 18-gauge 1-1/2 inch needles were used for blood collection. Eight weeks after the first injection all animals were sacrificed by exsanguination while under ether anesthesia and the blood collected.

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* Prolactin was obtained at no cost to the Air Force from the National Institutes of Health, Endocrinology Study Section, Washington, D.C.

** Ziegler, R.F., Bioassay of Borane Exposed Monkey Urine, 1966, Unpublished data.

Packed cell volumes were determined at each collection. The micro-hematocrit tubes were centrifuged for 4 minutes at 2400 rpm in an International Clinical Centrifuge and then read by the standard method. The blood for sera collection was allowed to clot for 1 to 2 hours at room temperature after which the tubes were rimmed, centrifuged, the raw sera removed, divided into 0.8 ml aliquots in plastic technicon cups, and placed in a low temperature freezer at -70C for storage.

SEROLOGICAL TESTING

Micro-Ouchterlony gel diffusion tests and a modification of the tanned sheep erythrocyte agglutination test (Read, 1960) were carried out (see Appendix). These tests were made to determine how much antiprolactin activity was present in the sera of the immunized animals. The micro-Ouchterlony gel diffusion test was used as a qualitative spot check while the tanned sheep erythrocyte agglutination test served as both a qualitative and quantitative analytical test. After standardization of the two above tests, samples of urine, serum, and urinary extract from the borane exposed monkey was tested by the micro-Ouchterlony method. An unknown prolactin standard, urine and urinary extract were also tested by the modified agglutination inhibition method of Read (1960).

SECTION III

RESULTS

Packed cell volume determinations carried out at the time of each sample collection, over the twelve week schedule, revealed no difference between the prolactin immunized rabbits and the controls. All animals injected with Freund's adjuvant developed necrosis at the injection site.

Micro-Ouchterlony test results on the 8-week post immunization sera samples are in tables I, II, III, and IV. Results of these tests showed that the saline and Freund's adjuvant control animals did not produce detectable precipitins specific for prolactin while 2 out of 3 animals injected with ovine prolactin exhibited a serum factor which produced a single precipitin line when reacted with the antigen in the agar (gel), and appeared to be specific for prolactin (see table II).

TABLE I

RESULTS OF MICRO-OUCHTERLONY TEST TO DETERMINE THE PROPER DILUTION OF 8-WEEK POSTINJECTION SERA FOR THE MICROGEL DIFFUSION TEST

mg/ml Concentration of Prolactin in PO_4	RAW SERA					
	Saline Injected			Prolactin Injected		
	u	1:2	1:4	u	1:2	1:4
1.6	-	-	-	+	+	-
0.8	-	-	-	+	+	-
0.4	-	-	-	+	+	-
0.2	-	-	-	+	+	-
0.0	-	-	-	-	-	-

u = Undiluted
+ = Precipitin reaction
- = No visible precipitin reaction

TABLE II

RESULTS OF MICRO-OUCHTERLONY TEST TO DETERMINE THE SPECIFICITY OF THE SERUM FACTOR APPEARING AFTER THE INJECTION OF PROLACTIN IN 8-WEEK POSTINJECTION UNDILUTED RAW RABBIT SERA

	RAW SERA							
	Saline Injected			Freund's Adjuvant Injected		Prolactin Injected		
Antigen	Rabbit A	B	C	D	E	F	G	H
Saline	-	-	-	-	-	-	-	-
Freund's Complete Adjuvant Undiluted	-	-	-	-	-	-	-	-
Prolactin - FA Undiluted 2.5 mg/ml	-	-	-	-	-	+	-	+

+ = Precipitin reaction
- = No visible precipitin reaction

TABLE III

RESULTS OF MICRO-OUCHTERLONY TEST TO DETERMINE THE ACTUAL LIMITS OF PROLACTIN DETECTION USING 8-WEEK POSTINJECTION SERA

mg/ml Concentration of Prolactin	RAW UNDILUTED RABBIT SERA			
	Saline Injected		Prolactin Injected	
	A	B	F	H
(in PO_4)				
2.56	-	-	+	+
1.28	-	-	+	+
0.69	-	-	+	+
0.35	-	-	+	+
0.18	-	-	+	+
0.09	-	-	+	+
0.05	-	-	+	+
(in 1/80 NGPS)				
1.6	-			+
0.8	-			+
0.4	-			+
0.2	-			+
0.1	-			+
0.05	-			+
0.025	-			+
0.013	-			+
0.0016	-			+
0.0010	-			-
0.0008	-			-
0.0007	-			-

+ = Precipitin reaction
- = No visible precipitin reaction

TABLE IV

RESULTS OF MICRO-OUCHTERLONY TEST TO DETERMINE PRESENCE OF PROLACTIN IN URINARY EXTRACT, SERA, AND UNCONCENTRATED URINE FROM A FEMALE MONKEY EXPOSED TO DECABORANE

	RAW UNABSORBED RABBIT SERA 8-WEEK POSTINJECTION (UNDILUTED)	
Antigen	Saline Injected A	Prolactin Injected H
Preexposure		
Extract Undiluted 1:2, 1:4, 1:8	-	-
Sera Undiluted 1:2, 1:4, 1:8	-	-
Urine Undiluted 1:2, 1:4, 1:8	-	-
Prolactin Standard		
0.2 mg/cc	-	+
0.1 mg/cc	-	+
0.05 mg/cc	-	+
Postexposure		
Extract Undiluted 1:2, 1:4, 1:8	-	-
Sera Undiluted 1:2, 1:4, 1:8	-	-
Urine Undiluted 1:2, 1:4, 1:8	-	-

+ = Precipitin reaction
- = No visible precipitin reaction

The raw, (untreated) undiluted and the raw 1:2 diluted positive serum produced excellent results in the microgel diffusion test (see table I).

Results shown in table III demonstrate that the sensitivity of the micro-Ouchterlony determination is 0.0016 mg/ml of 1/80 CGP-ovine in normal guinea pig serum (NGPS) and that no apparent difference was caused by suspending the prolactin in 1/80 inactivated-absorbed normal guinea pig serum as compared to Sorensen's phosphate buffer (PO_4) pH 7.3 (see details of procedure in appendix). The upper limits of detection of this test were not firmly established and the best definite precipitin lines occurred between the undiluted positive serum well and the 0.2, 0.1, and 0.05 mg/ml concentrations of prolactin (see figures 1, 2, 3, and 4).

Prolactin was not detected in either the urine, or sera of a monkey exposed to decaborane by the micro-Ouchterlony method (table IV). This indicated that the prolactin concentration was probably less than 0.0016 mg/ml.

Table V contains the results of the modified tanned sheep erythrocyte agglutination test carried out to standardize the known positive sera for quantitation procedures. A low agglutination titer was observed in the saline injected animal, but it was significantly less than that obtained from the prolactin injected animals. No serum factors specific for the treated cells could be detected in Freund's adjuvant control group. A 1:128 titer was the highest obtained from any serum sample tested.

The results from table VI indicate that we were able to show a quantitative difference in prolactin concentrations by the agglutination inhibition method. In this test all positive and negative controls reacted properly.

In all cases the titers obtained from the immunized animals were not as high as those reported by other investigators.

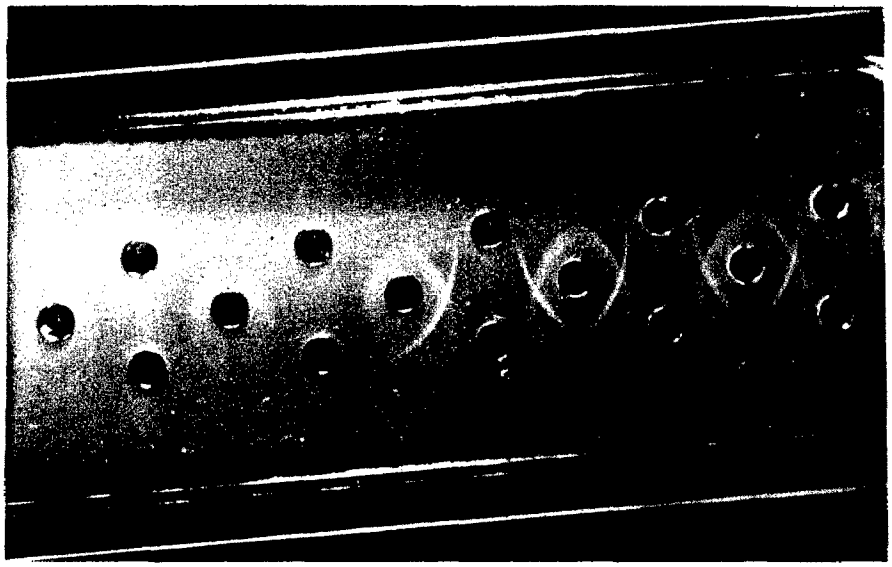

Figure 1. A Typical Micro-Ouchterlony Gel Diffusion Plate Testing the Antiprolactin Activity of 8-Week Postinjection Raw Undiluted Rabbit Sera Against Various Concentrations of Prolactin in 1/80 NGPS. Row A: From left to right contain prolactin 0.8, 0.4, 0.2, 0.1, 0.05 mg/ml concentration. Row B: From left to right contain prolactin immunized undiluted rabbit serum (H). Row C: From left to right contain prolactin 0.013 mg/ml, PO_4 buffer, and goat anti-rabbit sera in last three wells.

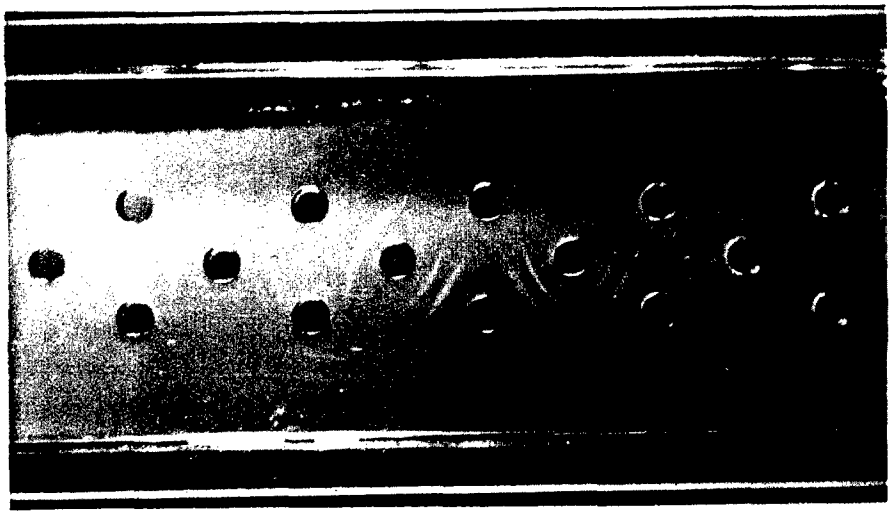

Figure 2. A Typical Micro-Ouchterlony Gel Diffusion Plate Testing the Pre- and Postborane Exposure Urinary Extract for Prolactin. Row A: From left to right contain prolactin 0.1 mg/ml, 0.05 mg/ml, preexposure extract undiluted, 1:2 and 1:4. Row B: From left to right contain prolactin immunized undiluted rabbit serum (H). Row C: From left to right contain postexposure extract 1:2, 1:4, and goat antirabbit sera in last three wells.

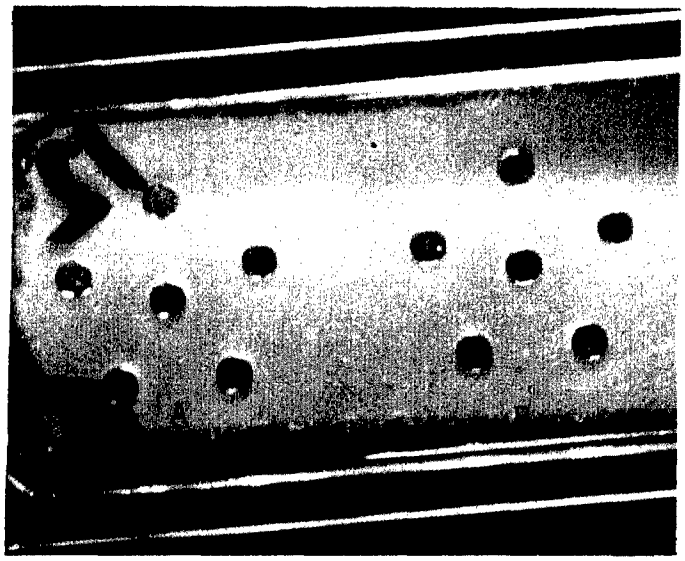

Figure 3. A Typical Micro-Ouchterlony Gel Diffusion Plate Testing Saline Injected Rabbit Serum for Antiprolactin Activity. Central wells contain normal rabbit sera. Group A: (clockwise from 7 o'clock) prolactin 0.1 mg/ml, post-exposure serum undiluted, 1:2, 1:4, and 1:8. Group B: (clockwise from 7 o'clock) prolactin 0.05 mg/ml, postexposure urine, undiluted, 1:2, 1:4, and 1:8.

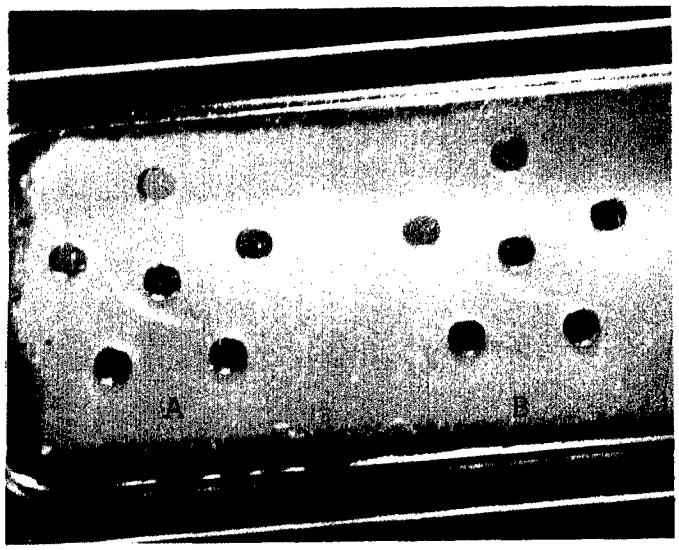

Figure 4. A Typical Micro-Ouchterlony Gel Diffusion Plate Using the Prolactin Positive Antiserum to Test for Prolactin in Monkey Urine and Serum After Borane Exposure. Central wells contain prolactin immunized undiluted rabbit serum. Group A: (clockwise from 7 o'clock) prolactin 0.1 mg/ml, post-exposure serum undiluted, 1:2, 1:4, and 1:8. Group B: (clockwise from 7 o'clock) prolactin 0.05 mg/ml, post-exposure urine undiluted, 1:2, 1:4, and 1:8.

TABLE V

RESULTS OF TANNED SHEEP ERYTHROCYTE AGGLUTINATION TEST TO DETERMINE THE LEVEL OF ANTIPROLACTIN FACTOR IN TEST RABBIT SERA

	INACTIVATED-ABSORBED SERA					
Weeks Post-injection	Saline Injected		Prolactin Injected			
	A	B	F	G	H	I
0	-	NT	NT	NT	-	NT
+2	-	NT	NT	NT	1:4	NT
+4	1:4	NT	NT	NT	1:64	NT
+6	1:4	NT	NT	NT	1:128	NT
+8	1:8	NT	NT	NT	1:64	NT
+4	-	-	1:32	1:30	1:32	-
+8	-	-	1:24	NT	1:48	NT

- = No agglutination occurred at any titer
1:4, 1:8, etc = Highest dilution (titer) at which agglutination occurred
NT = Not tested

TABLE VI

HIGHEST TITER OBTAINED IN TANNED SHEEP ERYTHROCYTE AGGLUTINATION INHIBITION TEST FOR PROLACTIN

Mg Prolactin/ml 1/80 NGPS Used to Absorb Test Sera	Saline Injected	Prolactin Injected
1.6	-	-
0.8	-	-
0.4	-	-
0.2	-	-
0.1	-	1:2
0.05	-	1:4
0.025	-	1:4
0.013	-	1:8
0.0	1:2	1:32
Urine Samples Used to Absorb Test Sera		
Preexposure urine	-	1:16
Postexposure urine	-	1:8
Extract of postexposure urine	-	1:8
Blind (0.1 mg/ml) PO_4 *	-	1:2

- = No agglutination occurred at any titer
* = Concentration of blind was not known until after the agglutination inhibition test had been completed.

SECTION IV

DISCUSSION

Our preliminary work to develop a practical immunoassay procedure for determining prolactin levels in body fluids indicates feasibility. Additional work must be done to determine whether the inhibition observed in urine was due to prolactin or some biologically inert urinary protein with similar immunological specificity. The quantities of reactants used in this determination are consistent with those of Holm (1965) who used 10 μl quantities in his micro-Ouchterlony gel diffusion test.

The fact that only one band of precipitate was formed between the sera from immunized rabbits and that ovine prolactin used as antigen indicates that a definite specificity exists but does not entirely cope with the nonspecific urinary protein theory expressed by Hipkin (1965). Further work must be done in this area to make this differentiation.

The micro-Ouchterlony test is apparently qualitative and at best semi-quantitative. The agglutination inhibition test, while more complex, seems to have the best potential as a quantitative test. All fluids or sera which may contain complement should be inactivated to prevent hemolysis of the indicator system. Apparent inconsistencies between the bioassay and immunoassay methods of hormone detection leaves the door open for improved detection procedures. Bioassay requires relatively large numbers of pigeons or mice and several days to complete the assay, while the immunological approach requires only one day, previously prepared vials of sera, and an indicator system (latex particles or tanned erythrocytes) for the agglutination inhibition test. No indicator system is used in the micro-Ouchterlony gel diffusion test which requires 12-36 hours to complete. The immunologic approach appears to be the more practical of the two, at this point, for detecting prolactin in body fluids.

Further work also should be done to incorporate the use of latex particles or formalin treated erythrocytes as the indicator systems of choice in the agglutination test. This would enable a standard indicator system to be developed and thus eliminate one of the inherent weaknesses of the agglutination inhibition test.

The fact that lower titers were obtained from our immunization schedule than were reported by Grumbach and Kaplan (1961) caused some concern. Several factors such as the quality of the prolactin used to immunize the rabbits and the type and quality of rabbit used could have produced this variance. Our rabbits were the same size and weight as Grumbach's. Also the method of reading the test could have caused apparent differences in immunological response. However, our results were consistent from day to day indicating validity of our test system.

APPENDIX

IMMUNOASSAY METHODS AND MATERIAL

I. Sorensen's Phosphate Buffer Preparation (PO_4):

(Hawk, Oser, and Summerson, Practical Physiological Chemistry, 13th Edition, The Blakeston Company, Inc., New York, 1954).

Solution A: M/15 anhydrous KH_2PO_4 Mwt. 136 (9.078 grams)
Solution B: M/15 Na_2HPO_4 Mwt. 142 (9.469 grams)
pH Solution A: 4.6
pH Solution B: 9.18
Stock Sorensen's Solution pH 7.3, 6.8, and 6.3

II. Micro-Ouchterlony Gel Diffusion Plates:

The micro-Ouchterlony plates were purchased from the Hyland Laboratories of Los Angeles, California. The rectangular, disposable, polystyrene dishes with snap on lids, have a 1 x 3 inch cavity which contains 4 ml of agar with the following composition:

Difco Special Noble Agar	0.2%
Glycine	7.5%
Sodium Chloride	1.0%
Sodium Azide	0.1%
pH	7.0-7.2%

The wells in these plates were empirically determined to have a capacity of 10 μl.

III. Micro-Ouchterlony Gel Diffusion Test Procedure:

A. Thaw the raw sera.

B. Make appropriate sera and antigen dilutions using serologically clean equipment.

C. Dispense reagents using a rubber eye dropper end and 5-3/4 inch "Dispopet" obtained from Scientific Products Company.

D. Place 10 μl of reactants in the desired wells.

E. Seal plate and incubate 12-48 hours at 37C in a moist environment and then 12 hours at 4C.

F. Read plates by using a view box with indirect light or hold them parallel to the light ray of a fluorescent lamp.

IV. The needles used were 5/8" and 1-1/2" Yale disposable. The disposable 1.5, 10, and 20 cc syringes were purchased from the Becton, Dickinson & Company of Rutherford, New Jersey.

V. The Complete Freund's Adjuvant used in the immunization was CS-145 Lot 4, produced by the Colorado Serum Company, Denver, Colorado.

VI. The normal Guinea Pig Sera (NGPS) (Lot No. 27740) used for the Tanned Sheep Cells Agglutination and Prolactin Standard solutions was obtained from the Courtland Laboratories, Los Angeles, California.

VII. Goat Antirabbit Sera was obtained from Pentex Inc., Kankakee, Illinois.

VIII. Tannic Acid:

Baker Tannic Acid (Lot No. 30523) was obtained from the J. T. Baker Chemical Company, Phillipsburg, New Jersey.

IX. A. Tannic Acid:

1 gm Baher's Analyzed Reagent/100 ml double distilled water, refrigerated until used (1:100) in a brown bottle. The Tannic Acid was made up within 3 days of the test.

For the tanned sheep erythrocyte test dilute the 1:100 tannic acid immediately before use to 1:20,000 with PO_4 saline pH 7.2.

B. Treatment of Sera before use:

1. Heat in water bath at 56C for 30 minutes.

2. Absorb with 3 times washed (in PO_4 saline) with normal aged sheep erythrocytes and 1 ml packed cells/1 ml serum at 37C for 30 minutes.

Sera are stored in refrigerator at 4C and diluted with 1/80 NGPS just prior to use. Sera for assay is kept frozen until the assay is performed.

C. Preparation of Hormone-conjugated Erythrocytes (Modified Method of Read, 1960):

1. Eight ml sheep erythrocytes in ACD "B" are aged 7-10 days following bleeding.

2. After aging, centrifuge at 2500 rpm for 10 minutes in a clean 12 ml graduated centrifuge tube.

3. Withdraw supernatant and resuspend cells in 8 ml of 0.9% saline and repeat 3 times. Don't shake cells too vigorously. On occasion, a glass stirring rod may be used gently to break up tightly packed cells.

4. After final wash, resuspend packed cells to a 10% concentration.

5. The 1.5 ml of the 10% suspension (4.) is placed in a clean tube containing 4.5 ml of 0.9% saline giving a 6 ml 2.5% concentration of the washed erythrocytes.

D. Add 6 ml tannic acid (1:20,000) in pH 7.2 buffered saline to each 6 ml of the washed 2.5% suspension of the sheep erythrocytes.

Mix well.

Incubate at 37C for 10 minutes.

Centrifuge at 2,000 rpm for 10 minutes, discard supernatant and leave a solid button of cells.

Wash cells 3 times with 6 ml of pH 6.3 Sorensen's buffered saline. Centrifuge at 2,000 rpm for 5 minutes.

After final wash, resuspend the cells in 6 ml of pH 6.3 Sorensen's buffered saline.

E. Dilute prolactin solution with a concentration of 1 mg/ml to 1.6 mg/ml to 6 ml with buffered saline pH 6.3.

Add to the cell suspension from 4 in a 1:1 ratio and incubate at 37C for 15 minutes in a water bath. Agitate at 5 and 10 minutes.

Centrifuge the coated tanned erythrocytes 2,000 rpm for 10 minutes and withdraw supernatant carefully.

F. Wash the coated cells 3 times in a 1/80 treated normal guinea pig serum and resuspend in the same medium (NGPS).

Before the final washing the cell suspension is transferred to a graduated centrifuge tube, centrifuged 10 min/2,000 rpm, the packed cell volume read and 1/80 NGPS added to give a 2.8% cell concentration.

G. PO_4 buffers are not used in the procedure at any point after the incubation of the hormone with the tanned sheep erythrocytes.

H. Normal Guinea Pig Sera Treatment:

1. Reconstitute normal guinea pig sera.

2. Let stand for 1 hour to stabilize.

3. Heat 56C for 30 minutes.

4. Absorb the inactivated sera with washed Normal Sheep Red Blood Cells in a 1:1 ratio.

5. Freeze in 1 dram screw top glass vials at -70C.

6. Just prior to using thaw at 37C and dilute 1/80 with 0.9% NaCl as needed.

X. Prolactin Determination Detection and Titration of Antisera:

A. Set up doubling dilutions ranging from undiluted to 1:512 in 12 x 75 mm test tubes (clean) with 1/80 reconstituted NGP serum as diluent to a total volume of 0.4 ml.

B. Add 0.05 ml of the coated cells to each tube, the final volume being 0.45 ml.

C. Set up appropriate controls:

1. Add 0.05 ml of 2.8% suspension of washed normal sheep erythrocytes to 0.4 ml of 1:4 dilution of the antiserum.

2. Add 0.05 ml of 2.8% suspension of washed tanned sheep erythrocytes to 0.4 ml of 1:4 dilution of the antisera.

3. Add 0.05 ml of 2.8% suspension of washed normal sheep erythrocytes to 0.4 ml of a 1/80 dilution of NGPS.

4. Add 0.05 ml of 2.8% tanned sheep erythrocytes to 0.4 ml of a 1/80 dilution of NGPS.

5. Add 0.05 ml of 2.8% suspension of the conjugated tanned sheep erythrocytes to 0.5 ml of a 1/80 dilution of NGPS.

D. Shake all tubes gently to disperse the cells evenly and incubate at 37C for 30 minutes.

E. Spin down 15 seconds in Clay-Adams serafuge or International Clinical bench top model. Then read for agglutination by the method of Dilley (1965).

F. Determine the titer of the antisera by observing the highest dilution that produces (a one plus) hemagglutination of the cells.

G. Record the data.

XI. Quantitation of Hormone in Unknown Samples of Body Fluids (modified method of Read, 1960):

A. The inhibitory effect of prolactin on antiserum.

1. Dilute the prolactin with 1/80 NGPS to: 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, and 0.013 mg/ml. Cover and refrigerate. The solutions will remain stable for several months at 2-4C.

2. Place 0.20 ml of each standard in a series of 75 x 100 mm tubes. Add 0.20 ml of diluted antisera giving a final volume of 0.4 ml, and a final concentration of the hormone equal to one-half of the original standards.

3. The previously prepared cells are centrifuged, resuspended in 1/80 NGPS and added to the hormone-antiserum mixture as per the titration of antisera.

4. Shake the tubes and incubate at 37C for 30 minutes.

5. Read and record the data.

B. Quantitative determination of hormone in unknown test urine or serum:

1. Substitute test inactivated serum or urine for the known standards in part A. Dilute the standard test serum with 1/80 NGPS to 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128.

2. Dilute 0.20 ml of the antiserum to the same extent as in Part A, producing a final volume of 0.4 ml.

3. Set up appropriate controls.

4. Add 0.05 ml of the proper indicator system to each tube (tanned sheep erythrocytes, tanned-conjugated sheep erythrocyte and normal sheep erythrocytes).

5. Incubate and read as above.

6. Measure simultaneously the tubes in part A and B, with part A serving as a control for part B.

7. The amount of inhibition of agglutination when compared with that produced by the standards gives the quantitation of prolactin in the unknown fluid.

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13. ABSTRACT

A practical immunologic assay to detect the hormone, prolactin, in body fluids was evaluated. Prolactin content was assayed using positive antiprolactin sera prepared in rabbits and test samples of serum, urine, and urinary extract from a borane exposed female monkey in micro-Ouchterlony gel diffusion and agglutination inhibition tests. The gel diffusion test gave qualitative results while the agglutination inhibition test allowed quantitation of prolactin content. Results of the later test indicated a possible difference in pre- and postborane exposure prolactin content of monkey urine. Further work in this area will need to be done to clarify this preliminary evidence and to expand on its applicability to human exposure to boranes.

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