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Immunologic Pregnancy Tests

Evaluation of Pregnosticon Dri-Dot and Pregnosticon Accuspheres

EMMET J. LAMB, MD, FACOG

Urine specimens from 50 pregnant and 50 nonpregnant women were tested with five immunologic pregnancy tests. Pregnosticon Accuspheres, a new hemagglutination inhibition test, was found to be more convenient when compared with UCG, an established tube test. Compared with two established rapid slide tests, Pregnosticon and Prequest, Pregnosticon Dri-Dot, a new latex agglutination inhibition test, was the most convenient to use, had an endpoint that could be detected easily and an acceptable degree of accuracy equal to the Pregnosticon Slide Test from which it was derived. The latter would be our choice as a pregnancy test for routine office use.

IMMUNOLOGIC TESTS for human chorionic gonadotropin (HCG) are popular and practical pregnancy tests.[1-3] They are more sensitive, specific and convenient than biologic tests in general use before 1960. Since little formal training is needed to use the immunologic test kits now available, many pregnancy tests are performed in physicians' offices rather than in hospital or clinical pathology laboratories. This is a report of our evaluation of two new reagent kits designed to provide the greatest possible convenience in use, Pregnosticon Accuspheres and Pregnosticon Dri-Dot.

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MATERIALS AND METHODS

Selection of Subjects

Urine specimens used for this study were submitted to the gynecology laboratory because a pregnancy test was clinically indicated; none were collected specifically for this study. An aliquot of each was stored at −20°C until clinical records had been reviewed 8–12 weeks later. Approximately half of the urine specimens tested were positive for human chorionic gonadotrophin (HCG). Those selected for study had been submitted by 50 pregnant and 50 nonpregnant women. With the following exceptions, they were consecutive specimens received by the laboratory: a) subsequent specimens from the same patient were not tested, and b) no urine specimen was tested if supplementary clinical information or followup data in the medical record were not suffcient to establish with, reasonable certainty, whether or not the patient had been pregnant at the time the urine was obtained. The technician performing the tests was not aware of the clinical information.

Principle of the Tests

All tests for HCG used in this study are based on the same principle: inhibition of agglutination of indicator particles, either

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sheep red blood cells or polystyrene latex particles. Two reagents are used: a) an aqueous suspension of red cells or latex particles the surface of which has been coated (sensitized) with purified HCG, and b) an anti-HCG serum derived from sensitized rabbit's serum. When the two reagents are mixed, the antiserum reacts with the HCG coated on the particle, causing agglutination. When performing the test, if urine containing sufficient HCG is added to the antiserum, urinary HCG neutralizes the antiserum, thereby inhibiting agglutination of the indicator particles. Agglutination is not blocked by urine from nonpregnant women, since HCG is not present and, therefore, cannot neutralize the antiserum.

Qualitative Tests

All urines were tested according to the manufacturer's recommendation, using each of the 5 reagent kits listed in Table 1. The sensitivity of the reagents was verified using serial dilutions of two freeze-dried urine samples containing 10,000 and 12,000 International Units (IU). All urines were tested for sugar and protein using Combistix reagent strips.

Semiquantitative Titers

If the qualitative test was positive, a semiquantitative titer was determined with the Accuspheres reagents. A urine diluent provided by the manufacturer was used for serial dilutions of urine to prevent the loss of HCG by adsorption on glassware. Twofold serial dilutions of the undiluted urine, a 1:10, 1:100 or 1:500 dilution, were tested. In tests with diluted urine, a phosphate buffer, prepared from a concentrated solution supplied by the manufacturer, was used instead of water to reconstitute the antiserum and red cell suspension in the Accusphere tubes. The buffer restores a proper ionic environment which, in the qualitative test, is provided by the undiluted urine. The last dilution with clearly discernible agglutination was recorded and the titer calculated.

RESULTS

Pregnant Patients

All five tests for urinary HCG were positive in 37 of the 50 pregnant patients. Of the 13 urine samples for which one or more of the tests were negative, 4 were from patients with threatened or missed abortion whose excretion of HCG is expected to be abnormally low (Table 2). Five other urine specimens had been obtained within 50 days of the last menstrual period—ie, at a time when HCG excretion is normally low. The test results with the remaining four urine specimens must be considered false negatives. The two new test kits, Pregnosticon Dri-Dot and Accuspheres, gave correct positive results with these and with all other urine samples from women with a normal

TABLE 1. SENSITIVITY OF THE REAGENTS USED IN THIS STUDY

Reagent	*Manufacturer*	*Sensitivity (IU/liter)*	
		Stated	*Observed*
Latex (slide)			
Prequest	Parke-Davis	5000	>5000 <6000
Pregnosticon	Organon	1000–2000	>1500 <2500
Dri-Dot	Organon	1000–2000	>1250 <1500
Red blood cell (tube)			
UCG	Wampole	1000	>375 <625
Pregnosticon Accuspheres	Organon	750	>750 <1250

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TABLE 2. NEGATIVE IMMUNOLOGIC TESTS FOR HCG: URINE FROM PREGNANT WOMEN

	Pregnancy test					
	RBC	Latex				
UCG	Accuspheres (IU/ml)	Prequest	Pregnosticon slide	Dri-Dot	Weeks	Followup
Spontaneous Abortion						
−	−	−	−	−	6	Aborted 2 weeks later; not bleeding at time of test
−	−	−	−	−	10	Missed abortion–Curettage
−	−	−	−	−	10	Missed abortion–Curettage
+	+(1.5)	−	+	+	11	Aborted 3 weeks later; bleeding at time of test
Early Pregnancy						
−	−	−	−	−	5	Clinically normal 7 weeks later
+	+(10)	−	+	+	6	Clinically normal 8 weeks later
+	+(10)	−	+	+	6	Clinically normal 7 weeks later
+	+(2.5)	−	+	+	6	Positive Prequest 2 weeks later
+	−	−	+	+	6	Positive Prequest 2 weeks later
False Negative						
+	+(1.5)	−	+	+	7	Clinically normal 7–12 weeks later
+	+(1.5)	+	−	+	7	Clinically normal 7–12 weeks later
−	+(15)	+	+	+	8	Clinically normal 7–12 weeks later
+	+(15)	−	+	+	18	Clinically normal 7–12 weeks later
5	5	11	5	4		TOTAL
10	10	22	10	8		NEGATIVE (%)
1	0	2	1	0		Normal pregnancy after 6 weeks
2	0	4	2	0		NEGATIVE (%)

pregnancy beyond 6 weeks. The other reagents had a 2–4% false-negative rate. As expected, the least sensitive (Prequest) had the highest number of negative results.

Among the pregnant patients were 7 with abnormal pregnancies. Each of the 4 who aborted within 8 weeks of the test had a negative result with at least one of the five tests (Table 2). Each of the 3 women with ectopic pregnancies excreted enough HCG at the time the urine specimen was obtained to have positive tests with all five reagent kits.

Semiquantitative titers were determined from serial dilutions of urine using Pregnosticon Accuspheres (Figure 1). The background shading in the figure depicts the normal range determined by Wide using a hemagglutination test on morning urine specimens. Many of the values in our sample are below this range, probably because most of the urine specimens were random samples, thus, less concentrated than a morning urine sample.

Nonpregnant Patients

All five tests were negative for HCG in 39 of the 50 urines obtained from nonpregnant patients. One or more of the reagent kits gave a positive test with urine specimens from 11 women (Table 3). Most of these patients were taking tranquilizers, corticosteroids or progestational agents. In 9, only one of the test kits gave a positive test which would indicate that these were false-positive tests. Considering the sensitivity of the slide tests and reported values for pituitary gonadotropin, it is not likely that the positive reactions are due to cross reaction with luteinizing hormone. Although it seemed unlikely when the record was reviewed before the results of the pregnancy tests were known, an early abortion cannot be abso-

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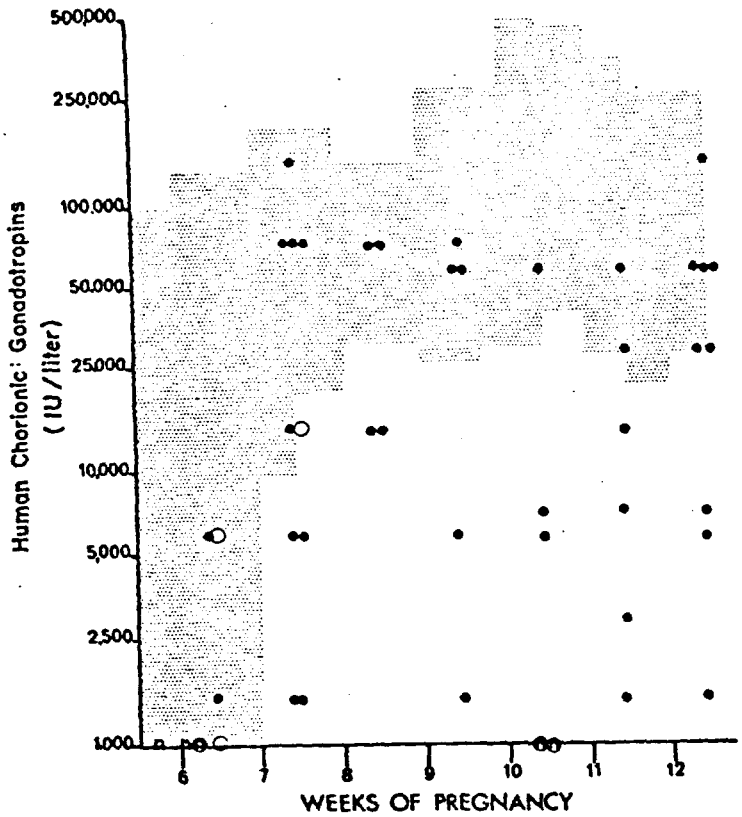

Fig 1. Semiquantitative titers during the first trimester of pregnancy as determined from random urine specimens using Pregnosticon Accuspheres. The shaded areas represent the fiducial limits of error ($P = 0.05$) of the immunologic HCG activity in morning urines from women with normal pregnancy as determined by Wide. Note log scale. Open circle = abnormal pregnancy; N = negative test, less than 1000 IU/liter.

lutely excluded in the 2 patients who had positive tests with more than one reagent kit.

DISCUSSION

Practicality

A new immunologic pregnancy test must be judged in terms of its use in a physician's office where only a few tests are done each day, usually one at a time, by someone without special laboratory skills. The ideal test should have a definite end point and be quick and easy to perform. The reagent kit should be self-contained and require a minimum of additional glassware, equipment or preparation of solutions.

The slide tests using latex particles have the major advantage of speed. The result is available while the patient is still in the office. Granular clumping of agglutinated latex particles is seen within 1 or 2 minutes when the slide containing the suspension of reagents and urine is rocked gently and observed under a good light against a black background (Figure 2). We noted no marked difference in ease of readability among the three slide tests used in this study, although a pale green dye added to the Prequest and Dri-Dot latex reagents aids slightly in detecting agglutination.

Without question, of the five reagent kits tested, the Pregnosticon Dri-Dot is the most convenient to use. The reagents are dried on a black cardboard slide and are brought into suspension with a drop of tap or distilled water. Only 30 seconds are required to redissolve the Dri-Dot reagents and allow the antigen-antibody reaction to occur. The reagents for the other slide tests are in aqueous suspension and must be refrigerated for storage.

Detection of agglutination of red cells in

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TABLE 3. POSITIVE IMMUNOLOGIC TESTS. URINE FROM NONPREGNANT WOMEN

Pregnancy test						
RBC		Latex				
UCG	Accuspheres (IU/ml)	Prequest	Pregnosticon slide	Dri-Dot	Drugs	Disease
−	+(0.7)	−	+	−	Narcotic Barbiturate Progestin	Proteinuria, pelvic infection
+	+(0.7)	−	−	−	None	Oligomenorrhea, bled after progesterone
−	+(1.5)	−	−	−	Corticosteroid Diuretic Tranquilizer	Autoimmune disease (vasculitis)
−	+(1.5)	−	−	−	Corticosteroid	Menorrhagia, dermatitis
−	+(0.7)	−	−	−	None	Menorrhagia, bled after progesterone
−	−	−	−	+	Barbiturate Chloral hydrate	Oligomenorrhea, age 48
−	−	−	−	+	Progestin	Lactating (post partum), bled after progesterone
−	−	−	−	+	Progestin	Oligomenorrhea, proteinuria, bled after progesterone
−	−	−	+	−	None	Post pill amenorrhea
−	−	+	−	−	Progestin Tranquilizer	Amenorrhea, age 50, hot flashes
−	−	+	−	−	None	Granulosa cell tumor of ovary, age 6
1	5	2	2	3		TOTAL
2	10	4	4	6		POSITIVE (%)

the tube tests requires 1 to 2 hours. Despite the obvious disadvantage of the longer time requirement, the overall reliability of the tube test makes it preferable whenever a clinical decision is to be aided by the test results. The clarity of the end point and the specificity of available red-cell tube test reagents is such that accurate results are obtained at lower concentrations of HCG than with slide tests using latex particles. Agglutination of the cells causes them to settle to the bottom of the test tube as a mat or diffuse yellow-brown sediment. In the absence of agglutination, the cells settle to the bottom as a brown ring; this doughnut pattern of cells is seen if no antiserum is added (control) or if agglutination is inhibited (positive test, Figure 3).

The Pregnosticon Accuspheres test is easily performed. The reagents are freeze-dried in the form of two pellets—one drop of antiserum and one of red cells. These are contained in a screw-capped round-bottom tube which is used as the reaction test tube, assuring that the precisely correct quantity of antiserum is present in each test. The reagents are reconstituted by adding 0.1 ml of urine and 0.4 ml of tap water. Unlike the Dri-Dot reagents, the freeze-dried Accuspheres reagents must be refrigerated for storage. For single tests, it is decidedly more convenient to use the screw-top tube for the reaction rather than separate test tubes and reagent containers. However, for semiquantitative assays or for testing several urine samples at once, this advantage is lost, since removing a cap from each test tube prolongs the setup time. However, reagents that have been dispensed with precision-dispensing machinery in a factory are an advantage over medicine droppers which have an inherent greater inaccuracy.

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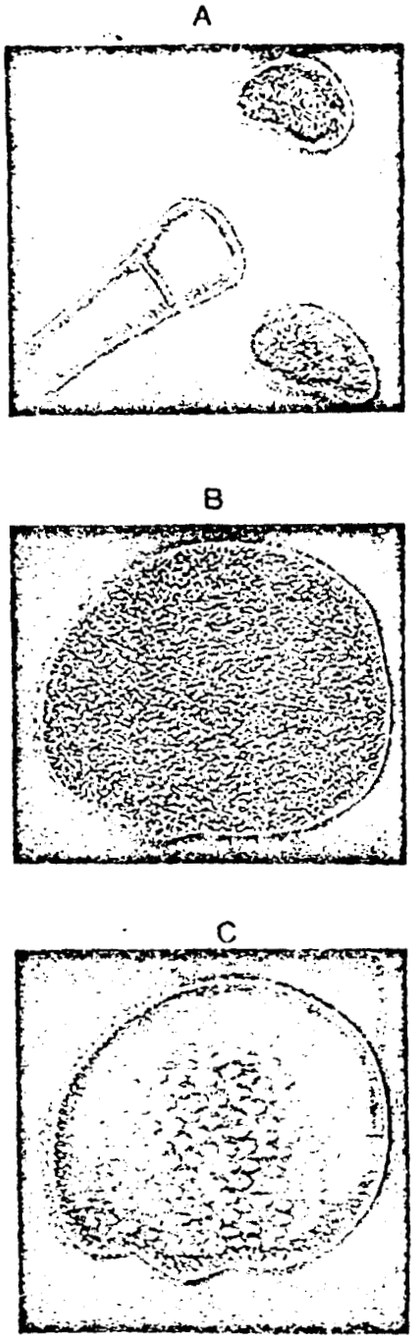

Fig 2. Dri-Dot latex test. A. Dried antiserum and latex particles. The reagents are reconstituted with a drop of tap water (latex) and a drop of urine. One end of the hollow plastic stirring rod is used to dispense the urine and the other end to mix the reagents. B. Positive test: in the absence of agglutionation the latex particles remain in a milky suspension. C. Negative test; granular clumping of agglutinated latex particles is evident in 2 minutes.

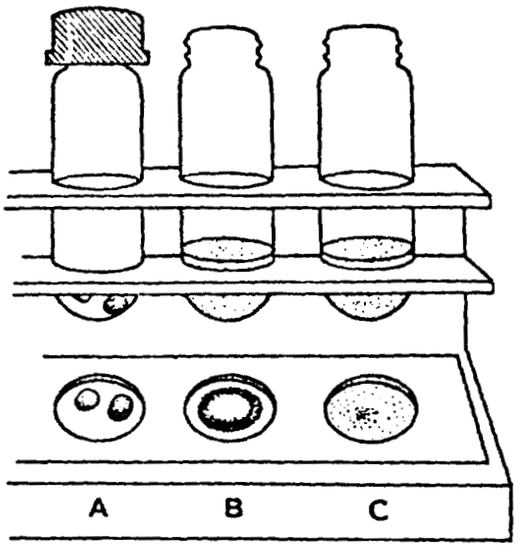

Fig 3. Pregnosticon Accuspheres tube test. A. Freeze dried pellets of antiserum and of red cells. The reagents are reconstituted by adding 0.1 ml of urine and 0.4 ml of tap water. B. Positive test: in the absence of agglutination the cells settle to the bottom as a ring. C. Negative test: agglutination of the cells results in their settling to the bottom of the tube as a mat within 2 hours.

Reliability

The ideal reagent kit should contain an antiserum capable of detecting the various concentrations of HCG present at each stage of pregnancy. Its end point should be specific enough so that it is not influenced by other urinary proteins, drug metabolites, etc.

Since more than 5000 IU of HCG/liter are required to give a positive test with Prequest, this test is often negative early in the first trimester or late in the second or third trimester of normal pregnancy when the urinary excretion of HCG may fall below this level. This lack of sensitivity was evidenced in this study by the large number of false-negative tests (Tables 2 and 4). The other reagent kits, each of which is designed to detect levels in the range of 500–2000 IU/liter, had far fewer false negatives. The Accuspheres and Dri-Dot tests gave correct positive tests for all women with normal pregnancies beyond 6 weeks. A false-nega-

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TABLE 4. PERCENT FALSE RESULTS

Test	Negative test (pregnant)	Positive test (not pregnant)
UCG	10%	2%
Accuspheres	10	10
Prequest	22	4
Pregnosticon slide	10	4
Dri-Dot	8	6

tive pregnancy test is usually less distressing to the clinician than is a false-positive one, since, in most instances, he can recheck the test several weeks later, induce progesterone withdrawal bleeding as a pregnancy test, or depend on his clinical findings. The number of false-negative immunologic pregnancy tests can be minimized by using a first, morning specimen.

A number of factors account for false-positive immunologic pregnancy tests. a) If present in large amounts, certain nonhormonal urinary proteins or drug metabolites may interfere with agglutination. Therefore, all urines tested for HCG should also be tested for protein. b) Errors in interpreting the end point account for some false-positive reactions. c) Anti-HCG serum will react with pituitary gonadotropins. The immunologic pregnancy test may be positive if urinary excretion of gonadotropins is increased, as during menopause or with the use of various psychotropic drugs.[4,5] The immunologic cross reaction of LH and HCG permits measurement of pituitary gonadotropins; using a sensitive antiserum, urine concentration procedures, or more sensitive detection methods (radioimmunoassay), gonadotropins can be detected at the level excreted by normal men and nonpregnant women. If the results of the pregnancy test are in conflict with clinical signs, the physician usually can resolve the conflict by using different immunologic tests or other pregnancy test methods.

The consistency of reagents from lot to lot is an extremely important factor not evaluated in this study. Slight variations in the sensitivity of antiserum are certainly acceptable, but some commercial kits, not those used in this study, are known to vary in stability from lot to lot.[2,4] The clarity of the end point obtained with the commercially available Accuspheres reagents was superior to that obtained with the lot reported here, which was tested before the Accuspheres were marketed.

SUMMARY AND CONCLUSIONS

1. Random urine samples from 50 pregnant women and 50 nonpregnant women were tested with five reagent kits designed as immunologic tests for pregnancy. The Pregnosticon Dri-Dot and Pregnosticon Accuspheres, two new kits, were evaluated and compared with two other slide tests and one other tube test.

2. Dri-Dot with dried reagents on a cardboard slide was the most convenient to use, had an easily detected end point, and an accuracy equal to that of the Pregnosticon slide test from which it has been derived. The Dri-Dot would be our choice as a pregnancy test for routine office use.

3. The Prequest slide test is relatively insensitive and, therefore, gives a high rate of false negative results.

4. The Pregnosticon Accuspheres test kit has freeze-dried reagents contained in a screw-capped tube which is used as the reaction test tube. This format is quite convenient especially if only one or two tests are to be done. Its sensitivity is such that there were no false-negative tests with urine from women with normal pregnancies after 6 weeks gestation.

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HAS FACILITATED THERE STUDIES MULTIPLE CHROMATOGRAPHIC STEPS

Isolierung und Charakterisierung des schwangerschafts-spezifischen β_1-Glykoproteins

Von Hans Bohn

In der menschlichen Plazenta wurde ein Protein aufgefunden, das normalerweise im Blut nicht vorkommt, sich aber bei Gravidität im mütterlichen Serum immunologisch nachweisen läßt und mit keinem der bereits bekannten schwangerschafts-spezifischen Enzyme oder Hormone identisch ist. Auf Grund seiner elektrophoretischen Beweglichkeit und seines Kohlenhydratgehalts wurde es als schwangerschafts-spezifisches β_1-Glykoprotein bezeichnet [1].

Das schwangerschafts-spezifische β_1-Glykoprotein wird wahrscheinlich in der Plazenta gebildet; es erscheint bald nach Beginn der Schwangerschaft im mütterlichen Blut und läßt sich auch im Urin gravider Frauen immunologisch nachweisen. Sein Nachweis im Serum bzw. Urin kann zur Erkennung der Schwangerschaft verwendet werden. Mit fortschreitender Gravidität nimmt die Konzentration dieses Glykoproteins im Serum stetig zu. Wahrscheinlich besteht ähnlich wie beim humanen Plazenta-Laktogen (HPL) und bei gewissen schwangerschafts-spezifischen Enzymen (Histaminase, Oxytocinase) auch beim schwangerschafts-spezifischen β_1-Glykoprotein eine Korrelation zwischen der Zunahme der Konzentration im Serum und dem Wachstum der Plazenta bzw. der fötoplazentaren Einheit. Eine quantitative Bestimmung dieses Proteins könnte daher eventuell für die Überwachung der Schwangerschaft von Bedeutung werden.

Im Nachfolgenden werden die Isolierung aus der Planzenta sowie die Eigenschaften dieses schwangerschafts-spezifischen Proteins näher beschrieben.

Material und Methoden

Isolierung

Ausgangsmaterial fur die Darstellung des schwangerschafts-spezifischen β_1-Glykoproteins (SP_1) war die Plazenta-Fraktion V. Sie wurde bei der Aufarbeitung des Plazentaextraktes nach Abscheidung der mit Diaminoäthoxyacridin-Laktat* fallbaren Proteine aus dem Überstand dieser Fällung durch Präzipitation mit Ammonsulfat bei 50% Sättigung erhalten [1].

Die Paste dieser Fraktion (1 kg) wird in Wasser gelöst und gegen 0,01 M Tris-HCl-Pufferlösung (pH 8,0) dialysiert. Durch Zusatz von DEAE-Zellulose (DE 32) wird SP_1 an den Austauscher gebunden und so von der Hauptmenge der Gammaglobuline, die in Lösung bleiben, abgetrennt.

Nach der Elution von der DEAE-Zellulose mit einem 0,02 M Trispuffer in physiologischer Kochsalzlösung bei einem pH-Wert von 6,5 wird das schwangerschafts-spezifische β_1-Glykoprotein durch Gelfiltration an Sephadex G-150 (Säule 20 × 100 cm) weiter gereinigt. Als Puffer dient eine 0,1 M Tris-HCl-Lösung pH 8,0, die 1,0 mol/l Kochsalz enthält.

Der Nachweis von SP_1 im Eluat erfolgt immunologisch mit einem spezifischen Antiserum. Die SP_1-haltigen Fraktionen werden vereinigt und mit 30% festem Ammonsulfat gefällt.

Zur Weiterreinigung wird die Fraktion der präparativen Zonenelektrophorese [2] unterworfen. Als Trägersubstanz wurde dabei Polyvinylchlorid (Geon X 427), als Puffer eine 0,075 M Ammoniumhydrocarbonatlösung verwendet. Nach Auftrennung wird SP_1 aus der β-Zone eluiert und lyophilisiert.

* siehe Seite 295.

Eingegangen am 13. 3. 1972.

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Eine weitere Fraktionierung erfolgt an DEAE-Sephadex A-50 (Säule 4 × 17 cm) unter Verwendung von 0,01M Trispufferlösung (pH 7,0) und einem linearen Kochsalzgradient von 0,2 bis 2,0%. Nach einer nochmaligen Gelfiltration an Sephadex G-150 (5 × 100 cm) erhält man das schwangerschafts-spezifische β_1-Glykoprotein mit einer Reinheit von etwa 95% (Ausbeute 50 bis 150 mg). Das Präparat wird nach Dialyse gegen Wasser lyophilisiert.

Abspaltung der Neuraminsäure mit Neuraminidase

100 mg SP_1 wurden bei pH 5,5 mit 500 Einheiten Neuraminidase (Behringwerke, Marburg a. d. Lahn) 2 h bei Zimmertemperatur und anschließend 20 h bei 4° C inkubiert. Nach Einstellen des pH-Wertes auf 7,0 und Zugabe von etwas EDTA (Äthylendiamintetraessigsäure) wurde das durch Abspaltung der Neuraminsäure modifizierte Schwangerschaftsprotein an DEAE-Sephadex (Säule 3 × 15 cm) unter Anwendung eines linearen Salzgradienten 0 bis 2% NaCl in 0,01M Trispuffer (pH 7,0) isoliert. Das Protein fällt mit Ammonsulfat bei 70% Sättigung aus. Der Niederschlag wird gegen Wasser dialysiert und anschließend aus salzfreier Lösung lyophilisiert.

Analytische Methoden

Die in der Arbeit verwendeten elektrophoretischen und immunologischen Methoden sind bereits früher [1] mitgeteilt worden.

Für die Untersuchung in der Ultrazentrifuge wurden die Substanzen in einem Phosphat-Puffer (0,05M Phosphat-Puffer pH 6,8 mit 0,2 mol/l NaCl) ohne bzw. mit 5 M Harnstoff gelöst und unter Verwendung einer Spinco-Apparatur (Modell E) sedimentiert. Die angegebenen Sedimentationskoeffizienten sind auf Wasser als Lösungsmittel umgerechnet, aber nicht auf die Konzentration Null extrapoliert worden.

Die Bestimmung der Kohlenhydrate erfolgte nach der Methode von *Schultze* u. a. [3].

Hormonteste

Das humane Choriongonadotropin (HCG) wurde mit dem Latex-HCG-Reagens Chorignost* bestimmt.

Im Tierversuch wurde durch einen Hormonscreening die Wirkung des Schwangerschaftsproteins auf Uterus, Ovarien, Hoden, Samenblase, Prostata, Nebennieren, Schilddrüse und Thymus untersucht. Als Versuchstiere dienten jugendliche Ratten (Wistar) mit einem Durchschnittsgewicht von etwa 60 g. Die Ratten erhielten an 12 aufeinanderfolgenden Tagen täglich jeweils 0,3 mg SP_1, gelöst in physiologischer Kochsalzlösung, die 0,1% Haemaccel* (Warenzeichen der Behringwerke AG) enthielt, s.c. injiziert. Einen Tag nach der letzten Injektion wurden die Tiere getötet, die Organe entnommen und gewogen und mit dem Gewicht der entsprechenden Organe der Kontrolltiere verglichen. Die statistische Auswertung erfolgte im t-Test.

Ergebnisse

1. Isolierung

Die im methodischen Teil beschriebenen Isolierungsschritte sind in Tab. 1 nochmals schematisch dargestellt. Die Tabelle enthält außerdem Angaben über Ausbeute und Reinheit des schwangerschafts-spezifischen β_1-Glykoproteins in den einzelnen Fraktionen.

Abb. 1 zeigt die erste Auftrennung an Sephadex G-150, wobei der vordere Peak Makroglobuline, der zweite im wesentlichen Immunglobuline und β_1A-Globulin enthält; anschließend kommt SP_1, gefolgt von α_2HS-Glykoprotein und anderen niedrigermolekularen Plasma- bzw. Plazenta-Proteinen.

* Eingetragenes Warenzeichen der Behringwerke AG.

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Fraktionierungsschritte	Ausbeute mg SP_1	Reinheit in %
1. Plazenta-Fraktion V (1 kg)	1000—2000	0,3—0,5
2. DEAE-Cellulose	800—1000	3—4
3. Sephadex G-150	300— 600	20—40
4. Zonenelektrophorese	200— 400	60—70
5. DEAE-Sephadex	100— 200	85—95
6. Sephadex G-150	50— 150	>95

Tab. 1.

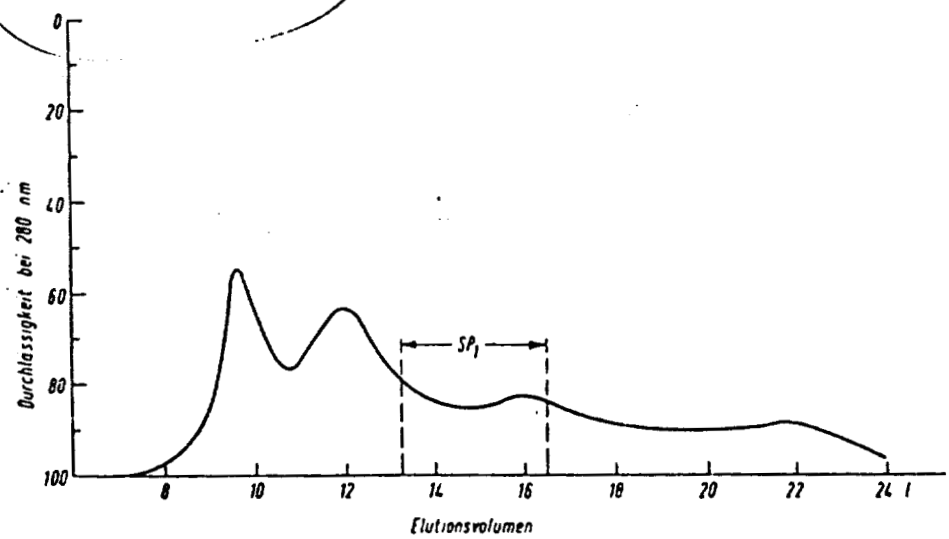

Abb. 1: Gelfiltration an Sephadex G-150. Saule 20 × 100 cm. Puffer: 0,1M Tris-HCl, pH 8,0, mit 1 mol/l NaCl.

Bei der präparativen Zonenelektrophorese lassen sich die noch vorhandenen Immunglobuline sowie das α_2 HS-Glykoprotein vom SP_1 abtrennen. Die nachfolgende Ionenaustauschchromatographie an DEAE-Cellulose trennt SP_1 vom β_1A-Globulin. Nach einer nochmaligen Auftrennung an Sephadex G-150 erhält man ein Produkt mit einer Reinheit von über 95%.

Ein immunologisch vollkommen reines, aber chemisch modifiziertes Präparat erhält man nach Abspaltung der Neuraminsäure mit Neuraminidase. SP_1 besitzt einen hohen Kohlenhydratgehalt und wird durch Neuraminidasebehandlung wesentlich stärker basisch als die noch in geringer Menge vorhandenen Begleitproteine. Das modifizierte Protein läßt sich so durch Rechromatographie an DEAE-Sephadex rein isolieren.

Die Fällungseigenschaften des schwangerschafts-spezifischen β_1-Glykoproteins ändern sich bei der Aufarbeitung mit zunehmender Reinheit des Produktes. Sie sind nach der Elution von der DEAE-Cellulose anders als im rohen Plazentenextrakt bzw. in der Plazenta-Fraktion V. SP_1 liegt im Ausgangsmaterial offensichtlich salzartig an Gammaglobuline gebunden vor und zeigt daher zunächst das gleiche

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Fällungsverhalten wie diese, d. h. es bleibt bei der Fraktionierung mit Diaminoäthoxyacridin-Laktat* zur Hauptsache im Überstand, wird aber mit Äthanol bereits bei einer Endkonzentration des Alkohols von 25% quantitativ ausgefällt. Nach der Abtrennung des Schwangerschaftsproteins von den basischen Gammaglobulinen durch DEAE-Cellulose liegen die Verhältnisse gerade umgekehrt; SP_1 wird dann mit der Acridinbase ausgefällt, aber nicht mehr durch Alkohol (auch nicht bei 40%) präzipitiert. Weniger stark ändert sich das Fällungsverhalten gegenüber Neutralsalzen. Mit Ammoniumsulfat wird das schwangerschafts-spezifische β_1-Glykoprotein aus dem Plazenten-Extrakt bzw. aus Fraktion V bei 50% Sättigung vollständig abgeschieden. Das gereinigte isolierte Protein benötigt zur quantitativen Ausfällung eine etwas höhere Salzkonzentration (60% Sättigung). Das durch Abspaltung der Neuraminsäure modifizierte Schwangerschaftsprotein dagegen fällt mit Ammoniumsulfat erst zwischen 50 bis 70% Sättigung aus.

2. Physikalisch-chemische Charakterisierung

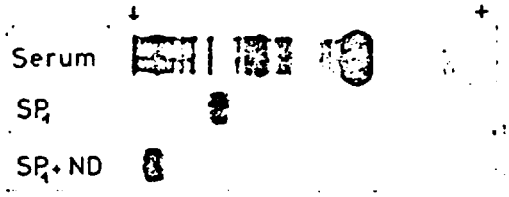

Abb. 2. Elektrophorese im PAA-Gel bei pH 8,6.
SP_1 + ND = SP_1 nach Einwirkung von Neuraminidase.

Abb. 2 zeigt die Wanderung des aus der Planzenta isolierten Schwangerschaftsproteins im PAA-Gel im Vergleich mit den Plasmaproteinen. Häufig ist außer der Hauptkomponente, die sich langsamer als Transferrin bewegt, noch eine schwächere Zone, die etwas schneller als Transferrin wandert, zu sehen. Diese schwächere Komponente präzipitiert mit einem spezifisch gegen SP_1 gerichteten Antiserum und ist wahrscheinlich eine Untereinheit oder ein Spaltstück des Schwangerschaftsproteins.

Nach Abspaltung der Neuraminsäure mit Neuraminidase wird das Glykoprotein in seiner Beweglichkeit stark verändert; es bleibt dann fast am Start zurück (Abb. 2).

In der Ultrazentrifuge zeigen die Präparate des Schwangerschaftsproteins ebenfalls eine Auftrennung in mindestens 2 Komponenten. Die Hauptmenge des schwangerschafts-spezifischen β_1-Glykoproteins sedimentiert mit 4,6 S. Für das mit Neuraminidase behandelte Glykoprotein wurde ein Sedimentationskoeffizient von 5,6 S ermittelt.

Abb. 3 zeigt die Auftrennung eines solchen Präparates. Die kleinere, langsamer sedimentierende Komponente ist vermutlich wieder die Untereinheit; sie sedimentiert mit 3,0 S und verhält sich damit ähnlich wie das in 5 M Harnstoff gelöste neuraminidasebehandelte Glykoprotein, das einheitlich mit 2,8 S sedimentiert. Auch bei der elektrophoretischen Auftrennung in harnstoffhaltigem Polyacrylamidgel

* Von den Farbwerken Hoechst AG unter dem Warenzeichen Rivanol® im Handel.

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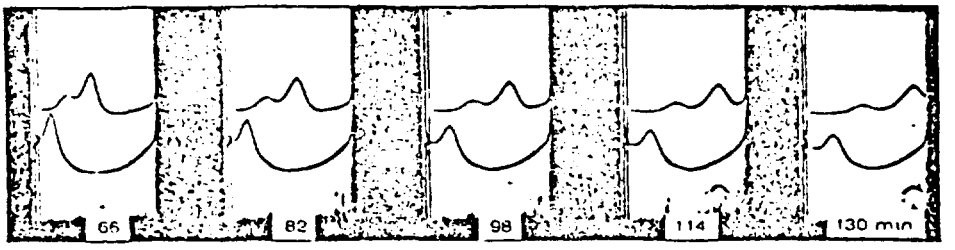

Abb. 3. Sedimentation des mit Neuraminidase behandelten Schwangerschaftsproteins in der Ultrazentrifuge. Oben: in NaCl-Phosphat-Puffer, pH 6,8, c = 1,0%. Unten: in Phosphatpuffer mit 5M Harnstoff, c = 1,0%. 60000 U/min.

zeigt SP_1 nur eine Zone. Aus dem Verhalten in der Ultrazentrifuge, bei der Gelfiltration und im PAA-Gel läßt sich schließen, daß das schwangerschafts-spezifische β_1-Glykoprotein ein Molekulargewicht von etwa 120000 besitzt und aus zwei offensichtlich identischen Untereinheiten zusammengesetzt ist.

Auf Grund der starken Veränderung, die das Schwangerschaftsprotein in der elektrophoretischen Wanderung nach Abspaltung der Neuraminsäure erleidet, war zu erwarten, daß der Kohlenhydratgehalt in diesem Protein sehr hoch ist. Die Analyse bestätigte diese Vermutung. Wie die Ergebnisse in Tab. 2 zeigen, beträgt die Summe der Kohlenhydrate 28,1%.

Kohlenhydratanalysen	
Hexosen	10,8%
Hexosamin (N-acetyl-)	9,9%
Neuraminsäure (N-acetyl-)	6,8%
Fucose	0,6%
	28,1%

Tab. 2.

Die spezifische Extinktion des β_1-Schwangerschaftsproteins ist trotz des großen Kohlenhydratanteils erstaunlich hoch; der Wert für $E^{1\%}_{1cm}$ betrug 11,2, gemessen in 1/15M Phosphatpuffer, pH 7,0, beim Maximum der Extinktion von 278 mμ.

3. Immunologischer Nachweis

Das aus Plazenten isolierte schwangerschafts-spezifische Glykoprotein wandert bei der Elektrophorese auf Acetatfolie und in Agar(ose) wie die β_1-Globuline, nach Abspaltung der Neuraminsäure aber wie die γ-Globuline des menschlichen Serums. Abb. 4 zeigt die durch Immunreaktion sichtbar gemachte Auftrennung der beiden Proteine in Agar und Agarose. Das Schwangerschaftsprotein im Schwangerenserum wandert genauso wie das isolierte Protein.

Die Immunreaktion nach Auftrennung der Proteine im PAA-Gel ist in Abb. 5 dargestellt. Im Schwangerenserum wird SP_1 wahrscheinlich durch die basischen γ-Globuline etwas zurückgehalten. Beim isolierten SP_1 ist deutlich vor der Hauptkomponente das schneller wandernde Spaltstück (Untereinheit?) zu erkennen.

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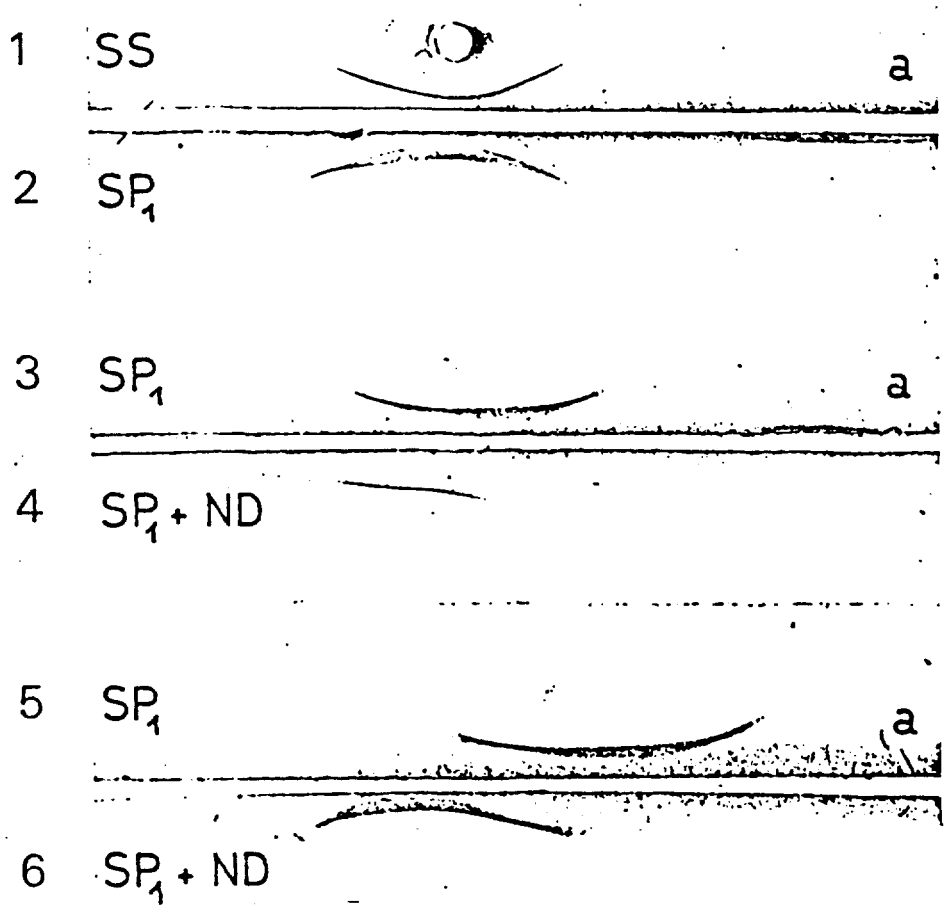

Abb. 4. Immunelektrophorese in Agar und Agarose bei pH 8, 6.
SS = Schwangerenserum, ND = Neuraminidase, a = Anti-SP_1. 1–4 in Agar, 5–6 in Agarose.

Durch Abspaltung der Neuraminsäure wird das Schwangerschaftsprotein in seinen Antigendeterminanten nicht verändert; im Geldiffusionstest nach *Ouchterlony* gehen die Präzipitatlinien des mit Neuraminidase behandelten Proteins und des unbehandelten Proteins ohne Sporn ineinander über.

Dagegen ist das Protein im Urin schwangerer Frauen, das auch mit einem gegen SP_1 gerichteten Antiserum reagiert, gegenüber dem Schwangerschaftsprotein im Serum immunchemisch verändert [1]; es hat einen Teil seiner Antigendeterminanten verloren und gibt im Geldiffusionstest mit dem Serumprotein einen Sporn (Abb. 6).

In der Immunelektrophorese wandert das Schwangerschaftsprotein aus dem Urin wie das Serumprotein im β-Bereich der Globuline; bei der PAA-Gelelektrophorese dagegen ist es etwas schneller als dieses und erscheint etwa in Höhe des Transferrins.

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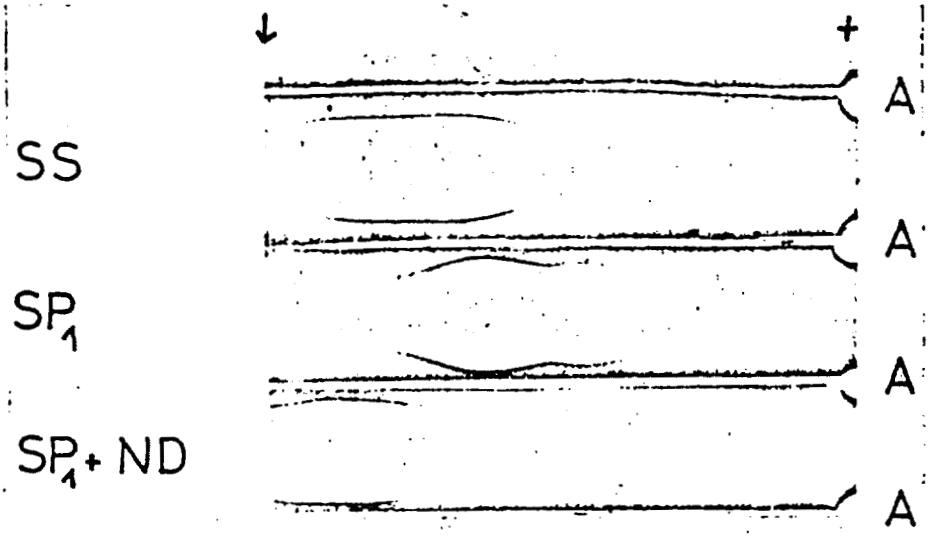

Abb. 5: Auftrennung im PAA-Gel mit Immunreaktion.
SS = Schwangerenserum, ND = Neuraminidase. A = Anti-SP_1.

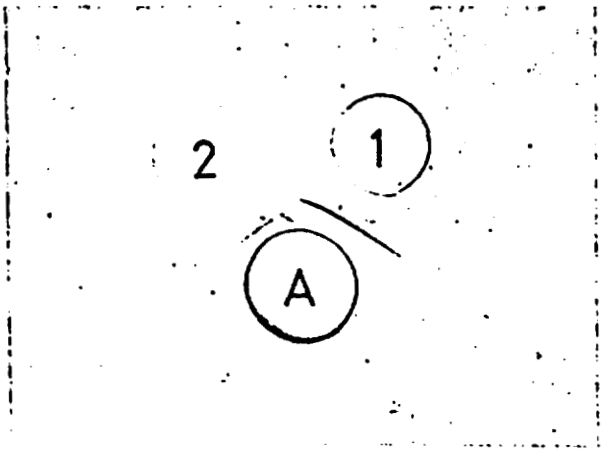

Abb. 6: Geldiffusionstest.
A = Anti-SP_1, 1 = Schwangerenserum, 2 = Schwangerenurin (konz.).

Das Molekül im Urin hat offenbar eine ähnliche Ladung, aber ein kleineres Molekulargewicht als das Schwangerschaftsprotein im Serum.

Seren von trächtigen Tieren (Hund, Ratte) zeigten, so weit sie untersucht wurden, im Geldiflusionstest keinerlei Reaktion mit dem Antiserum gegen das menschliche schwangerschafts-spezifische β_1-Glykoprotein.

Auch eine Reihe von menschlichen Seren von Patienten mit den verschiedensten Tumoren wurden im Geldiffusionstest mit Anti-SP_1 geprüft; sie waren alle negativ. Es standen uns aber leider noch keine Karzinomseren von Patienten mit tropho-

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blastischen Tumoren für diese Untersuchung zur Verfügung. Das Auftreten von SP_1 im Serum solcher Patienten wäre naheliegend und zugleich ein Hinweis dafür, daß dieses Schwangerschaftsprotein tatsächlich im Gewebe der Plazenta gebildet wird.

Über die quantitative immunologische Bestimmung des schwangerschafts-spezifischen β_1-Glykoproteins im Serum schwangerer Frauen wurde bereits berichtet [*1*]. Die Konzentration im Serum zeigte dabei eine stetige Zunahme mit fortschreitender Schwangerschaft bis zur Entbindung hin. Als Bezugsserum wurde damals ein Retroplazentarserum mit überdurchschnittlich hohem SP_1-Gehalt ausgewählt. Mit Hilfe eines hochgereinigten Schwangerschaftsproteins konnte jetzt die absolute Menge in diesem Bezugsserum ermittelt werden. Für das Bezugsserum ergab die Absolutbestimmung einen Gehalt an schwangerschafts-spezifischem β_1-Glykoprotein von 33 mg pro 100 ml. Abb. 7 zeigt nochmals den Anstieg des β_1-Schwangerschaftsproteins in verschiedenen Seren während der Gravidität, jetzt aber mit Absolutwerten als Ordinate dargestellt.

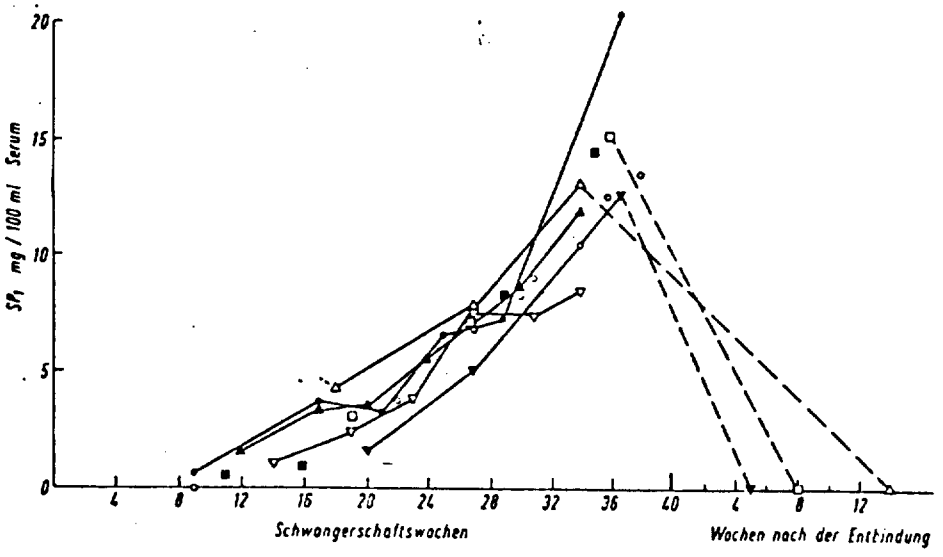

Abb. 7: Gehalt an SP_1 im Serum schwangerer Frauen.

Wie aus der Abbildung ersichtlich, erreicht der SP_1-Gehalt im Serum bereits zwischen der 8. und 16. Schwangerschaftswoche 1 mg% und liegt im letzten Trimester zwischen 5 und 20 mg pro 100 ml. Nach der Entbindung verschwindet das schwangerschafts-spezifische β_1-Glykoprotein innerhalb von 5 Wochen wieder vollständig aus dem mütterlichen Blut.

Biologische Teste

Wir vermuteten, daß das schwangerschafts-spezifische β_1-Glykoprotein eventuell mit der von *Beas* und *Flores* [*4*] aus menschlichen Plazenten isolierten und als uterotrophes Plazentahormon bezeichneten Substanz identisch sein könnte. In einem

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Hormonscreening an Ratten wurde geprüft, ob SP_1 eine Wirkung auf den Uterus, auf Ovarien, Hoden, Samenblase, Prostata, Nebennieren, Schilddrüse oder Thymus besitzt. Bei Verwendung des hochgereinigten Proteins zeigte sich jedoch bei einer täglichen Dosierung von 0,3 mg SP_1 pro Tag und pro Tier nach 12 Tagen keine signifikanten Veränderungen im Gewicht dieser Organe. Weniger reine Präparate (die Fraktion nach der ersten Gelfiltration mit einer Reinheit von 20 bis 40% SP_1) zeigten unter gleichen Bedingungen (je 0,3 mg SP_1 an 12 Tagen subkutan) bei männlichen Tieren eine signifikante Zunahme des Gewichtes von Samenblase und Prostata, während der Thymus verkleinert war. Bei den weiblichen Ratten waren zuweilen (nicht bei allen Präparaten) Ovarien und Uterus gegenüber den Kontrollen vergrößert.

Die Fraktionen nach der ersten Sephadex-Gelfiltration enthalten aber alle noch 10 bis 20 Einheiten HCG pro 1 mg SP_1 als Verunreinigung. Die Hormonwirkung dieser Präparate ist wahrscheinlich auf diese Beimengungen an HCG zurückzuführen.

Möglicherweise ist das schwangerschafts-spezifische β_1-Glykoprotein am Zustandekommen jener immunologischen Toleranz beteiligt, die verhindert, daß die fetoplazentare Einheit als Allograft während der Schwangerschaft von der Mutter abgestoßen wird. Vermutlich spielen solche kohlenhydratreichen Proteine (Mukoproteine) auch beim Aufbau der sogenannten „Fibrinoidschicht" in der Plazenta, welche die Mutter immunologisch vom Feten abschirmen soll, eine Rolle [*6,7*].

Besprechung

Das schwangerschafts-spezifische β_1-Glykoprotein ist mit keinem der bisher bekannten schwangerschafts-spezifischen Enzyme, Inhibitoren oder Hormone identisch [*1*]. Seine biologische Funktion ist noch unbekannt.

In einem an infantilen Ratten durchgeführten Hormontest zeigte das hochgereinigte Protein weder eine Wirkung auf den Uterus noch auf Ovarien, Hoden, Samenblase, Prostata, Nebennieren, Schilddrüse oder Thymus. Zwischen SP_1 und dem von *Beas* und *Flores* [*4*] in der menschlichen Plazenta nachgewiesenem uterotrophen Hormon scheint daher offensichtlich kein Zusammenhang zu bestehen.

Das schwangerschafts-spezifische β_1-Glykoprotein besitzt einen ähnlich hohen Gehalt an Kohlenhydraten wie das humane Choriongonadotropin. Die Konzentration von SP_1 im Serum schwangerer Frauen zeigt aber einen anderen Kurvenverlauf als die von HCG [*5*]. Die Hauptausschüttung des Choriongonadotropins erfolgt im 2. und 3. Monat der Schwangerschaft, während die Konzentration von SP_1, ähnlich wie die von HPL und Oxytocinase, im Serum mit fortschreitender Gravidität ständig zunimmt. Der Gehalt im Serum erreicht in der 8. bis 16. Schwangerschaftswoche bereits 1 mg/100 ml und liegt in den letzten beiden Monaten der Schwangerschaft zwischen 10 und 25 mg/100 ml. In Retroplazentarseren wurden Werte bis zu 33 mg/100 ml gemessen. Die Konzentration von SP_1 übertrifft damit die aller anderen bisher bekannten schwangerschafts-spezifischen Proteine im Blut.

Als Bildungsort für SP_1 ist in Analogie zu anderen Schwangerschaftsproteinen mit ähnlichem Kurvenverlauf wahrscheinlich das Synzytium der menschlichen Plazenta anzunehmen. Beweise dafür stehen noch aus.

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Bei der Isolierung des Schwangerschaftsproteins hat F. *Stutzinger* mitgearbeitet. Den Test auf Hormonwirkung hat Dr. H. *Rommeberger* durchgeführt. Herrn Dr. Th. *Kranz* verdanken wir die Untersuchungen in der Ultrazentrifuge, die Kohlenhydratanalysen wurden von J. *Schoning* ausgeführt.

Zusammenfassung

Beschrieben wird die Isolierung und physikalisch-chemische Charakterisierung eines kohlenhydratreichen Proteins aus der menschlichen Plazenta, das auch im Serum schwangerer Frauen vorkommt und als schwangerschafts-spezifisches β_1-Glykoprotein bezeichnet wurde.

Dieses Schwangerschaftsprotein sedimentiert in der Ultrazentrifuge mit 4,6 S bzw. nach Abspaltung der Neuraminsäure mit 5,6 S. Es besitzt ein Molekulargewicht von etwa 120000. In 5M Harnstoff zerfällt es in zwei offensichtlich identische Untereinheiten, die mit 2,8 S sedimentieren.

Der Kohlenhydratgehalt des Glykoproteins ist sehr hoch und beträgt 28,1% (Hexosen 10,8%, Hexosamin 9,9%, Neuraminsäure 6,8%, Fucose 0,6%).

Die Absolutwerte der Konzentration des Proteins wurden in mehreren Seren schwangerer Frauen quantitativ mit der einfachen radialen Immundiffusion bestimmt. Sie liegen in der 8. bis 12. Schwangerschaftswoche im Durchschnitt bei 1 mg/100 ml und erreichen am Ende der Schwangerschaft Werte zwischen 10 bis 25 mg/100 ml.

Die biologische Funktion des schwangerschafts-spezifischen β_1-Glykoproteins ist noch unbekannt.

Summary

The isolation and characterization of a carbohydrate-rich protein from human placentas is described. This protein which was found to occur in sera from pregnant women, too, has been termed pregnancy-specific β_1-glycoprotein.

In the ultrazentrifuge for the pregnancy protein a sedimentation constant of 4,6 S was determined. Its molecular weight apparently approximates to 120000. After treatment with neuraminidase the molecules sediment with 5,6 S. When dissolved in 5M urea they dissociate into subunits which have a sedimentation constant of 2,8 S.

The pregnancy protein was found to contain hexoses (10,8%), hexosamines (9,9%), sialic acid (6,8%) and fucose (0,6%); the total carbohydrate content thus amounts to 28,1%.

The results of a quantitative determination of the pregnancy-specific β_1-glycoprotein in sera taken from women during pregnancy are given. The concentration increases progressively from approximately 1 mg/100 ml between the 8th and 12th week of pregnancy to 10 to 25 mg per 100 ml serum in the third trimester.

The physiological function of the protein is unknown.

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Literatur: 1 Bohn, H.: Nachweis und Charakterisierung von Schwangerschaftsproteinen in der menschlichen Plazenta, sowie ihre quantitative immunologische Bestimmung im Serum schwangerer Frauen. Arch. Gynak. *210,* 440 (1971). — 2 Heimburger, N. und R. Schmidtberger: Moderne Verfahren der Chromatographie und Elektrophorese zur Präparation von Plasmaproteinen. Behringwerke-Mitteilungen *43,* 119 (1964). — 3 Schulze, H. E., R. Schmidtberger und H. Haupt: Untersuchungen über die gebundenen Kohlenhydrate in isolierten Plasmaproteinen. Biochem. Z. *329,* 490 (1958). — 4 Beas, F. and H. Flores: Characterization and Properties of a New Placental Protein. Nature (Lond.) *221,* 574 (1969). — 5 Diczfalusy, E. and P. Troen: Endocrine Functions of the Human Plazenta „Vitamins and Hormones" *19,* 229 (1961). Academic Press, New York. — 6 Kirby, D. R. S., W. D. Billington, S. Bradbury and D. J. Goldstein: Antigen barrier of the mouse placenta. Nature (Lond.) *204,* 548 (1964). — 7 Douthwaite, R. M. and G. I. Urbach: In vitro antigenicity of trophoblast. Amer. J. Obstet. Gynec. *109,* 1023 (1971).

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PREGNANCY DIAGNOSIS BY A HEMAGGLUTINATION INHIBITION TEST FOR URINARY MACAQUE CHORIONIC GONADOTROPIN (mCG)

Gary D. Hodgen and Griff T. Ross with the technical assistance of C. K. Turner, D. L. Barber, and A. M. O'Connor. Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014.

ABSTRACT. An antiserum to the β subunit of ovine luteinizing hormone has been used to develop a simple, rapid, and specific hemagglutination inhibition test for urinary macaque chorionic gonadotropin (mCG). Reliable diagnosis of 20 pregnancies were made using neat urine specimens collected on all days between the 18th and 23rd day after fertilization. Only 0.2 ml of urine were needed for the test which required 2 hours to complete. When urinary concentrates were tested, diagnostic results were obtained on the 16th day of pregnancy. The rate of "false positive" tests was less than 1%.

We have produced an antiserum (A/S) which reacts with antigenic determinants common to chorionic gonadotropins of humans, gorillas, orangutans, chimpanzees, baboons and macaques, but not to follicle stimulating hormone (FSH) and luteinizing hormone (LH) of the latter two species. This A/S has been used to develop a simple, inexpensive, rapid and specific hemagglutination inhibition test for urinary mCG. Only 0.2 ml of neat urine (or an equivalent amount of urine extract) was required for testing and results were interpretable two hours after the reaccants had been mixed.

MATERIALS AND METHODS

A. Reagents: 1. A/S. The A/S was harvested from 1 of 5 rabbits (H-26) each given 150 μg of an ovine luteinizing hormone β-subunit preparation (DNW 8-161 generously provided by Dr. D. N. Ward, M. D. Anderson Hospital and Tumor Institute, Houston, Texas) at multiple intradermal sites (1). The immunogen had been mixed with complete Freund's Adjuvant (Difco) to which an additional 5 mg of heat killed, desiccated mycobacterium tuberculosis were added. At a separate subcutaneous site, 0.5 ml of pertussis vaccine was given on the same day. On the 140th day after the primary immunization, a total dose of 10 μg of the immunogen suspended in 2.5 ml of an emulsion consisting of 0.15 M saline, arlacel A (Hilltop Research, Miamiville, Ohio) and marcol 52 (Humble Oil and Refining Co.) in the proportions of 1 to 1 to 3 was given at multiple intradermal sites.

For 35 consecutive weeks after the booster injection, the A/S harvested remained specific and produced agglutination at a maximal dilution of 1:3000 under the assay conditions described below.

2. Antigen and diluents. Apart from the A/S and the reference preparation, all other reagents were obtained from Ortho Diagnostics Research Foundation. These consisted of sheep red blood cells to which human chorionic gonadotropin had been attached by a modification of the method of Onkelinx et al. (2), a 0.025 M phosphate buffer solution (pH 7.3), used to produce suspensions of these cells, and a buffered A/S diluent containing protein.

3. Urine specimens. All urine was collected daily or intermittently from cycling caged female rhesus monkeys (Macaca mulatta), from pregnant monkeys before, during and after biologically active chorionic gonadotropin could be detected in urine or serum, and from castrated, juvenile or hypophysectomized monkeys. Urine specimens were

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collected three ways: 1) continuously for 24 hr periods where the voided urine immediately drained into plastic bottles packed in dry ice, where it froze 2) analogous 24 hr specimens collected at room temperature (26C) and 3) single voidings collected on sheets of aluminum foil and immediately recovered by aspiration with disposable pipets.

Neat urine and urine extracts prepared either by a modification (3) of the kaolin-acetone procedure of Albert (4) or by the acetone precipitation method of Frank and Salmon (5) were tested. All specimens were centrifuged at 1000 X g for 10 minutes and only the supernatant was used for testing.

Kaolin acetone extracts of pooled pregnant rhesus monkey urine with a biological specific activity of 5 IU/mg (rat uterine weight assay, Second International Standard hCG) were used to prepare an ad hoc mCG reference preparation. A minimal effective dose of this preparation was included as a positive control in all assays.

B. Conduct of assays. Disposable glass culture tubes (Scientific Products), 12 mm by 75 mm, were placed in a rack over a mirror so that bottoms of tubes could be seen without disturbing the contents. The volume of mixed reactants was 0.5 ml and contained 0.2 ml of either a 1:2400 dilution of the A/S or A/S diluent, 0.1 ml of sample and 0.2 ml of a suspension of hCG coated sheep red blood cells. After shaking to thoroughly mix reagents, tubes were left undisturbed at ambient temperature (22 to 25C) for 2 hr.

C. Interpretation of results. Test results were read as either "positive", "inconclusive", or "negative" according to the following criteria.

1. "Positive" tests. In control tubes, lacking A/S, hemagglutination did not occur and the red blood cells settled out in a ring approximately 5 mm in diameter When a similar ring formed in both the absence and the presence of A/S the test was recorded as "positive".

2. "Inconclusive" tests. For reasons presently unknown, ring formation failed to occur in approximately 8% of the urine specimens tested without A/S and these tests were regarded as "inconclusive". Dilution of these specimens 1:3 (V/V) with A/S diluent resulted in ring formation when all such samples were retested without A/S.

3. "Negative" tests. A test was interpreted as "negative" when cells formed a ring pattern in the absence, but a diffuse, matted pattern in the presence of A/S.

D. Timing fertilization and diagnosing pregnancy. For timing events in cycling female rhesus monkeys, the onset of menstruation, detected by daily vaginal smears, was counted as day 1 of the menstrual cycle. On the morning of the 11th day of the cycle, females to be mated were caged with males for 48 hr and removed early in the 13th day. Detection of coagulated semen in the vagina indicated that inseminations had occured. Since ovulation occurs most frequently on the 13th day of the menstrual cycle of rhesus monkeys, we have arbitrarily designated day 13 as the day of fertilization.

To provide independent evidence of pregnancy, serum collected on the 18th day, and concentrates of 24 hour urine specimens collected on the 20th day of pregnancy (vide supra) were bioassayed for mCG (6).

RESULTS

Hemagglutination failed to occur at A/S dilutions in excess of 1:3000. To allow some margin for variation from lot to lot of hCG-coated erythrocytes and buffer, a 1:2400 dilution of the A/S was chosen for assays.

Specimens known to contain high

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Table 1. Hemagglutination tests on urine samples collected from female rhesus monkeys.

	Nonpregnant			Pregnant					
	Days after Onset of Menses			Days after Fertilization					Others**
	<10	11-20	>20	<12	12-17	18-23	24-30	>30	
Number	100	80	91	94	42	34	22	17	31
Positive	0	0	1*	1*	13	31	8	0	0
Negative	100	80	90	93	29	0	14	17	31
Initially Inconclusive†	8	6	7	6	4	3	2	2	2

† All "inconclusive" results were resolved by retesting after diluting 1:3 with A/S diluent so that final results were either "positive" or "negative" for all specimens tested.

* Interpreted as "false positives".

** Specimens collected randomly from castrated, hypophysectomized and juvenile animals.

concentrations of FSH and LH were tested for cross-reactivity of A/S with pituitary gonadotropins. Specimens from both unmated cycling monkeys and from pregnant females 6 weeks after biologically active gonadotropin was no longer measurable in urine were tested to determine susceptibility of the test to urinary substances giving "false positive" results. Test results, classified as "positive", "inconclusive", or "negative", using the criteria described, are summarized in Table 1. It can be seen that fewer than 1% "false positive" reactions were obtained in assays of 511 specimens. False positive tests were never observed in specimens collected from the same animal on two consecutive days.

Aliquots of a total of 36 specimens were tested with and without acetone precipitation of urine. There were two apparent advantages to this preliminary precipitation: 1) pregnancy was diagnosed about 48 hr earlier than in tests using neat urine; and 2) no "inconclusive" tests were encountered in this series.

To arrive at some estimate of the day of pregnancy on which neat urine might first give "positive" tests, specimens collected serially from 4 rhesus monkeys that conceived were tested. The earliest "positive" test was found in a specimen collected on day 14 after fertilization. All specimens collected from days 18-23 after fertilization were positive for all monkeys and from days 13-28 in one of these. This reflects the known variability in urinary mCG excretion from animal to animal (6-8) rather than unreliability of the test.

Because the peak period of mCG excretion is so brief, this diagnostic test becomes progressively less definitive when applied to neat urine specimens collected either prior to day 18 or after day 23 following fertilization. The test may not be reliable when examining neat urine specimens collected before or after the recommended period because of the incidence of false

1015619

negatives when hCG levels are low. The period of reliability can be extended two days or more when urine concentrates are tested.

An accurate diagnosis of pregnancy was made by tests of urine specimens from two baboons, two chimpanzees, one gorilla and one orangutan.

DISCUSSION

Traditional methods for diagnosing pregnancy in macaques and baboons depend upon either rectal palpation or bioassays of serum or concentrated urinary extracts. The time which must elapse post-coitally before reliable results can be obtained limits usefulness of both tests for some purposes. Diagnosis of pregnancy with biological assays for gonadotropins in urine or serum from mated rhesus monkeys becomes reliable about the 20th day and diagnosis by rectal palpation about the 30th day after fertilization. Bioassays are costly, require a minimum of 72 hours to complete, and suitable animals are not always available.

In contrast to these traditional tests for pregnancy, the method described here has been shown to provide a dependable diagnosis by the 18th day after fertilization when neat urine was tested or on day 16 when 5 fold concentrates were tested. Results were obtained less than 3 hr after the specimen had been collected. Each of the urine collection procedures used here was satisfactory. However, the collection of aliquots of freshly voided urine was most satisfactory because it minimized both the effort and time invested in sample collection and the debris in urine specimens.

We believe colleagues will find the assay useful in research where diagnosing or monitoring the course of early pregnancy in macaques and baboons is important. Accordingly, we have prepared a pool of 400 ml of the undiluted antiserum. The remainder of the reagents have been purchased and kits containing a quantity sufficient for 50 diagnostic tests have been prepared under the aegis of the Contraceptive Development Branch of the Center for Population Research, National Institute of Child Health and Human Development. Kits can be obtained by writing to The Hormone Distribution Officer, Office of the Director, NIAMDD, Building 31A, Room 9A47, National Institutes of Health, Bethesda, Md. 20014).

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1075620

Evaluation of four slide test kits for the detection of human chorionic gonadotropin in urine

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Summary: Three "indirect-type" slide tests utilizing the principle of hemagglutination inhibition and one new "direct-type" slide test employing direct agglutination were evaluated for their sensitivity in detecting human chorionic gonadotropin (HCG) in urine. The results of positive tests in a group of woman in very early pregnancy were correlated with the "days after last menses". In this series the direct slide test was the most accurate. A control must be used with each direct test to indicate interfering substances and when such are present a different test must be used. All tests were found to be of the relative sensitivity stated by the manufacturer.

Résumé: *Evaluation de quatre épreuves sur lame pour la détection de la gonadotrophine chorionique humaine dans l'urine*

Nous avons évalué la sensibilité de quatre épreuves sur lame quant à la détection de la gonadotrophine chorionique humaine (GCH) dans l'urine. Trois de ces épreuves sur la lame (du "type indirect") utilisaient le principe de l'inhibition de l'hémagglutination et une nouvelle épreuve sur lame (du "type direct") employait l'agglutination directe. Nous avons corrélationné les résultats des épreuves positives et les "jours après les dernières menstrues" chez un groupe de femmes tout au début de leur grossesse. Dans cette série, l'épreuve directe était la plus précise. Avec ce dernier test, il importe d'exercer un contrôle pour déceler des substances susceptibles d'interférence et, le cas échéant, recourir à un autre test. Tous ces types d'épreuves possédaient vraiment la sensibilité relative mentionnée par le fabricant.

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The presence of human chorionic gonadotropin (HCG) in serum and urine of a woman is generally interpreted as an indication of pregnancy. As is well known, it is also found in the urine in certain other conditions such as choriocarcinoma and hydatidiform mole. There are two types of immunologic test available to determine the presence of HCG in urine — tube tests and slide tests.

The tube test uses the hemagglutination-inhibition assay method first introduced by Wide and Gemzell in 1960.[1] The principle of this method is as follows. When HCG antiserum is mixed with erythrocytes coated with HCG, agglutination of these cells takes place. If, however, a urine sample containing HCG (e.g. one from a pregnant woman) is first added to the antiserum neutralization of the antiserum occurs, and when the coated erythrocytes are added there is inhibition of agglutination. If the urine sample contains no HCG, neutralization of the antiserum does not take place and the coated erythrocytes, when added, agglutinate. Characteristic settling patterns occur with time at the bottom of the tube and reflect whether agglutination has or has not occurred.

Slide tests, or latex particle tests, were first described by Robbins *et al* in 1962.[2] These tests for HCG in urine can be further divided into two types — indirect and direct.

The indirect type of slide test uses the same principle as the tube test already described except that latex particles coated with HCG are substituted for the coated erythrocytes.

The interpretation of results for both indirect-type tests is expressed in the following statements:

- No agglutination of coated erythrocytes = HCG present.
- Agglutination of coated erythrocytes = no HCG present.

The direct type of slide test uses latex particles coated with anti-HCG. Urine samples containing HCG will agglutinate the latex particles, and those samples with no HCG present will leave the particles unagglutinated.

The interpretation of results for direct-type tests is expressed as follows:

- Agglutination of coated latex particles = HCG present.
- No agglutination of coated latex particles = no HCG present.

As the manufacturers' instructions accompanying the test kits indicate, tube tests for HCG are more sensitive and more accurate than slide tests. Tube tests in general require a two-hour incubation period before a result is available while slide tests require only one to two minutes. Consequently, laboratories that perform tests for HCG are faced with a choice between speed and increased accuracy and sensitivity. Some use both types, a slide test for "stat" samples and a tube test for routine samples. If a slide test of comparable accuracy could be found the tube test could be eliminated.

This evaluation was performed to determine the accuracy of four slide tests for HCG — Brevindex® (Ortho Pharmaceutical [Canada] Ltd.), Pregnosis®

1075621

25

Slide (Denver Laboratories [Canada] Ltd.) and Dap (macro)® (Denver). Brevindex, Pregnosis and UCG-Slide are indirect-type tests and Dap (macro) is a direct-type test. All the test kits for HCG used in this survey were made available by the manufacturers.

Methods

The urine samples were all received at the laboratories for pregnancy testing. The sources were presurgical patients, outpatients and emergency admissions, e.g. for threatened abortion. If testing was not performed the same day the specimen was received, the sample was stored at –20°C with known positive and negative control specimens to ensure that storage did not affect the results. In 129 cases the samples were clinically confirmed as containing HCG and in 271 cases as containing no HCG.

Each sample of urine was tested with each of the kits, following the manufacturers' instructions. The results were related to the clinical evaluation of the patient including, if possible, the date of the last menses. Any samples that showed disagreement in the results of any of the kits were further tested by determining specific gravity using a hydrometer and the presence of protein or blood using Bili-labstix® (Ames Co.).

Because we believe that accuracy should be the most important consideration in routinely using a test kit for HCG, the following considerations were disregarded: (a) ease of performance of the test, (b) readability of the reaction, and (c) price of the kit. Regarding ease of performance, the Dap (macro) test kit requires filtration and an internal method control must be set up with each urine sample tested to rule out the presence of interfering substances. In our experience these steps take about 1½ minutes extra time.

Results

The percentage accuracy of the HCG test kits is shown in Table I. The figures agree quite closely with those for Gravindex* and Pregnosis obtained by Driscoll *et al*[5] but are considerably lower than the figures for UCG-Slide obtained by Goetschel.[4] Dap (macro) is a new product, so no other percentage accuracy figures are available.

For 16 urine samples there were disagreements either between one or more clinical evaluation. The results obtained with all the test kits and the results of further investigations performed on these samples are listed in Table II. The 16 samples were divided into six groups according to their reactions with the test kits.

Group 1 — specimens 1, 17, 84, 164, 307 and 372

These specimens were shown to contain HCG only by Dap (macro). Only one specimen (no. 372) contained protein and blood, so the failure of the other test kits to detect the HCG present in these specimens must be due to some other cause. The specific gravity of all these specimens was also normal, excluding insufficient concentration as a cause. The presence of Darvon® in Specimen 1 was not important; Lang[3] has shown that Darvon does not interfere with the accuracy of slide tests for HCG. The variations therefore, to be due to the relative sensitivities of the test kits used. The sensitivities reported in the manufacturers' instructions inserts are given in Table III.

The sensitivities of all the test kits used are relatively close with the exception of Brevindex, so some other factor must be involved. The methodology for indirect-type slide tests for HCG requires the addition of a standard amount, depending on the sensitivity of the test kit, of anti-HCG. This is neutralized by HCG present in the

Table III—Sensitivities of the test kits as reported in the manufacturers' instructions inserts

Brevindex	3.5 IU/ml
Pregnosis	1.5-2.5 IU/ml
Dap (macro)	2.0 IU/ml
UCG-Slide	2.0 IU/ml

Table I—Percentage accuracy of the four pregnancy test kits

Clinically confirmed samples	Total no. of samples	Brevindex		Pregnosis		Dap (macro)		UCG-Slide	
		No. detected	%	No. detected	%	No. detected	%	No. detected	%
HCG-positive	129	115	89.1	121	93.8	127	99.2*	118	91.5
HCG-negative	271	270	99.6	271	100	271	100	271	100

*Calculated on the basis of 128 samples; one test sample was excluded because its control sample gave a positive reaction, indicating the presence of interfering substances, and invalidating the results.

Table II—Results of further investigations performed on 16 urine samples giving variable results with the test kits or with the clinical evaluation

Specimen no.	Results of tests	Specific gravity	Protein	Blood	Days after last menses	Medication
1	Positive by Dap (macro) only	1.015	negative	negative	29	Darvon
17	Positive by Dap (macro) only	1.017	negative	negative	31	none
84	Positive by Dap (macro) only	1.010	negative	negative	31	none
164	Positive by Dap (macro) only	1.014	negative	negative	31	none
307	Positive by Dap (macro) only	1.014	negative	negative	32	none
372	Positive by Dap (macro) only	1.011	+	moderate	31	none
111	Positive by Dap (macro) and Pregnosis only	1.012	negative	negative	33	none
243	Positive by Dap (macro) and Pregnosis only	1.011	negative	negative	34	none
35	Negative by Brevindex only	1.012	negative	negative	42	none
67	Negative by Brevindex only	1.013	negative	negative	NA	NA
72	Negative by Brevindex only	1.012	negative	negative	42	none
201	Negative by Brevindex only	1.015	negative	negative	41	none
64	Negative by UCG-Slide only	1.014	+	large amount	39	none
292	Negative by UCG-Slide only	1.014	+	large amount	39	none
357	Positive by Brevindex only	1.010	++	small amount	NP	none
97	Positive specimen not picked up by any test method	1.011	negative	negative	NA	none

NA = not available
NP = not pregnant

*Gravindex and Brevindex are the same product, Brevindex being the name used for marketing purposes in Canada.

1075622

urine sample, so that when the coated latex particles are added no agglutination takes place. In a case of early pregnancy there may not be enough HCG present in the urine sample to neutralize all of the anti-HCG added, so the amount remaining agglutinates the coated latex particles, indicating to the observer that no HCG is present in that sample.

In direct-type slide tests no neutralization of anti-HCG is required, so the above problem does not arise. This could explain why these six specimens reacted only with Dap (macro), the only direct-type test of the four kits. All were cases of early pregnancy according to "days after last menses".

Group 2 — specimens 111 and 243

These specimens showed the presence of HCG by Dap (macro) and Pregnosis. No protein or blood was detected and the specific gravity of both was normal. The problem is probably the same as that already discussed for Group 1. Because Pregnosis has greater sensitivity and therefore requires the addition of less anti-HCG than the other indirect-type tests used, failure of agglutination as described for Group 1 will be observed at a lower urinary level of HCG with Pregnosis. The "days since last menses" of these two patients, again, show them to be cases of relatively early pregnancy. The date on which a sample is obtained may be significant; for example, the level of HCG in the urine during pregnancy increases rapidly between the 30th and 40th days since the last menses (Fig. 1).

Group 3 — specimens 35, 67, 72 and 201

These specimens showed absence of HCG by Brevindex only. No protein or blood was present and the specific gravities were normal. The reason for the false-negative results obtained is probably related to the sensitivity of the test. According to the manufacturer's instructions insert for Brevindex the urinary level of HCG is not sufficiently high to be detected by Brevindex until 41 days after the last menses. The intervals since the last menses of three of these four patients are all close to 41 days (not available for no. 67).

Group 4 — specimens 64 and 292

These specimens showed absence of HCG by UCG-Slide only. Both specimens contained a large amount of blood and it has been the authors' experience that in the UCG-Slide test agglutination of the latex particles occurs in the presence of blood, giving a false-negative result. The interference may not be caused by the blood alone but by some product formed by interaction of blood with other constituents of the urine.[6]

Group 5 — specimen 357

This specimen, which showed the presence of HCG by Brevindex only, was from a clinically confirmed nonpregnant woman. The specimen contained a small amount of blood and 2+ protein. The instructions insert of Brevindex states that interference by protein is unlikely. However, there appears to be no other cause for the false-negative results than protein interference.

Group 6 – specimen 97

This specimen, which was negative for HCG by all four test kits, was shown by quantitation† to contain 1000 IU HCG/litre of urine. This is simply too low a level of HCG to be detected by any of the test kits used. The specimen was taken from a patient considered to be a case of incomplete abortion three hours before curettage was performed. The surgical specimen showed decidual tissue only.

†UCG® tube test, Denver Laboratories (Canada) Ltd.

Conclusions

In this survey the Dap (macro) was the most accurate of the HCG test kits, followed in order of accuracy, by Pregnosis, UCG-Slide and Brevindex. The direct-type Dap (macro) test requires filtration of the urine and use of a control to detect interfering substances, and when such substances are present a different type of test must be used. In this series, in only 1 of 400 tests was an interfering substance detected by the Dap (macro). The four tests were found to possess the relative sensitivity claimed by the manufacturer.

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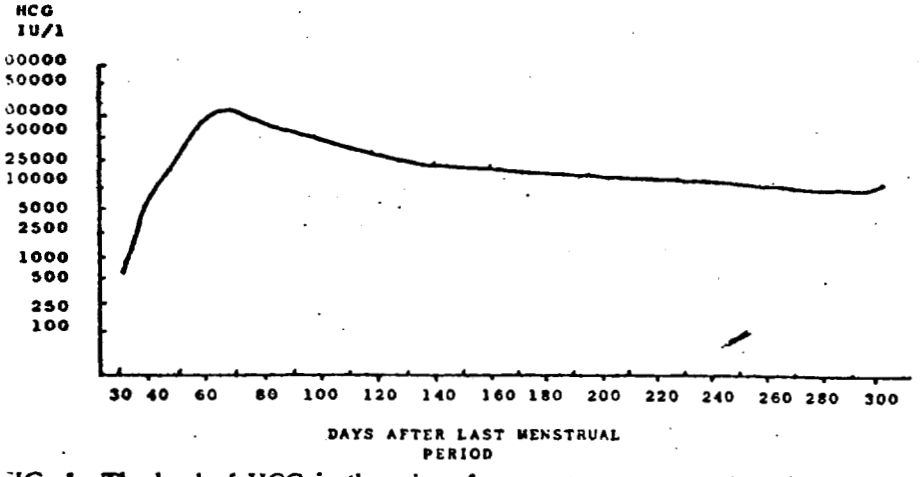

FIG. 1—The level of HCG in the urine of pregnant women at various times after last menstrual period (after Wide[1]).

1075623

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Comparative study of immunological tests for pregnancy diagnosis

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ɔrsis The reliability of five commercially produced immunological pregnancy diagnosis ıods has been investigated. The tests used were Pregnosticon (a tube test) and four slide tests, ınd, Pregslide, Gravindex, and Planotest. In the series described, Planotest gave 0·5% false tives, Gravindex had 2·1%, Pregnosticon 2·6%, and Pregslide 4·7%, Hyland A (sensitivity) iu/l.) gave 3·6% and Hyland B (sensitivity 2 to 3,000 iu/l.) had 8·7% false positives. Pregnosticon 0·5% false negatives, Planotest 2·0%, Gravindex 3·5%, Hyland B 6·5%, Pregslide 9%, and ·nd A 19·5% false negatives.

.ınotest and Pregnosticon were found to be less influenced by protein and blood in the urine ; the other pregnancy tests investigated.

ɔugh many papers have been written on indi- ıl immunological pregnancy tests, such as the vindex slide test,[1] and such tube tests as ıosticon,[2] UCG,[3] Prepuerin,[4] and the Ortho there is a lack of papers comparing these tests each other. In many papers also, clinical con- ıtion of the diagnosis was not obtained and the ·nts were not divided according to the state of ɔregnancy. Recently, some new slide tests, which advantages over the tube tests in simplicity time taken to obtain a result, have been produced these have not been adequately investigated. these reasons it was thought that it would be of .est to compare all the available slide tests with tube test, Pregnosticon, which had been in use ıis laboratory for some years.

METHODS

ıancy tests were carried out on 686 early morning imens of urine. Final diagnoses on all patients were ined either from the case notes or by correspondence the general practitioner. Four hundred and eighty- ırines were tested with four different pregnancy diagnosis tests: Pregnosticon[2] (a haemoagglutination inhibition tube test); and three-complement fixation slide tests, Hyland HCG-test,[5] Pregslide,[3] and Gravindex.[1] In addition, 238 of these urines were tested with Planotest,[2] another complement-fixation slide test. Two hundred further urines were tested with Planotest alone.

The sensitivities of the tests were:

Gravindex, 3,000 to 3,500 iu/l. (stated on the product).

Pregnosticon, 1,000 iu/l. (stated on the product).

Planotest, 2,500 iu/l. (manufacturer's personal communication).

Pregslide, 3,000 iu/l. (manufacturer's personal communication).

Hyland, 2,000 to 6,000 iu/l. (manufacturer's personal communication).

It was noted during the preparation of this paper that the pattern of results with the Hyland HCG test had changed during the course of the work, the earlier results showing fewer false positives and more false negatives than the later results. Baxter laboratories were contacted and informed us that they had changed the sensitivity of their test from 6,000 iu/l. through 4,500 iu/l. to 2 to 3,000 iu/l., which they are currently supplying. We were unable to obtain the exact dates when these changes were made and are thus unable to include all the results with the Hyland slide test in this paper. Few tests were carried out with the Hyland test at a sensitivity of 6,000 iu/l. and these have been excluded. The results obtained using the Hyland test at a sensitivity of 4,500 iu/l. are referred to as type A and with a sensitivity of 2 to 3,000 iu/l. as

o Diagnostics, Raritan, New Jersey, USA (English address, derton, Bucks.).

. Organon, Oss, Holland (English address, Organon Laboratories, ı House, Morden Surrey).

ıole Laboratories, Stanford, Connecticut, USA (English ss, Denver Laboratories, 12 Carlisle Road, London, N.W.9).

ɔughs Wellcome & Co., Euston, London, N.W.1.

[5]Hyland Laboratories, Los Angeles, California, USA (English address, Baxter Laboratories Ltd, Thetford, Norfolk).

ıed for publication 16 May 1968.

1075624

type B. The importance of pregnancy diagnosis kits carrying an approximate estimate of their sensitivity cannot be overemphasized and users should be notified if a change is made. Without this, mistakes and confusion can arise when interpreting results.

All urines were filtered before testing and the urines were tested for protein by the salicyl sulphonic acid test and Albustix,[5] blood by Haemostix[5] and for salicylates with ferric chloride. No urine was tested earlier than 35 days after the stated date of the last menstrual period. All urines not tested immediately were refrigerated and all glassware used in the tests was thoroughly washed in tap water followed by distilled water. Soap and detergents were not used.

SLIDE TESTS The tests were carried out according to the manufacturers' instructions. It was found easier to read the result in daylight but when artificial light was used it was found best to use a Tungsten bench lamp, the slide being held near, but not directly under, the light. The results were read as positive (no agglutination), negative (agglutination present), and inconclusive (doubtful agglutination).

TUBE TEST The test was carried out according to the manufacturers' instructions. The result was read as positive (definitive, well formed brown ring), negative (no ring formation), and inconclusive (a very open, thin or irregularly formed ring). The end point of the tube test was easy to read and tests could be carried out either in daylight or artificial light.

RESULTS

UNCOMPLICATED PREGNANCIES Table I shows the results in 203 uncomplicated pregnancies. Pregnosticon was the most sensitive test used, giving only one false negative (0·5%). The patient giving the false negative result was tested at 37 days after the date of the last menstrual period, and gave a positive result with Pregnosticon and Gravindex, an inconclusive result with Pregslide and a negative result with Hyland A when tested 21 days la Planotest and Gravindex had a similar sensiti Planotest giving 2·0% false negatives and Gravi 3·5%. Pregslide had 9% false negatives. Hylan was the most insensitive test, giving 19·5% f negatives, Hyland B gave 6·5% false negatives.

COMPLICATED PREGNANCIES AND PATHOLOGIC CONDITIONS All pregnancy tests were positive the patient with a hydatidiform mole and nega in the follow up patients who were tested betw three and 18 months after the original diagnosis treatment of the mole. The latter patients had evidence for recurrence of the mole.

Four patients were diagnosed clinically as ect pregnancies. With one of these patients no chori tissue was found histologically and the diagnos therefore in doubt. This patient had a nega pregnancy test by all five methods. One patient a positive test with Pregnosticon and a nega result with the other tests. This was presumably to the greater sensitivity of Pregnosticon. patient was positive with all tests and the other all tests except Hyland A. Sixteen patients h threatened abortion. The patient with a nega result by all tests was tested at 40 days after the of the last menstrual period; this patient is still p nant. Of the 11 patients with a positive result each of the tests carried out, seven aborted withi to 33 days of the test, while four are still pregn The remaining four patients were positive Pregnosticon and had varying results with the o tests. A single pregnancy test, therefore, appear be of little help in the management of a threat abortion.

The four patients with incomplete abortion had positive results, while patients with comp abortions generally gave negative results. Pregn con and Pregslide, however, gave inconclusive re

[5]Ames Co., Inc., Elkhart, Indiana, USA (English address, Division of Miles Laboratories Ltd, Stoke Poges, Slough, Bucks.).

TABLE I

RESULTS OF PREGNANCY TESTS IN PREGNANT PATIENTS (EXCLUDING URINES CONTAINING BLOOD AND/OR PROTEIN

Days after Last Menstrual Period	*Pregnosticon*					*Hyland A*					*Hyland B*				
	+	−	±	*False Negative* (%)	*Inconclusive* (%)	+	−	±	*False Negative* (%)	*Inconclusive* (%)	+	−	±	*False Negative* (%)	*Inconc* (%
35-49	42	1	3	2·2	6·5	10	6	1	35	6	15	2	1	11	6
50-59	52	0	1	0	1·9	13	2	1	12	6	20	2	1	8·7	4
60-69	45	0	0	0	0	13	4	0	24	0	11	1	0	8·3	0
70-79	18	0	0	0	0	5	1	0	17	0	4	0	0	0	0
80-89	13	0	0	0	0	4	0	1	0	20	5	0	0	0	0
90-99	7	0	0	0	0	2	0	0	0	0	5	0	0	0	0
100-199	13	0	0	0	0	3	0	0	0	0	8	0	0	0	0
>200	3	0	0	0	0	1	0	0	0	0	2	0	0	0	0
Longstanding amenorrhoea	2	0	0	0	0	1	0	0	0	0	1	0	0	0	0
No amenorrhoea	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Postpartum amenorrhoea	[illegible]	0	0	0	0	1	0	0	0	0	1	0	0	0	0

1015625

with one patient each, two and three days respectively after the abortion.

NON-PREGNANT PATIENTS EXCLUDING URINES WITH PROTEIN AND BLOOD This group of patients was divided into menopausal and premenopausal because of the observation (Wide, Roos, and Gemzell, 1961; Lunenfeld, Isersky, and Shelesnyak, 1962; Hamburger and Arquilla, 1963; Taymor, Goss, and Huytendorp, 1963) that antisera to human chorionic gonadotrophin will crossreact with pituitary gonadotrophin. Patients with postpartum amenorrhoea were considered separately because of the observation (Bertini, 1965) that false positives are found in these patients. There were eight patients with postpartum amenorrhoea; all results were negative except one inconclusive with Pregnosticon.

Pregnosticon gave two inconclusive results in the menopausal group which became negative on diluting the urine. These were presumably due to the greater sensitivity of Pregnosticon causing pituitary gonadotrophins to be detected. In the premenopausal group, 0·5% false positives and 0·5% inconclusive were found with Planotest; 2·1% false positives, 4·1% inconclusive with Gravindex; 2·6% false positives, 6·2% inconclusive with Pregnosticon; 4·7% false positives, 8% inconclusive with Pregslide; 3·6% false positives none inconclusive with Hyland A, and 8·7% false positives, 1·4% inconclusive with Hyland B.

The 32 patients who gave false positive or inconclusive results with one or more of the pregnancy tests were thoroughly investigated to find if any cause for these false positives could be found. Three patients were taking steroids, two patients were on contraceptive pills, two on barbiturates, two on penicillin, and one each on chlordiazepoxide and Prochloroperazine and Nitrazepam. Eight patients had infertility, six menstrual irregularities, and two chronic active hepatitis. There were, however, 25 patients on barbiturates, seven patients on a contraceptive pill, four on chlordiazepoxide and three on penicillin, who did not give false positive results, so these compounds do not appear to be the cause of the false positives seen. No false positives were found in patients on salicylates.

NON-PREGNANT PATIENTS WITH URINE CONTAINING BACTERIA, PROTEIN, AND/OR BLOOD In this group of patients, Planotest was the only pregnancy test which did not give a false positive result. False positives with the other pregnancy tests appeared to be related more to protein in the urine than to bacteria but some urines with an *E. coli* infection gave a positive or inconclusive pregnancy test even in the absence of protein with Pregslide or Gravindex.

PREGNANCY TESTS ON BACTERIAL CULTURES Preparations of cultured staphylococci, streptococci, and of an *E. coli*-Proteus mixture were prepared with a bacterial count several times higher than that which would be present in a highly infected urine. These cultures were then tested undiluted and at a dilution of 1 in 10 and 1 in 20. The only pregnancy test to give a positive was Hyland B which reacted down to a 1 in 10 dilution with the staphylococci and gave an inconclusive result with undiluted streptococci.

EFFECT ON PREGNANCY TESTS OF ADDITION OF PROTEIN TO URINE Human albumin,[7] γ globulin,[7] and glycoprotein[8] were added to urines at varying

[7]A.B. Kabi, Stockholm, Sweden (English address, Bilton House Uxbridge Road, W.5).
[8]Koch-Light Laboratories Ltd, Colnbrook, Bucks.

TABLE I—*continued*

RESULTS OF PREGNANCY TESTS IN PREGNANT PATIENTS (EXCLUDING URINES CONTAINING BLOOD AND/OR PROTEIN)

Days after Last Menstrual Period	*Pregslide*					*Gravindex*					*Planotest*				
	+	−	±	*False Negative* (%)	*Inconclusive* (%)	+	−	±	*False Negative* (%)	*Inconclusive* (%)	+	−	±	*False Negative* (%)	*Inconclusive* (%)
35–49	36	6	4	13	9	41	5	0	11	0	48	3	4	6	8
50–59	44	5	4	9	7·5	52	1	0	1·9	0	55	1	0	2	0
60–69	36	6	3	13	6·7	44	1	0	2·2	0	37	0	0	0	0
70–79	18	0	0	0	0	18	0	0	0	0	10	0	0	0	0
80–89	11	0	2	0	15	13	0	0	0	0	18	0	0	0	0
90–99	7	0	0	0	0	7	0	0	0	0	6	0	0	0	0
100–199	13	0	0	0	0	13	0	0	0	0	9	0	0	0	0
>200	2	1	0	—	0	3	0	0	0	0	2	0	0	0	0
Longstanding amenorrhoea	2	0	0	0	0	2	0	0	0	0	1	0	0	0	0
No amenorrhoea	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Postpartum amenorrhoea	2	0	0	0	0	2	0	0	0	0	2	0	0	0	0

Overall results	*Pregnosticon*	*Hyland A*	*Hyland B*	*Pregslide*	*Gravindex*	*Planotest*
No. tested	203	65	78	203	203	195
False negative (%)	0·5	19·5	6·5	9	3·5	2
Inconclusive (%)	2·0	4·5	2·6	6	0	2

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concentrations to investigate the relative non-specificities of the pregnancy tests. Using human albumin at a concentration of 2 g/100 ml urine, positive results were obtained with Hyland B, Pregslide, and Gravindex but not with Pregnosticon or Planotest. At a concentration of 1 g/100 ml negative results were found with all tests. Using human γ globulin, Planotest and Pregnosticon gave inconclusive results at concentrations of 2 and 1 g γ globulin/100 ml. Pregslide and Gravindex gave positive results down to 250 mg/100 ml and Gravindex gave a further two inconclusive results at 125 and 62·5 mg/100 ml. Hyland B gave positive results at concentrations down to 125 mg/100 ml. Human glycoprotein (Cohn fraction VI) gave positive results only with Gravindex and Hyland B. Gravindex and Hyland B gave positive results down to 500 mg/100ml. Gravindex also gave an inconclusive result at 250 mg/100 ml.

TESTS ON FILTERED AND UNFILTERED URINE Although all tests in this series were on filtered urine, a series of tests was carried out with the slide methods to find if filtering was absolutely necessary. Six urines with varying degrees of cloudiness were tested. The only difference found was with one moderately cloudy urine, which gave a positive result with Pregslide on the filtered urine and an inconclusive result on the unfiltered urine. It would appear from this small series that filtration may not be necessary before pregnancy testing with Planotest, Hyland, Pregslide, or Gravindex.

DISCUSSION

To be completely satisfactory a pregnancy test should give neither false positive nor false negative results.

In this survey Planotest was the most satisfactory method for eliminating false positives, giving only 0·5% false positives in protein and blood-free urine from non-pregnant patients compared with 2·1% for Gravindex, 2·6% with Pregnosticon, 4·7% with Pregslide, 3·6% with Hyland A, and 8·7% with Hyland B. False positive immunological tests could be due to a number of different reasons: technical factors (Noto and Miale, 1964), impurity of reagents (Lunenfeld *et al*, 1962; Loewitt, 1966), cross reaction of the antihuman chorionic gonadotrophin (anti-HCG) preparation with antigens other than HCG (Wide *et al*, 1961; Wide and Gemzell, 1962; Goss and Lewis, 1964), and other as yet undetermined causes. Tests using albumin, γ globulin, glycoprotein, and various bacteria have shown that Pregnosticon and Planotest contain the purest reagents, only giving inconclusive results with 1 g γ globulin/100 ml. Since a fairly high proportion of pregnant females may have non-symptomatic bacteriuria and/or proteinuria, a pregnancy test which does not give false positives under these circumstances is wanted (Godts and Mighorst, 1964; Kew, Seftel, and Bloomberg, 1967), otherwise it is necessary to maintain a biological test. The lower limit of sensitivity of immunological pregnancy tests needs to be set at such a level that cross reactions with pituitary gonadotrophins do not interfere. Wide and Gemzell (1962) found that the level of luteinizing hormone in fertile and menopausal urine would be detected as 100 to 400 iu of HCG and it has been suggested that the lower limit of sensitivity should therefore be set at 1,000 iu which is the limit for Pregnosticon. It would appear however, from the two inconclusive results seen in the menopausal urine and the inconclusive results seen in some patients with infertility that even at these level pituitary gonadotrophins may sometimes interfere.

A test which does not give a large number of false negatives is also required, especially in such cases as ectopic pregnancies and threatened abortions when the level of HCG may be much lower than that in normal pregnancy. In this series Pregnosticon was the most suitable method, giving only 0·5% false negatives in normal pregnancies. Planotest 2·0%, Gravindex 3·5%, Pregslide 9%, Hyland A 19·5%, and Hyland B 6·5%. False negative results using immunological pregnancy tests have been reported in ectopic pregnancies (Southam, Sultze, and Cohen, 1963; Gusdon, 1964; Islami, Fisher, and Kupfer, 1964) and in threatened abortions (Hutchinson, Schwartz, and Bates, 1964; Sato and Greenblatt, 1965; Fink and Frie, 1966). Pregnosticon gave the smallest number of false negatives in these conditions in the present work.

The type of pregnancy test used will depend on the type of pregnancy being tested. As a general pregnancy test, Planotest would appear to be the most efficient. In ectopic pregnancies and threatened abortions it may be preferable to use a more sensitive test, such as Pregnosticon or a similar test, but the number of false positives will then be greater.

My thanks are due to the local general practitioners who were so helpful in supplying final diagnoses and other relevant information on their patients, to the Obstetric Department for access to their notes, and to Professor D. N. Baron for valuable help and criticism.

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The November 1968 Issue

THE NOVEMBER 1968 ISSUE CONTAINS THE FOLLOWING PAPERS

Bone marrow in nine cases of clinical glandular fever and a review of the literature J. F. BOYD and D. REID

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Role of erythrocytes in the acute platelet response to operation P. N. BENNETT

Methodological study and a recommended technique for determining the euglobulin lysis time R. CHAKRABARTI, M. BELAWILC, J. F. EVANS, and G. R. FEARNLEY

Clotting factor V activity in plasma cryoproteins A. VON FELTIN, P. W. STRAUB, and P. G. FRICK

Semi-automatic method for routine evaluation of fibrinolytic components D. COLLEN, G. TYTGAT, and M. VERSTRAETE

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Plesiomonas shigelloides in South Australia R. G. COOPER and GERALDINE W. BROWN

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Staining method for the examination of the small intestinal villous pattern in necropsy material IAIN W. DYMOCK and BRENDA GRAY

Mitral stenosis together with a giant cell myocarditis limited to the left atrium I. GILLIE and H. FOX

Porotic hyperostosis and the Gelligaer skull JOHN CULE and I. LYNN EVANS

Semi-automated method for the differential determination of plasma catecholamines HELEN MCCULLOUGH

A stable starch preparation for amylase determinations G. P. FRASER and J. C. B. FENTON

Assessment of thyroid status in pregnant women and in patients taking oral contraceptives by a free thyroxine index T. M. D. GIMLETTE and A. PIFFANELLI

Residual formaldehyde after low-temperature steam and formaldehyde sterilization G. L. GIBSON, H. P. JOHNSTON, and V. E. TURKINGTON

Technical methods

Effect of unconjugated bilirubin on the estimation of serum iron by a modified Sanford technique PAUL M. SMITH, JOHN E. MIDDLETON, and ROGER WILLIAMS

Improved automated methods of protein determination D. F. GIBBS and C. M. J. BRIGHT

Micro modification of the dilute blood clot lysis time for determining fibrinolytic activity C. R. SPITTLE, N. M. LANSDOWN and C. M. HAWKEY

Erythrocyte mechanical fragility test R. G. COOPER, R. A. KAHN, C. N. CORNELL, and M. E. MUHRER

Differentiation of foetal and maternal erythrocytes in formol-fixed tissues C. G. PAINE

Mechanical rotary device for plating out bacteria on solid medium R. FRASER WILLIAMS and JENNIFER M. BAMBURY

Letter to the Editor

Book reviews

The Association of Clinical Pathologists: 81st general meeting

Index for 1968

Contents for 1968

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1075628

Klin. Exp. Ophthalmol. 1975, 194(2), 125-31 (Ger). Human aq. humor contained an av. of 11.8 mg α1-antitrypsin/100 ml and 0.3 mg ceruloplasmin/100 ml, as detd. by immunochem. methods, but no secretory immunoglobulin A was found. The absence of the latter indicates that the aq. humor is mainly a filtration product of the serum.

25554k Coagulation of insoluble and basic proteins from rat seminal vesicle secretion with vesiculase. Influence of collagenase-like peptidase from rat testis. Koren, E.; Schon, E.; Lukac, J. (Lab. Exp. Med., Univ. Zagreb, Zagreb, Yugoslavia). *J. Reprod. Fertil.* 1975, 42(3), 401-5 (Eng). Proteins from the secretion of rat seminal vesicles were fractionated to yield: (1) insol. protein, (2) basic protein insol. in the presence of urea, and (3) neutral plus acidic proteins. The 1st 2 were necessary for the formation in vitro of a coagulum which was very similar to the copulatory plug. Collagenaselike peptidase from rat testis degraded both protein fractions and prevented coagulum formation.

25555m Isolation and characterization of lactoferrin from sow milk and boar seminal plasma. Roberts, T. K.; Boursnell, J. C. (Unit Reprod. Physiol. Biochem., Agric. Res. Counc., Cambridge Engl.). *J. Reprod. Fertil.* 1975, 42(3), 579-82 (Eng). Lactoferrin isolated from sow milk (~0.6 mg/ml) was chromatog. homogeneous, an observation supported by electrophoresis and by reaction against monosp. antilactoferrin antiserum. Isoelec. focusing showed multiple forms of the protein (isoelec. point 9.3 - 10.0) converted by neuraminidase to 1 form (isoelec. point 9.65). Boar seminal plasma contained immunologically identical lactoferrin (0.1 to 0.5 mg/ml) which was bound strongly to boar sperm.

25556n Identification and estimation of plasma proteins in human dentine. Thomas, M.; Leaver, A. G. (Sch. Dent. Surg., Univ. Liverpool, Liverpool, Engl.). *Arch. Oral Biol.* **1975**, 20(3), 217-18 (Eng). The total noncollagenous matrix (NCM) of human dentine was isolated by EDTA extn. followed by collagenase digestion, both procedures being done in a dialysis sac. The presence of serum albumin, transferrin, and the immunoglobulins (IgG, IgA, and IgM) was demonstrated by double diffusion on Ouchterlony plates. Quantitation of the amts. of these serum proteins in human dentine was achieved by the single radial immunodiffusion technique. One gram of dentine NCM contained 20·1 mg of albumin, 2·1 mg of transferrin, and 4·0 mg of IgG; IgA and IgM each occurred at a level of 0·24 mg/g.

25557p Quantitative histochemistry of γ-aminobutyric acid in the human substantia nigra and globus pallidus. Kanazawa, Ichiro; Toyokura, Yasuo (Fac. Med., Univ. Tokyo, Tokyo, Japan). *Confin. Neurol.* **1974**, 36(4-6), 273-81 (Eng). In the normal adult human substantia nigra, there was a marked unevenness of the γ-aminobutyric acid (I) distribution, with I being most highly concd. in the pars reticulata. In the human globus pallidus, there were high levels of I both in the external and internal segments at almost the same concn. The I level was higher in the dorsal than in the ventral parts of the pallidum. Caudate destruction in the monkey decreased the I concn. in both external and internal segments of the pallidum and the medial 67% of the nigra, suggesting that the caudate nucleus sends I-operated fibers to these brain regions.

25558q Comparison of G-, Q-, and EM-banding patterns exhibited by the chromosome complement of the Indian muntjac, Muntiacus muntjak with reference to nuclear DNA content and chromatin ultrastructure. Green, Richard J.; Bahr, Gunter F. (Armed Forces Inst. Pathol., Washington, D. C.). *Chromosoma* **1975**, 50(1), 53-67 (Eng). When the chromosomes of the Indian muntjac were compared following treatment with 2 presently used banding methods, trypsin-Giemsa (G) and quinacrine-HCl (Q), with structural bands as seen in the electron microscope, definite correlations were obsd. with respect to the nos. and positions of individual bands. Wts. obtained for the individual chromosomes were: No. 1 9.89 pg, No. 2 4.10 pg, No. 3 4.43 pg, No. 3-X 5.05 pg and Y 0.55 pg. Avg. diameters and wts. for individual fibers were 193 Å and 8.74×10^{-16} g/μ, resp., for stimulated metaphase chromosomes and 185 Å and 8.73×10^{-16} g/μ, resp., for unstimulated chromosomes. Fibers of interphase nuclei exhibited an av. diameter of 191 Å and a wt. of 5.87×10^{-16} g/μ. The total amt. of nuclear DNA present in interphase nuclei was 3.88 pg.

25559r Immunochemical determination of soluble antigens in protein fractions from placental tissue. Brock, J.; Straube, W.; Hofmann, R.; Klausch, B.; Friemel, H. (Inst. Physiol. Chem., Univ. Rostock, Rostock, E. Ger.). *Zentralbl. Gynaekol.* **1975**, 97(5), 281-7 (Ger). Protein fractions were prepd. from placental tissue by homogenization, $(NH_4)_2SO_4$ fractionation, gel filtration, and ion-exchange chromatog. Some of antigens detected in these fractions by absorbed antiserums against placental tissue and pregnancy serum were present in pregnancy and fetal serum, also. The other antigens, which were found neither in pregnancy serum nor in fetal serum, could be specific for placental tissue. Their possible significance is discussed.

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25560j Physiology and biochemistry of oviducal fluid with special reference to the presence of sulfated glycoproteins in the oviducal fluid of the rabbit. Yamazaki, K. (Dep. Gynecol. Obstet., Fukushima Med. Coll., Fukushima, Japan). *Fertil. Steril., Proc. World Congr., 7th* 1971 (Pub. 1973), 206 (Eng). Edited by Hasegawa, Toshio; Hayashi, Motoyuki; Ebling, F. J. G. Excerpta Med.: Amsterdam, Neth. The sexual cycle, estrogen, progesterone, and autonomic nervous system drugs were shown to affect the secretion of oviducal fluid in rabbits. The chem. compn. of the oviducal fluid, ascites, and blood serum from estrous, pregnant, and castrated rabbits was also analyzed. Glycoproteins isolated from the Pronase digest of the oviducal fluid were essentially different from those in ascites and blood serum.

25561k Acrosome of the human spermatozoon. Morphology and enzyme content. Pedersen, H.; P., H. (Dep. Anat., Harvard Med. Sch., Boston, Mass.). *Fertil. Steril., Proc. World Congr., 7th* 1971 (Pub. 1973), 471-2 (Eng). Edited by Hasegawa, Toshio; Hayashi, Motoyuki; Ebling, F. J. G. Excerpta Med.: Amsterdam, Neth. In acrosomal material isolated from human spermatozoa, there was a trypsin-like enzyme which digested benzoyl-L-arginine Et ester and p-tosyl-L-arginine Me ester and was inhibited by soybean trypsin inhibitor, seminal plasma, and fresh serum.

25562m Human fallopian tube fluid. Identification of proteins and amino acids. Moghissi, K. S. (Sch. Med., Wayne State Univ., Detroit, Mich.). *Fertil. Steril., Proc. World Congr., 7th* 1971 (Pub. 1973), 774-7 (Eng). Edited by Hasegawa, Toshio; Hayashi, Motoyuki; Ebling, F. J. G. Excerpta Med.: Amsterdam, Neth. The pattern of proteins and amino acids in human fallopian tubal fluid suggested both transudation and active secretion, as supported by a similar distribution of major protein fractions, the absence of immunoglobulin M, the presence of a slightly higher concn. of certain protein bands in immunoelectrophoretic studies (haptoglobulin and ceruloplasmin), and the finding of a specific tubal protein (X-glycoprotein) absent in serum.

25563n Placental γ-glutamyl transpeptidase. Landon, M. J. (Med. Res. Counc. Growth Unit, Princess Mary Matern. Hosp., Newcastle upon Tyne, Engl.). *Int. J. Biochem.* 1975, 6(5), 341-4 (Eng). γ-Glutamyl transpeptidase activity was measured in a series of normal full term placentas. The pH optimum of this enzyme was 8·5 and the activity ranged from 107 to 333 munrTs/g wet wt.

25564p Mineral content of various tissues in cattle. 4. Dry matter, calcium, magnesium, sodium, and potassium contents of mucosa and muscular layers of various segments of the gastrointestinal tract. Dittrich, Bernd; Kolb, Erich (Sekt. Tierprod. Veterinaermed., Karl Marx Univ. Leipzig, Leipzig, E. Ger.). *Arch. Exp. Veterinaermed.* **1975**, 29(2), 171-9 (Ger). Among various parts of the gastrointestinal tract of cattle, there were considerable differences in mineral content. Forestomach mucosa contained high concns. of Ca and Mg, attributable to accumulation and sepn. of mineral salts in this tissue. There was generally more Mg than Ca in the 3 segments of the small intestine, and high concns. of Na and K were present. The results are intended as a basis for comparison with pathol. states.

25565q Alkaline phosphatase activity in blood serum, bile, and urine of cattle and calves. Hadlich, Manfred; Kolb, Erich (Sekt. Tierprod. Veterinaermed., Karl Marx Univ. Leipzig, Leipzig, E. Ger.). *Arch. Exp. Veterinaermed.* **1975**, 29(2), 181-4 (Ger). Alk. phosphatase (I) activities (in King-Armstrong units) in the serum of calves, cattle, and bulls were 909, 131, and 325, resp., and these values were statistically significant. Values for bile I activity were: calves 416, cattle 546, and bulls 738; these differences were not statistically significant. In urine, I activities were: calves 0.77, cattle 0.?? and bulls 1.69. The relations between the I activity in the serum and urine are discussed.

25566r Activities of potassium and sodium ions in rabbit heart muscle. Lee, Chin Ok; Fozzard, Harry A. (Pritzker Sch. Med., Univ. Chicago, Chicago, Ill.). *J. Gen. Physiol.* **1975**, 65(6), 695-708 (Eng). Activities of intracellular K^+ and Na^+ in rabbit ventricular papillary muscles were detd. with cation-selective glass microelectrodes and concns. were estd. with flame photometry. The concn. and activity of K^+ of the muscles were 134.9 and 82.6 m*M*, resp., at 25°. The corresponding concn. and activity of Na^+ were 32.7 and 5.7 m*M*, resp. The apparent intracellular activity coeffs. for K^+ (γ_K) and Na^+ (γ_{Na}) were 0.612 and 0.175, resp. Similar results were obtained at 35°. γ_K was substantially lower than the activity coeff. (0.745) of extracellular fluid (Tyrode's soln.), which might be expected on the basis of a different intracellular ionic strength. γ_{Na} was much lower than that of extracellular fluid, thus much of the Na^+ was compartmentalized or sequestered. For external K^+ concns. >5 m*M*, the resting membrane potentials agreed well with the potential differences calcd. from the K^+ activity gradients across the cell membrane as a K^+ electrode. Thus, K^+ equil. potentials in heart muscle should be calcd. by activities rather than concns.

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agitation. Thus, the application of agitation to the CPD-H blood storage system was of no great benefit in preventing metabolic intermediate levels.

25700e Potassium load in CPD citrate phosphate dextrose-preserved whole blood and two types of packed red blood cell [illegible]

[illegible] conservation of stored blood [illegible]

25701f Hemolysis and lysosomal activation by solid state tyrosine. [illegible] Duke Univ., Durham, N. [illegible] 1975, [illegible] (L-Tyrosine crystals hemolysed human erythrocytes. Erythrocytes with [illegible] were [illegible] L-Tyrosine crystals [illegible] lysosomal β-glucuronidase from rat liver lysosomal preps. Sucrose, glucose, propylene glycol, Me_2SO, polyethylene glycols, serum, albumin, globulins, and cyclic AMP protected against the effect of tyrosine. Tyrosine analogues with a lower pK of the phenolic hydroxyl group than tyrosine, or with steric blockage of the phenolic hydroxyl group, had less hemolytic activity than tyrosine. Apparently, tyrosine crystals in vitro possess membranolytic effects by donation of phenolic hydrogens to membrane components.

25702g Pregnancy-specific β_1-glycoprotein (SP-1). Clinical aspects. Tatra, G.; Placheta, P.; Breiteneckar, G. (Gynäkol. Univ. Frauenklin. Wien, Vienna, Austria). *Wien. Klin. Wochenschr.* **1975**, 87(8), 279-81 (Ger). Single radial immunodiffusion provided a simple and highly reproducible clin. assay for pregnancy-specific β_1-glycoprotein SP-1 in maternal serum. The half-time for elimination of SP-1 from the serum was 34 hr postpartum. There was a direction correlation between SP-1 concns. in maternal serum and placental wt. and birth wt. of the infant.

25703h Role of potassium ion loss in the anoxic impairment of respiration of rat cerebral-cortex slices. Patel, Noshirwan J.; Fixter, Leslie M. (Dep. Biochem., Univ. Glasgow, Glasgow, Scot.). *Biochem. Soc. Trans.* **1975**, 3(1), 101-2 (Eng). Preincubation of rat cerebral cortex slices with high-speed brain homogenate supernatant at 37° for 60 min prevented the anoxic impairment of subsequent respiration obsd. after anaerobic preincubation. K^+ was involved as preincubation in isoosmotic KCl-Tris-HCl (pH 7.4, 30 m*M* KCl) was nearly as effective as the homogenate. K^+ was not replaced by Na^+, Li^+, or Tris. Aerobically preincubated slices had a K^+ concn. of 26.5 μequiv/g final wet wt., whereas anaerobically preincubated slices had a K^+ concn. of 6.9 μequiv/g final wet wt. Conditions of preincubation that protected against anoxic impairment of respiration maintained the K^+ concn. of slices at 20 μequiv/g final wet wt. or higher.

25704j Age-related changes in lipid peroxidation in rat liver mitochondria. Barrett, Michael C.; Horton, Alan A. (Dep. Biochem., Univ. Birmingham, Birmingham, Engl.). *Biochem. Soc. Trans.* **1975**, 3(1), 124-6 (Eng). The capacity of rat liver mitochondria for undergoing peroxidn. increased ~2.3 fold as the age of the rat increased from 3 to 24 months, whereas the degree of unsatn. of the mitochondrial lipids fell from 3 to 12 months of age and rose again from 12 to 24 months. This may be due to an age-related increase in the proportion of polyunsatd. fatty acids and a decrease in the proportion of monounstad. fatty acids in the mitochondrial lipids.

25705k Effect of confinement of the circadian rhythm of ovine cortisol. Holley, D. C.; Beckman, D. A.; Evans, J. W. (Dep. Anim. Sci., Univ. California, Davis, Calif.). *J. Endocrinol.* **1975**, 65(1), 147-8 (Eng). The circadian rhythm of plasma cortisol was similar in sheep before and during a 10-day period of cage restraint (single animals in small cages). Two peaks in cortisol concns. were obsd., at 4:00 a.m. and 4:00 p.m., the latter possibly resulted from feeding at that time.

25706m Role of plasma volume in the increase of aldosterone secretion rate during sodium deprivation. Dalakos, T. G.; Streeten, D. H. P. (Dep. Med., State Univ. New York, Syracuse, N. Y.). *Clin. Sci. Mol. Med.* **1975**, 48(3), 161-5 (Eng). Redn. of Na intake from 200 to 10 mmoles/day increased the aldosterone secretion rate without altering plasma vol. With a Na intake of 10 mmoles/day, the aldosterone secretion rate was unchanged by salt-poor infusion of albumin (75 g in 150 ml), although plasma vol. was increased by this infusion. This indicated that Na restriction did not stimulate aldosterone secretion by a redn. in plasma vol.

25707n Partial reversal by adenosine triphosphate of the rigor-induced decrease in the diameter of skeletal muscle fibers. [illegible] 1975, 50(4A), [illegible] reversal of the [illegible]

25708p Effect of a ultrahigh frequency field on the activity of certain enzymes and the level of pyridoxine in the organs of albino rats. [illegible] Zlatin'ych, N. V.; [illegible] Med. Inst., Perlovka, Tomsk, USSR). *Gig. Sanit.* **1975**, [illegible] 11-14 (Russ). In rats after chronic exposure to ultrahigh-frequency electromagnetic field (2 GHz, [illegible] μW/cm²), levels of pyridoxine were decreased in blood, brain, liver, kidney, and heart and increased in skeletal muscle 1.5-2 fold. In all the organs, activity of succinate dehydrogenase decreased. Pyridoxine deficiency did not worsen the effect of the field on this enzyme. In blood, aspartate aminotransferase decreased and alanine aminotransferase increased. Thus, chronic exposure to ultrahigh frequency field may affect some nutritional requirements of the animal.

25709q Effects of diet, lipolysis, and limb ischemia on the distribution of plasma tryptophan in the rat. Stoner, H. B.; Cunningham, V. J.; Elson, Patricia M.; Hunt, A. (Toxicol. Unit, Med. Res. Counc., Carshalton, Engl.). *Biochem. J.* **1975**, 146(3), 659-66 (Eng). A nonlinear relation was found between nonesterified fatty acids concn. in blood plasma and the percentage of free plasma tryptophan in rats in different nutritional states, but this was not seen in rats injured by limb ischemia. (−)-Isoprenaline bitartrate (200 μg/kg, s.c.), which is known to increase plasma nonesteritied fatty acid concn., decreased total plasma tryptophan concn. Dichloroisoprenaline-HCl (17 mg/kg, i.v.) produced a sustained increase in nonesterified fatty acids accompanied by an increase in free plasma tryptophan concn., which was followed by a fall in total tryptophan concn. The loss of plasma tryptophan was attributed to altered distribution in the extracellular space rather than to increased metab.

25710h DNA-to-protein crosslinking in synchronized HeLa cells exposed to ultraviolet light. Han, Antun; Korbelik, Mladen; Ban, Jasna (Lab. Exp. Cancerol., Cent. Inst. Tumor Allied Dis., Zagreb, Yugoslavia). *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med.* **1975**, 27(1), 63-74 (Eng). The max. yield of DNA-to-protein crosslinking in uv-irradiated HeLa cells was obsd. during the S phase with much smaller yields in the G_1 and G_2 phases. Exposure of mid-S cells to 250 ergs/mm² rendered ~22% of the DNA unextractable due to crosslinking. Susceptibility to crosslinking correlated with the lethal effects of uv-irradn. on the cells. Up to ~500 ergs/mm², the amt. of DNA recovered from S cells decreased by ~8% for every 100 ergs/mm² of uv light. No recovery from crosslinking damage was obsd. for 8 hr after exposure.

25711j Dependence of differential staining of heterochromatin on the time of its replication and the degree of condensation. Tupitsina, L. P.; Zakharov, A. F.; Tsvetkova, T. G. (Lab. Gen. Cytogenet., Inst. Med. Genet., Moscow, USSR). *Tsitologiya* **1975**, 17(3), 261-5 (Russ). A comparison of the chromosomes banding pattern after G- and C-staining with the time of DNA replication and the degree of chromosome condensation, was carried out using Chinese hamster metaphase chromosomes. Chromosome condensation was studied during 5-bromodeoxyuridine and 5-bromodeoxycytidine treatment. All the chromosomal segments stained with the C-technique were also stainable with G-technique, whereas only some G-pos. segments were able to be C-bands. C-bands were heterochromatic segments characterized by extremely late replication with great delay in condensation under the action of the DNA nucleotide analogs, while G-bands were segments with earlier labeling and irregular decondensation. There is apparently a close correlation between chromosomal G- and C-staining ability of different regions and the degree of heterochromatinization. J. R. Mase

25712k Study of renaturation of the DNP [deoxyribonucleoprotein] complex of cellular nuclei by means of microfluorimetry. Artyushin, S. K.; Lideman, R. R.; Zlobina, G. P. (Lab. Gen. Pathol. Physiol., Inst. Psychiatry, Moscow, USSR). *Byull. Eksp. Biol. Med.* **1975**, 79(3), 44-6 (Russ). The intensity of fluorescence of the DNP of lymphocyte cell nuclei upon transfer of the cells to a cold citrate salt soln. after previously heating to various temps. was measured as a function of time by a modification of the method of R. Rigler and D. Killander (1969). No renaturation occurred in prepns. heated to 65°, but renaturation did occur in prepns. heated to 75°, 85°, or 100°, as judged by the characteristic fluorimetric profiles. A. Kuby

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Immunochemischer Nachweis löslicher Antigene in Proteinfraktionen aus Plazentagewebe

Von J. Brock, W. Straube, R. Hofmann, B. Klausch und H. Friemel

Mit 5 Abbildungen

Zusammenfassung: Proteinfraktionen aus Plazentagewebe wurden durch Homogenisieren, Ammoniumsulfat-Fällung, Gelfiltration und Ionenaustauschchromatographie gewonnen. Die in diesen Fraktionen mit absorbierten Antiseren gegen Schwangerenserum bzw. Plazentaextrakt nachweisbaren Antigene sind zum Teil auch im mütterlichen oder fetalen Serum vorhanden. Die übrigen Proteine, die weder im mütterlichen noch fetalen Serum vorkommen, könnten plazentaspezifisch sein. Ihre mögliche Bedeutung wird diskutiert.

Summary: Protein fractions were prepared from placental tissue by homogenization, ammonium sulfate fractionation, gel filtration and ion exchange chromatography. The antigens detected in these fractions by absorbed antisera against placental tissue and pregnancy serum partly occur in the pregnancy and fetal serum too. The other antigens, which are found neither in pregnancy serum nor in fetal serum, could be specific for placental tissue. Their possible significance is discussed.

Die Schwangerschaft kann nach Billingham [4] als eine parabiotische Verbindung von 2 genetisch differenten Individuen aufgefaßt werden. Mütterlicher und fetaler Organismus befinden sich in einem Zustand der immunologischen Homöostase; die Plazenta scheint hierbei eine zentrale Rolle zu spielen [20, 23, 26, 37].

Die immunologische Bedeutung der Plazenta ist im wesentlichen durch 4 Aspekte gekennzeichnet [36]:

1. Plazenta als immunologische Barriere für Antigene, antigentragende und immunkompetente Zellen sowie Immunglobuline.
2. Immunogenität der Plazenta.
3. Immunologische Kompetenz der Plazenta.
4. Immunologie der Trophoblasttumoren.

Die vorliegenden Untersuchungen sollen ein Beitrag zum 2. Aspekt, d. h. zur Frage der Immunogenität der Plazenta, darstellen. In diesem Zusammenhang ist besonders die Immunogenität des Trophoblasten von Interesse, der fetalen Ursprungs ist und damit wie der Fetus per definitionem als allogenes Transplantat im mütterlichen Organismus angesehen werden könnte. Auf der Ebene der Trophoblastenschicht dürften sich fetale Antigene, die zu 50% väterliches Erbgut enthalten, und die mütterlichen immunologischen Erkennungsmechanismen unmittelbar gegenüberstehen. Nach unserem heutigen Wissen enthält die Plazenta Transplantationsantigene, mütterliche und fetale Serumproteine sowie organspezifische und nichtorganspezifische zytoplasmatische Proteine, wobei letztere möglicherweise ebenfalls immunogen wirken können [5, 13, 22, 36]. Bohn [7] konnte in Proteinfraktionen aus Plazentagewebe insgesamt 6 lösliche Antigene immunchemisch darstellen, die sich weder im mütterlichen noch im fetalen Serum nachweisen ließen. Unter den in der Plazenta reichlich vorhandenen Serumproteinen

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befinden sich auch Schwangerschaftsproteine, die ausschließlich oder vorzugsweise bei Schwangeren vorkommen [1, 6, 10, 17, 24].

Kenntnisse über Natur, physikalisch-chemische Eigenschaften und biologische Funktionen von den in der Plazenta vorkommenden löslichen Antigenen könnten einen Beitrag zum Verständnis immunologischer Reaktionsabläufe in der Schwangerschaft leisten.

Im folgenden wurde der Versuch unternommen, die in Proteinextrakten aus Plazentagewebe enthaltenen Antigene immunchemisch nachzuweisen und teilweise zu reinigen. Aus dem Verhalten dieser Antigene bei der Fraktionierung lassen sich ferner einige Erkenntnisse über ihre physikalisch-chemischen Eigenschaften ableiten.

Material und Methoden

Plazentaextrakt: Die Herstellung des Plazentaextraktes erfolgte aus menschlichen Plazenten, wie sie nach normalem Schwangerschaftsverlauf anfallen, durch Homogenisieren im Ultra-Turrax (Janke und Kunkel, Staufen, BRD) mit M/15 Phosphat-Puffer pH 7,4 und nachfolgendem Zentrifugieren bei 20 000 g [15].

Ammoniumsulfat-Fraktionierung: Dieser Extrakt wurde durch stufenweisen Zusatz von Ammoniumsulfat bei pH 7,0 und 4 °C bis zum Sättigungsgrad 0,3, 0,4, 0,5, 0,6, 0,7 und 0,8 fraktioniert [21]. Die Präzipitate wurden abzentrifugiert, in Sörensen-Puffer pH 7,4 gelöst und erneut bei der entsprechenden Sättigung präzipitiert. Danach wurde gegen Sörensen-Puffer pH 7,4 oder 0,15 M NaCl dialysiert und mit Hilfe von Polyäthylenglykol 20 000 (Fluka, Buchs, Schweiz) eingeengt.

Gelchromatographie und Ionenaustausch: Die Gelchromatographie erfolgte an Sephadex G-200, der Ionenaustausch an DEAE-Sephadex A-50 mit Hilfe eines linearen Gradienten 0,05 M Tris HCl pH 8,0 bis 0,5 M Tris HCl pH 8,0 [17].

Immunodiffusion und Immunelektrophorese: Die Fraktionen wurden mit Hilfe der Immunodiffusion nach Ouchterlony [2, 28] und der Immunelektrophorese nach Scheidegger [12, 30] mittels absorbiertem Anti-Plazentaserum (aAPl) sowie absorbiertem Anti-Schwangerenserum (aASS) getestet.

Immunseren: Absorbiertes Anti-Plazentaserum [15] bzw. Anti-Schwangerenserum [14] wurden durch Immunisierung mit Plazentaextrakt bzw. Schwangerenserum und nachfolgender Absorption mit Humanserum nach Böszörmenyi [2, 11, 14] erhalten.

Ergebnisse

Der stufenweise Zusatz von Ammoniumsulfat zum Plazentaextrakt ergab, daß die mit aASS nachweisbaren Antigene im wesentlichen bei einem Sättigungsgrad von 0,4 Ammoniumsulfat präzipitieren. Bei der Erhöhung auf einen Sättigungsgrad von 0,5 fällt nur noch eine geringe Menge Antigenmaterial aus, das mit aASS reagiert. Der Ouchterlony-Test mit aAPl zeigt auch mit den oberhalb des Sättigungsgrades 0,4 erhaltenen Fraktionen Präzipitationslinien (Abb. 1). Der Ouchterlony-Test sowohl mit aASS als auch mit aAPl läßt erkennen, daß durch eine Präzipitation mit Ammoniumsulfat bei einem Sättigungsgrad von 0,4 eine günstige Vorfraktionierung erzielt werden kann. Daher wurde eine bei dem Sättigungsgrad 0,4 erhaltene Fraktion weiter an Sephadex G-200 fraktioniert [17]. Es resultierten 4 Peaks (Abb. 2). Die immunchemische Analyse ergab folgende Ergebnisse (Abb. 3 und 4):

1. Im 1. Peak waren je 2 Antigene mit aAPl und aASS nachzuweisen.
2. Bei Peak 2 präzipitierte ein Antigen mit aAPl und ein Antigen mit aASS. Im Ouchterlony-Test mit aASS war in geringer Konzentration ein 2. Antigen nachweisbar, das in höherer Konzentration im Peak 1 enthalten war.
3. Im 3. Peak waren mit aAPl 2 Antigene präzipitierbar. Mit aASS wurden keine Präzipitationslinien beobachtet. Auf Grund der Ouchterlony-Teste nach der fraktionierten

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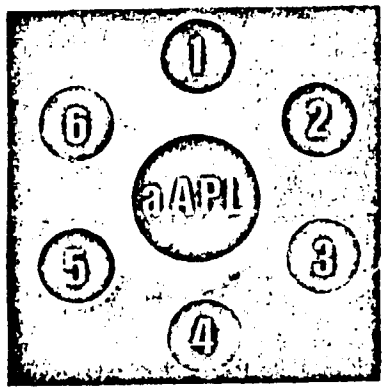


Abb. 1. Ouchterlony-Test nach fraktionierter Ammoniumsulfat-Fällung des Plazentaextraktes. 1:0 – 0,3, 2:0,3 – 0,4, 3:0,4 – 0,5, 4:0,5 – 0,6, 5:0,6 – 0,7, 6:0,7 – 0,8 Sättigung aAPL: absorbiertes Anti-Plazentaserum

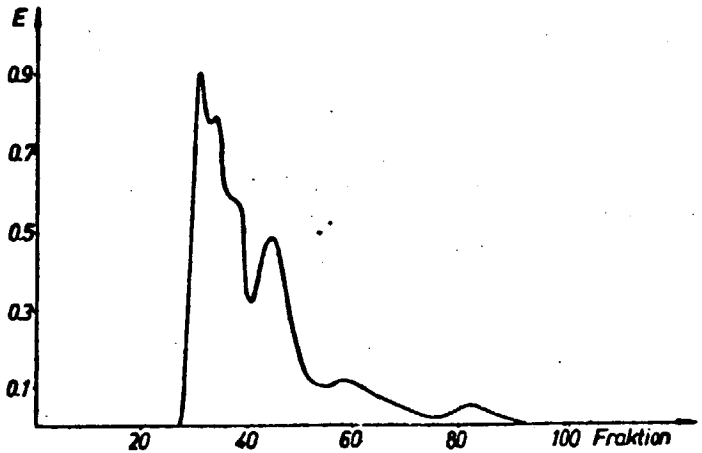


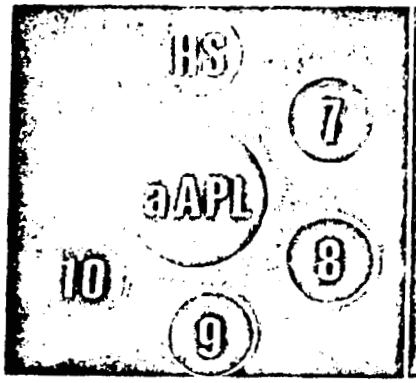
Abb. 2. Elutionskurve des Ammoniumsulfat-Präzipitats (0 bis 0,4 Sättigung) an Sephadex G-200. Fraktionen 28 – 40: 1. Peak, Fraktionen 41 – 53: 2. Peak, Fraktionen 54 – 74: 3. Peak, Fraktionen 78 – 91: 4. Peak

Fällung mit Ammoniumsulfat kann angenommen werden, daß diese Antigene nur zu einem geringen Prozentsatz bei einem Sättigungsgrad von 0,4 ausgesalzt werden.

4. Der 4. Peak enthielt weder mit aAPl noch mit aASS präzipitierbare Proteine.

Die im 3. Peak mit Anti-Plazentaserum nachgewiesenen Antigene ließen sich durch Ionenaustausch an DEAE-Sephadex A-50 voneinander isolieren [17], (Abb. 5).

An Hand des Ouchterlony-Tests und der Immunelektrophorese ließ sich ein Antigen im Peak 1 nach der Gelchromatographie an Sephadex G-200 als Protein der „pregnancy



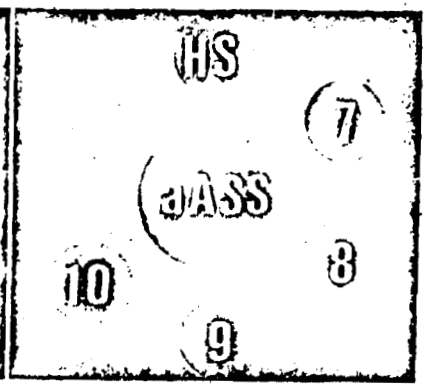


Abb. 3. Ouchterlony-Test nach der Gelfiltration an Sephadex G-200 7: 1. Peak, 8: 2. Peak, 9: 3. Peak, 10: 4. Peak. HS: Humanserum, aAPL: absorbiertes Anti-Plazentaserum. — Abb. 4. Ouchterlony-Test nach der Gelfiltration an Sephadex G-200. 7: 1. Peak, 8: 2. Peak, 9: 3. Peak, 10: 4. Peak. HS: Humanserum, aASS: absorbiertes Anti-Schwangerenserum

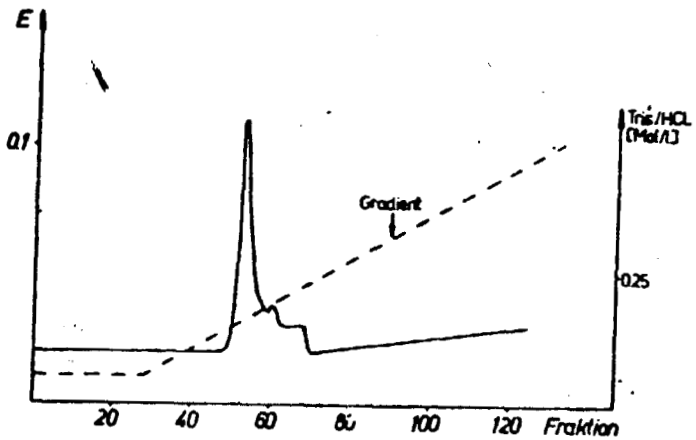


Abb. 5. Elutionsprofil nach Chromatographie an DEAE-Sephadex A-50

zone" (schwangerschaftstypisches Antigen Nr. 2 nach Hofmann und Mitarb. [16]) identifizieren, das im α_2-Bereich wandert. Dieses Protein präzipitiert sowohl mit aAPl als auch mit aASS. Das 2. Antigen dieses 1. Peaks nach der Gelfiltration ist das schwangerschaftstypische Antigen Nr. 3 nach Hofmann und Mitarb. [16]. Es wandert im β-Bereich und ist ebenfalls mit aAPl und aASS präzipitierbar. Dieses Antigen ist auch im Schwangerenserum, Wöchnerinnenserum und Fruchtwasser enthalten [16]. Das Antigen im 2. Peak nach Gelchromatographie ist ebenfalls ein im β-Bereich wandernde Protein. Während die Antigene des 1. und 2. Peaks sowohl mit aAPl als auch mit aASS reagieren, präzipitieren die beiden mit aAPl nachweisbaren Antigene des 3. Peaks nicht mit aASS, das heißt sie sind fetus- bzw. plazentaspezifisch. Eines dieser beiden Antigene

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wurde als Nabelschnurserum-Antigen oder schwangerschaftstypisches Antigen Nr. 4 nach Hofmann und Mitarb. [16] identifiziert [17]. Das 2. Antigen ist weder im Fruchtwasser noch im Nabelschnurserum nachzuweisen. Da es auch nicht mit aASS reagiert, könnte es in der Plazenta gebildet werden.

Diskussion

Mit aAPl waren in der Immunelektrophorese bis zu 8 Präzipitationslinien zu identifizieren. Der Nachweis dieser Präzipitationslinien bedeutet aber aus mehreren Gründen nicht, daß alle nachgewiesenen Antigene plazentaspezifischer Natur sind:

1. Einige dieser Antigene lassen sich auch im Schwangerenserum bzw. Nabelschnurserum sowie in Fraktionen daraus nachweisen [33].
2. Die Serumkonzentration mancher Antigene ist so gering, daß sie sich dem Nachweis im Ouchterlony-Test bzw. in der Immunelektrophorese entziehen, was z. B. für das humane Plazentalaktogen zutrifft
3. Es besteht außerdem die Möglichkeit, daß die Antigene in anderen Organen ebenfalls vorhanden sind. So ist bekannt, daß Plazentaantigene z. B. mit Antigenen der Niere und Leber kreuzreagieren können [7,22].

Das nach Ammoniumsulfat-Fällung (0,4 Sättigung) bei der Gelchromatographie an Sephadex G-200 im 1. Peak wandernde Protein der „pregnancy zone" läßt sich sowohl in der Plazenta als auch im Schwangerenserum nachweisen. Darüber hinaus konnte dieses Protein in Übereinstimmung mit anderen Autoren [1, 6, 10] in Seren von nichtschwangeren Frauen nachgewiesen werden, die unter dem Einfluß hormonaler Kontrazeptiva standen [34]. Bohn [8] fand auch in männlichen Normalseren nach einer Konzentrierung in fast allen Fällen dieses Protein, ein Befund, den wir nicht bestätigen können. Die Tatsache, daß das „pregnancy zone"-Protein auch bei Patienten mit Tumoren und entzündlichen Erkrankungen gefunden wurde, führte zu der Vorstellung, daß es zu den Reaktanten der akuten Phase zu rechnen sei [8, 10]. Im Nabelschnurserum kann das „pregnancy zone"-Protein nicht nachgewiesen werden. Das Molekulargewicht wird von v. Schoultz und Mitarb. [31] mit 326 000 Dalton angegeben. Nach Untersuchungen anderer Arbeitsgruppen [24, 32] gibt es Hinweise für ein wesentlich höheres Molekulargewicht, was mit unseren Erfahrungen übereinstimmt. Inwieweit das Protein der „pregnancy zone" mit dem von Krieg [22, 23] beschriebenen trophoblastspezifischen α_2-Globulin identisch ist, kann z. Z. noch nicht entschieden werden. Krieg konnte dieses trophoblastspezifische α_2-Globulin auch nach intensiver Perfusion der Plazenta isolieren. Nach unserer Erfahrung verarmt die Plazenta mit zunehmender Perfusionsdauer an Antigenen, so daß nach längerer Perfusionsdauer mit 0,15 M Kochsalzlösung mittels aAPl keine Antigene mehr nachgewiesen werden können.

Das ebenfalls im 1. Peak nachgewiesene Antigen (schwangerschaftstypisches Antigen Nr. 3 nach Hofmann und Mitarb. [16]) präzipitiert ebenfalls sowohl mit aAPl als auch mit aASS. Wie das „pregnancy zone"-Protein ist es also nicht plazentaspezifisch. Das Molekulargewicht dürfte geringer sein als das des „pregnancy zone"-Proteins, da es auch im 2. Peak nach der Gelfiltration auftritt, wenn auch in geringerer Konzentration. Es könnte mit dem schwangerschaftsspezifischen β_1-Glykoprotein (SP_1) nach Bohn identisch sein [6, 9, 10]. Doch spricht das vom gleichen Autor ermittelte Molekulargewicht von 20 000 Dalton [9, 10] dagegen, das zu niedrig erscheint.

Das Hauptantigen im 2. Peak nach der Gelfiltration an Sephadex G-200 läßt sich ebenfalls im Schwangerenserum nachweisen, das heißt es ist nicht plazentaspezifisch. Das Molekulargewicht ist geringer als das der beiden vorher beschriebenen Antigene. Es besteht daher die Möglichkeit, daß es mit dem von Bohn [6, 10] als schwangerschaftsassoziiertes β_1-Glykoprotein (SP_2) bezeichneten Protein mit einem Molekulargewicht von ~ 100 000 Dalton identisch ist. Letzteres ist mit dem Steroid-bindenden β-Globulin identisch [10] und soll ebenso wie das Protein der „pregnancy zone" zu den Reaktanten der akuten Phase gehören [8].

Die im 3. Peak nach der Gelfiltration an Sephadex G-200 gefundenen Antigene lassen sich an DEAE-Sephadex A-50 auftrennen. Ein Antigen ist mit dem Nabelschnurserum-Antigen (schwangerschaftstypisches Antigen Nr. 4 nach Hofmann und Mitarb. [16]) identisch. Das 2. Antigen dieses Peaks kann plazentaspezifisch sein.

Die vorliegenden Untersuchungen haben gezeigt, daß die Plazenta neben den Transplantationsantigenen der Trophoblastzellmembran weitere potentielle Antigene enthält. Es bleibt die Frage zu beantworten, inwieweit diese potentiellen Antigene tatsächlich während der Schwangerschaft im mütterlichen Organismus immunogen wirken. Für eine solche immunogene Wirkung gibt es mehrere Hinweise. Antikörper gegen Plazentaantigene konnten im Schwangeren- und Wochnerinnenserum nachgewiesen werden [19, 23, 27]. Auch war es im Tierexperiment möglich, mit Hilfe von Anti-Plazentaserum Abortus auszulösen [3]. Daß es trotz der nachgewiesenen Antigenität der Plazenta nicht zur Abstoßung dieses allogenen Gewebes kommt, läßt verschiedene Deutungsmöglichkeiten zu (Übersichten bei [18, 36]). Einmal könnte die bereits in der Frühschwangerschaft beginnende Einschwemmung von Trophoblast-Sproßlingen in die mütterliche Zirkulation zur Induktion einer Toleranz und oder Bildung blockierender Enhancement-Antikörper führen. Darüber hinaus gibt es Hinweise dafür, daß die Oberfläche des Trophoblasten mit einer azellulären Deckschicht („Fibrinoid", Sialomuzin) „maskiert" und damit funktionell antigenfrei ist. Schließlich besteht die Möglichkeit einer immunsuppressiven Wirkung bestimmter Plazentaproteine [29, 35], wie sie bereits für α-Globuline im Serum beschrieben worden ist [25].

Inwieweit der qualitative und quantitative Nachweis der beschriebenen Plazentaantigene im mütterlichen Serum für die klinische Diagnostik einer gestörten Schwangerschaft anwendbar ist, muß vorerst offen bleiben.

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Lin (1)

Characterization of four human pregnancy-associated plasma proteins

4 Preg-Assoc. Proteins like Preg. Plasma with Lactogen

None is

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Four proteins have been detected in third-trimester human pregnancy plasma by gel immunodiffusion methods, using antisera to pregnancy plasma that had been exhaustively absorbed with nonpregnancy plasma. These antisera failed to react with plasma from untreated normal nonpregnant females or from males. Two of the pregnancy-associated plasma proteins (PAPP-B and PAPP-C) migrated as β-globulins and the other two (PAPP-A and PAPP-D) as α2-globulins in immunoelectrophoresis. PAPP-B was regularly detected only by crossed-immunoelectrophoresis with the reagents available and it was not further studied here. Proteinases destroyed the immunologic reactivities of PAPP-A, C, and D, but none of them was inactivated by ribonuclease and deoxyribonuclease, and they did not appear to contain lipids. They were all stable to heat at 60° C. for 30 minutes, but were variably labile at higher temperatures. The three PAPP's also showed varying lability at pH extremes. They were well separated from each other in gel filtration on Sephadex G-200 and Sepharose 6B, in DEAE-cellulose column chromatography, and in sucrose density gradient ultracentrifugation, but not by step-wise salting out precipitation or gradient solubilization with ammonium sulfate. Molecular weights estimated by gel filtration were found to be 750,000 for PAPP-A, 110,000 for PAPP-C, and 20,000 for PAPP-D. Their isoelectric points were found by isoelectric focusing in ampholine-sucrose gradients: PAPP-A, pH 4.4; PAPP-C, pH 3.8; PAPP-D, pH 5.7. PAPP-C contained iron detectable by histochemical staining methods; it was heterogeneous and had minor components of different charge and isoelectric point (pH 6.0).

ALTERATIONS in the composition of plasma proteins during normal human pregnancy have been described increasingly during recent years.[1-5] In a number of instances, constituents have been found which are not detectable in normal untreated nonpregnant female subjects or in males. With the use of immunochemical methods, one or two such pregnancy-associated plasma proteins had been previously reported with antisera to pregnancy plasma[6-10] and four such proteins had been found with antisera to placental extracts.[11, 12]

In our laboratory, four pregnancy-associated plasma antigens also have been detected, but by means of hyperimmune antisera to third-trimester pregnancy plasma.[13] The latter antigens were absent from cord blood serum and from amniotic fluid concentrates, and they were shown to be un-

Not

antisera to third-trimester pregnancy serum

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related to several human hormones (placental lactogen, chorionic gonadotropin, oxytocin), as well as to a number of enzymes associated with pregnancy (oxytocinase and alkaline phosphatase). The present report further characterizes some of the properties of the pregnancy-associated plasma antigens, indicating their protein nature and describing methods applicable to their purification, as well as demonstrating their lack of relationship to several additional known human proteins.

Materials and methods

Human plasma. Plasma samples were harvested from citrated blood obtained during the third trimester of pregnancy; the specimens were kept frozen at −20° C. until use. Only those showing a minimum of three pregnancy-associated antigens when tested against potent antiserum concentrates were used. All of the studies were carried out with pools of two to five individual pregnancy plasmas. Normal nonpregnant female and male plasma samples were obtained from volunteers working in the medical school.

Antisera. Rabbit antisera against third-trimester human pregnancy plasma were prepared as described elsewhere.[13] They were absorbed at the rate of 120 to 180 mg. per milliliter with lyophilized nonpregnancy plasma pool (male or female) and were tested individually against pooled pregnancy and nonpregnancy plasma by immunodiffusion. The most potent antisera were pooled, and the immunoglobulins were concentrated by salting out with 50 per cent saturated ammonium sulfate followed by dialysis. The immunoglobulins were reconstituted at three times their original concentration and were tested for pregnancy-associated specificity by immunodiffusion against a panel of 36 or more nonpregnancy female and male plasmas as well as several pools of third-trimester pregnancy plasmas. Two such pools of antibody which showed equivalent reactions were used throughout this study. These produced somewhat different patterns from the antibody pool used previously.[13] They showed three pregnancy-associated precipitin systems by immunodiffusion, showed four by crossed-immunoelectrophoresis, and failed to show any reactions with nonpregnancy plasmas. The antibodies were stored in 1 ml. aliquots in the frozen state at −20° C. until use.

Immunoassay. The sensitive two-directional immunodiffusion (IDF) method using 1 per cent agarose was described elsewhere[13] but in some instances, templates with wells of 3.5 mm. edge-to-edge distances (instead of 8 mm.) were used to speed development time (Cordis, Miami, Fla.). Similar plates prepared with 1.5 per cent Bacto-Agar yielded appreciable nonspecific haloes around the antigen wells.

In screening fractions obtained from the various separation procedures for the presence of the pregnancy proteins, counter-electrophoresis (CEP) was employed for more rapid read-out. In this technique, 5 ml. of 1 per cent agarose in veronal-acetate buffer, pH 8.6, $\mu = 0.025$, was spread on 5 by 5 cm. glass slides. Wells for antigen and antibody, 2 and 4 mm. diameter, respectively, were cut in rows 4 mm. apart edge to edge. Ten μl volumes were used, with antibody in the larger wells. The buffer reservoirs contained the same veronal-acetate buffer, but at twice the ionic strength. A current of 0.5 to 1.0 ma. per centimeter was applied across the slide (1 to 2 v. per centimeter) for two hours, with examinations at 15 minute intervals. Most of the reactions were apparent early within the two-hour period, but the final examinations were carried out after incubation at room temperature for one to two days, dialysis, drying, and staining with Amido Black.

Immunoelectrophoresis (IEP), radial immunodiffusion (RID), "rocket" electroimmunodiffusion (EID), and crossed immunoelectrophoresis (CIE) have been described elsewhere.[13-15] Either RID or EID, or both, were used to quantitate the peaks of antigen activity in various fractions for estimation of molecular weights, isoelectric points, and

against third trimester pregnancy plasma

Concentrated by 3X

tested against third trimester plasma

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assay of their separation during chromatography.

Normal human plasma proteins were identified in IDF and IEP with various antisera: goat anti-human whole serum, rabbit anti-human serum albumin, rabbit anti-human ceruloplasmin, goat anti-human lactoferrin, goat anti-human α-1-fetoprotein (Cordis, Miami, Florida); rabbit anti-human transferrin, rabbit anti-human α-2-macroglobulin, rabbit anti-human IgG, and rabbit anti-human IgM (Behringwerke, New York).

To enhance immunoprecipitate lines in fractions obtained from gel filtration and other procedures, in some instances a second antibody (an appropriate anti-immunoglobulin) was used to refill the antiserum well or trench after the developed plate had been carefully dialyzed against 0.01M phosphate-buffered saline, pH 7.1 (PBS). The plate was then further developed for several days and processed for staining as usual.[16]

Characterization techniques. Details of the heat and pH lability tests, enzyme susceptibility, immunoenzyme assays, sucrose density gradient centrifugation (Beckman L4 ultracentrifuge), estimation of molecular weight by gel filtration, determination of isoelectric point by isoelectric focusing in ampholine-sucrose gradient columns, salting-out with ammonium sulfate, DEAE (diethylaminoethyl) -cellulose and hydroxyl apatite column chromatography have been described elsewhere.[15, 17] Crystalline enzymes were used and Naphthol As-BI sulfate (potassium salt) was used as substrate for arylsulfatase activity.

The ammonium sulfate gradient solubilization technique of King[18] was followed. When plasma (10 ml.) was mixed with [illegible]dex G-25 (25 Gm. of dry [illegible] the mixture was made to 80 [illegible]ation with ammonium sulfate [illegible]ry was packed into a 2.5 by [illegible] It was then eluted with a [illegible]nt of ammonium sulfate [illegible] to 0 per cent saturation) [illegible]ction was diluted tenfold with distilled water and scanned at 280 nm. Fractions were pooled at ten-tube intervals, concentrated by precipitation with saturated ammonium sulfate, and redissolved to the original volume with distilled water for immunodiffusion assay.

Lipid staining with Sudan Black and iron staining with Bathophenanthroline were done as described by Uriel.[19] To stain for iron with ferrocyanide,[20] the dried immunodiffusion slides were covered with a fresh mixture of equal volumes of 20 per cent HCl and 10 per cent sodium ferrocyanide for 2 hours, with occasional examination for the appearance of blue stain.

Unless otherwise indicated, all experiments were done with whole plasma. The partially purified antigens studied were prepared by fractionating the plasma first with DEAE-cellulose column chromatography and then with Sephadex C-200 gel filtration.

Results

Identification and electrophoretic mobility. Four antigens in pregnancy plasma were detected with the antiserum concentrates used in this study. They were designated in the reference systems alphabetically according to the position of their precipitate lines in the immunodiffusion pattern starting from the antigen well (Fig. 1, *a* and *b*). The precipitin line of the pregnancy-associated plasma protein A (PAPP-A) was regularly straight and relatively crisp. The PAPP-C system showed up earliest and was thickest; it often appeared to split on continued development (Fig. 1, *b*) and was thought either to consist of two components or to represent overlapping with the PAPP-B system. The latter was reproducibly visualized only by crossed immunoelectrophoresis (Fig. 1, *d*). The PAPP-D system was revealed by a concave line very close to the antibody well in immunodiffusion. As shown in Fig. 1, *b*, the pregnancy plasma could be diluted at least eightfold and the PAPP's remained detectable in immunodiffusion. With the standard templates and whole pregnancy plasma, the PAPP-C system appeared first

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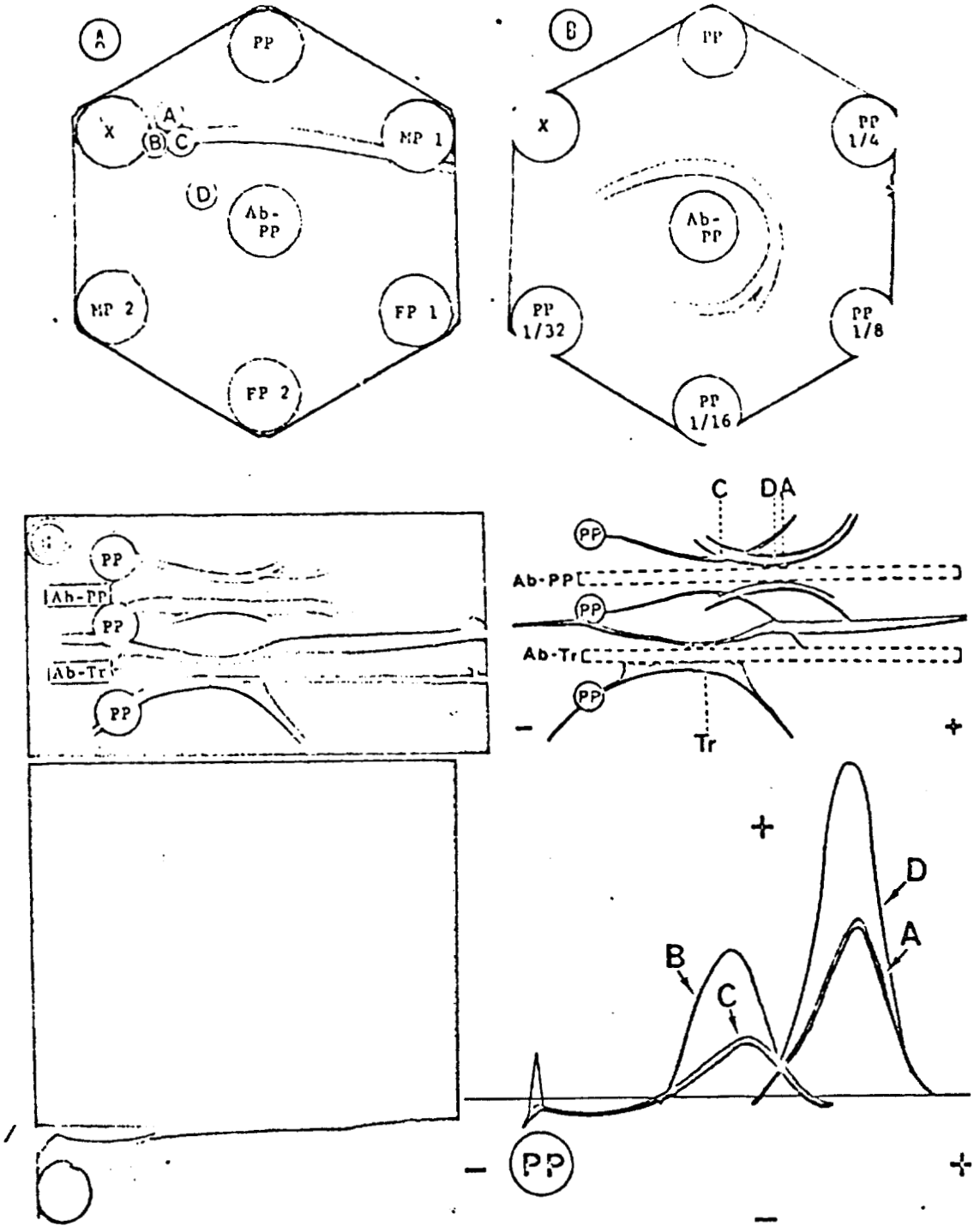

Fig. 1. Immunodiffusion *(a, b)*, immunoelectrophoresis *(c)*, and crossed immunoelectrophoresis *(d)* of the reference system of the pregnancy-associated antigens. *PP:* pregnancy plasma; *MP 1, MP2:* individual male plasmas; *FP 1, FP 2:* individual female nonpregnancy normal plasmas; *X:* empty; *PP 1/4, PP 1/8, . . . :* twofold serial dilutions of third trimester pregnancy plasma; *Tr:* human transferrin precipitin arc; *Ab-PP:* antibody concentrate (threefold) of rabbit anti-human pregnancy plasma exhaustively absorbed with normal nonpregnancy plasma; *Ab-Tr:* rabbit anti-human transferrin serum. *A, B, C, D:* the immunoprecipitate lines of pregnancy-associated antigens A, B, C, and D, respectively.

(overnight), the PAPP-A required two days, and the PAPP-D at least three days for development.

The electrophoretic mobilities of these antigens were compared in parallel by immunoelectrophoresis (Fig. 1, *c* and *d*) with those of several well-known plasma proteins with the use of monospecific antisera or lipid staining for lipoproteins (transferrin, albumin, ceruloplasmin, immunoglobulin G and M, α2- and β-lipoproteins). It was revealed that both PAPP-B and PAPP-C migrated with the β-globulins, slightly faster than transferrin (Fig. 1, *c*). PAPP-A moved slightly ahead of PAPP-D, both with the α2-globulins, and somewhat faster than ceruloplasmin. Since PAPP-B was not regularly identifiable by these techniques, it was not studied further below.

Physical stability. Heating pregnancy plasma to 60° C. for 30 minutes did not render any of these antigens nonreactive. Partial destruction of this property occurred at higher temperatures, i.e., PAPP-A at 70° C., PAPP-C at 85° C., and PAPP-D at 100° C. (Table I). All antigens were stable at pH levels between 4 and 10 at 0° C. for two hours. PAPP-A was completely destroyed at pH 2 and 12; PAPP-C was stable at pH 12, but was affected at pH 2; PAPP-D resisted all the pH levels (2 to 12) tested. Alternate freezing (−70° C.) and thawing (room temperature) for at least ten cycles did not have any obvious effect on the reactivities of these antigens.

Enzyme susceptibility. Trypsin (1 mg. per milliliter) had no gross effects on the pregnancy antigens in whole plasma. It did, however, destroy the reactivities of partially purified PAPP-A and PAPP-C (Table II). Pepsin inactivated both PAPP-C and PAPP-D in whole pregnancy plasma. Neither RNase nor DNase had any effect on any of the PAPP's under similar conditions.

Salting-out. PAPP-A remained soluble in [illegible] pregnancy plasma exposed to 1/3 saturated [illegible] sulfate solution (pH 7.1) at 4° [illegible] was precipitated in a 1/2 saturated [illegible] of this salt. PAPP-C was partially sedimented at 1/5 and completely so at 2/3 saturation with ammonium sulfate. PAPP-D was salted out at 2/3 but not at 1/3 saturation.

Table I. Sensitivity of pregnancy-associated plasma proteins to heat

Temperature* (C)	Residual reactivity† of antigen		
	A	C	D
50	+	+	+
56	+	+	+
60	+	+	+
70	±	±	±
85	−	±	±
100	−	−	±

*The whole plasma was exposed to these temperatures for 30 minutes.

†+, Reactions equivalent to control unheated pregnancy plasma; ±, reactions significantly decreased as compared to controls; −, no reactions.

A plasma pool from normal males was salted out in the same way with ammonium sulfate; no PAPP was detected in any of the fractions, even after they had been reconstituted in solutions fourfold concentrated from the starting volumes.

Solubilization in gradient ammonium sulfate. Virtually all of the plasma proteins including the PAPP's were precipitated and trapped in Sephadex G-25 gel suspension 80 per cent saturated with ammonium sulfate. Elution of these precipitated pregnancy plasma proteins from the gel column with decreasing concentration of this salt resulted in four major protein peaks (Fig. 2). All but the first contained the pregnancy-associated proteins, as indicated by the horizontal bars. PAPP-C showed a wide range of distribution, being solubilized between 60 and 5 per cent saturated ammonium sulfate solution; PAPP-A was eluted between 65 and 35 per cent; PAPP-D came out between 55 and 40 per cent saturated salt solution.

Sucrose density gradient ultracentrifugation. Whole plasma (0.5 ml.) was layered on the surface of 5 to 20 per cent sucrose gradient in duplicate (total volume, 10.5 ml.; height, 6 cm.). After centrifugation at 100,000 ×g for 16 hours at 8° C., with a

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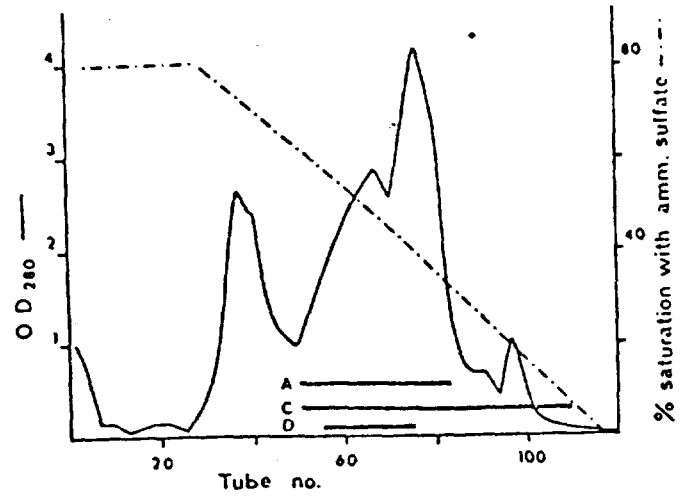

Fig. 2. Solubilization of human pregnancy plasma in a decreasing concentration gradient of ammonium sulfate. The thick horizontal bars localize the designated pregnancy associated proteins in the chromatography (4.5 ml. per tube).

Table II. Susceptibility of pregnancy-associated plasma proteins to enzymes at 37° C.

Enzyme	pH	Time exposure (hr.)	Residual activity* A	C	D
Trypsin	7.1	0†	+‡	+	ND
		1	–	±	ND
		4	–	–	ND
Pepsin	4.0	0†	–	+	+
		1	–	+	–
		4	–	±	–
RNase	7.1	1	+	+	+
		4	+	+	+
DNase	7.1	1	+	+	+
		4	+	+	+

*In whole plasma, except when trypsin was the enzyme.
†Control in the absence of enzyme, incubated 4 hours at the same pH and temperature as experimental conditions.
‡+, ±, –, as in Table I. ND, not tested.

fixed-angle No. 65 rotor, 0.9 ml. fractions were collected from a pinhole at the bottom of the tube. These fractions were assayed by immunodiffusion for the presence of the PAPP's as well as for several well-known normal plasma proteins. The sucrose gradient was estimated by substituting PBS for plasma in control tubes and the harvested fractions were measured for their sucrose concentrations by weighing and comparing with standards. The results are shown in Fig. 3: PAPP-A settled to the bottom; PAPP-C sedimented a bit more slowly than human serum albumin; PAPP-D was recovered in the upper portion, which also contained heavy lipoproteins.

Gel filtration and molecular weights. The three pregnancy-associated antigens in whole plasma could be effectively separated from each other by various types of gel filtration. As shown in Fig. 4, PAPP-A was eluted in Sepharose 6B well ahead the majority of plasma proteins. PAPP-C was best separated from other plasma proteins in Sephadex G-200 gel, as it appeared in the trough between the two major protein peaks (Fig. 5). Isolation of PAPP-D from plasma was also effectively achieved in Sephadex

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)00 gel, being considerably more retarded
most plasma proteins, and appearing
actions containing very low protein con-
trations. Similar data from calibrated
mns (Fig. 6) indicated that the molecu-
weights of PAPP-A, PAPP-C, and
PP-D were 750,000, 110,000, and 20,000,
ectively. Both PAPP-A and PAPP-C
ld be detected in the eluates with-
concentration, whereas PAPP-D often
ded to be concentrated for immuno-
y.

on-exchange column chromatography. A
cal chromatogram of pregnancy plasma
a DEAE-cellulose column is shown in
7. It can be seen that PAPP-C eluted
much lower salt concentrations than the
er two pregnancy proteins, which over-
ped with each other and ceruloplasmin.
er concentration of an early eluted frac-
by salting out with ammonium sulfate,
ntigen (PAPP-C') was detected which
nunologically identical to but elec-
horetically different from PAPP-C, as
trated in Fig. 8. The elution patterns
ned with gradients of Tris-phosphate
NaCl-phosphate buffers were similar.

romatography of pregnancy plasma on
xyl apatite columns, with potassium
ate used as the eluting salt, yielded
patterns for the pregnancy-associated
proteins shown in Fig. 9.

pools from normal untreated males
arly chromatographed on DEAE-
ed eluate fractions similarly har-
concentrated as above for im-
In no case was any pregnancy-
ntigen activity detected in any
entrates.

points. Rough estimations of
e points of these PAPP's were
whole plasma in polyacrylamide
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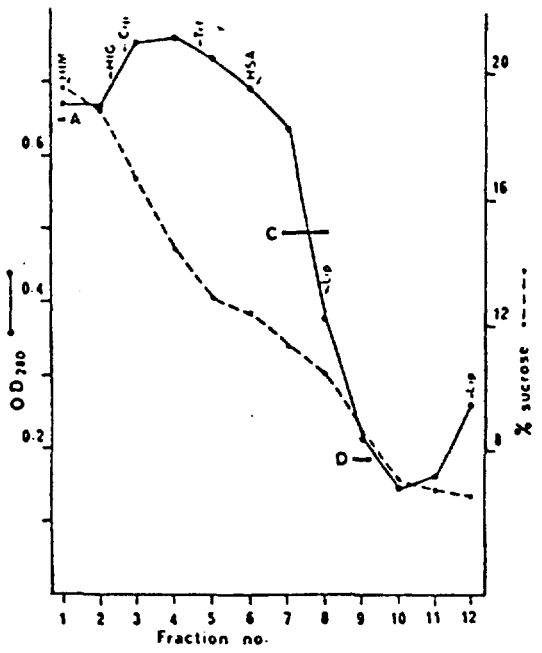

Fig. 3. Sucrose density gradient ultracentrifugation of human pregnancy plasma (100,000 ×g for 16 hours). The thick horizontal bars localize the designated pregnancy-associated proteins in the eluates. For reference purposes, the following normal human plasma proteins were identified with specific antisera or staining (arrows): *HIM*, human immunoglobulin M; *HIG*, human immunoglobulin G; *Crp*, ceruloplasmin; *Trf*, transferrin; *HSA*, human serum albumin; *Lip*, Lipoproteins.

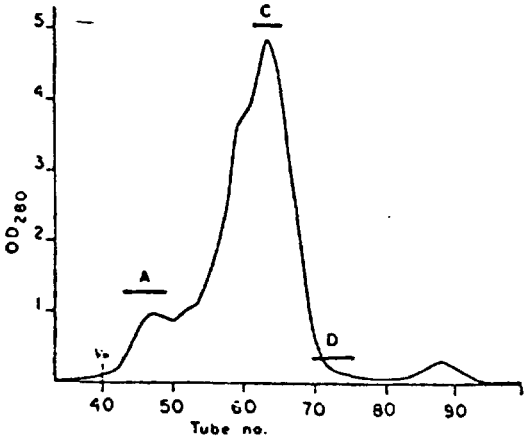

Fig. 4. Sepharose 6B gel filtration of pregnancy plasma. A 6 ml. quantity of sample was applied onto the surface of a 2.5 by 83 cm. gel column and eluates (5 ml. per tube) were collected at a flow rate of 10 ml. per hour. The fractions containing the pregnancy-associated antigens A, C, and D are marked with thick horizontal bars. *Vo*: void volume. A similar pattern was obtained with a 2 ml. sample.

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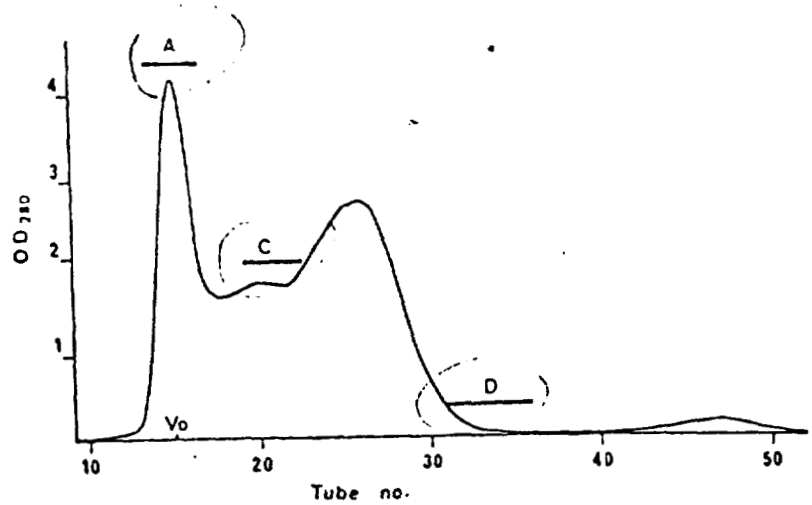

Fig. 5. Sephadex G-200 gel filtration of human pregnancy plasma. A 4 ml. quantity of sample was applied onto a 2.5 by 90 cm. column and eluates were collected (10 ml. per tube) at a flow rate of 30 ml. per hour. Fractions containing the pregnancy-associated antigens A, C, and D are marked with thick horizontal bars. *Vo:* void volume.

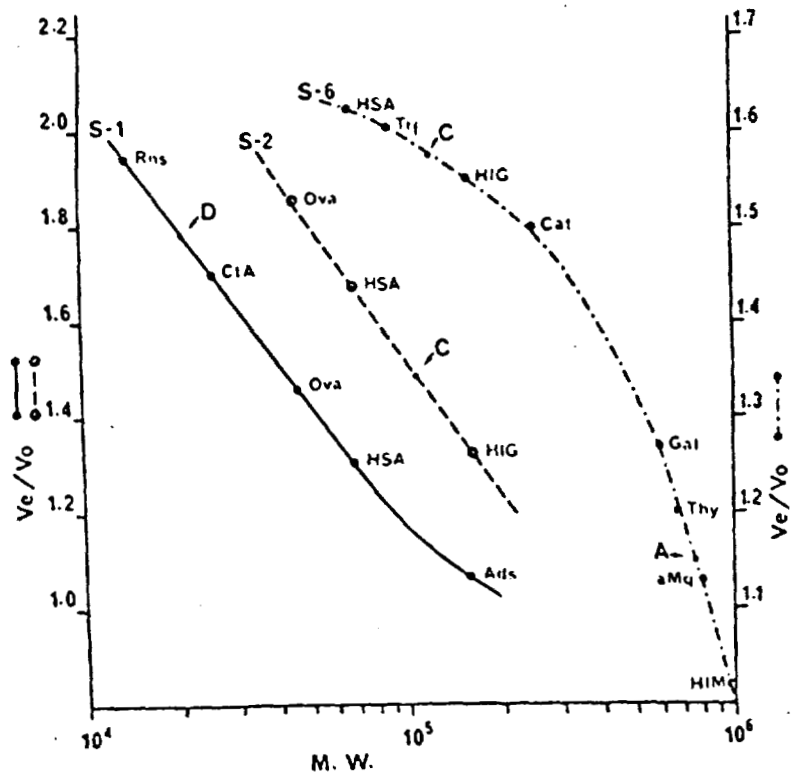

Fig. 6. Estimation of molecular weights of pregnancy-associated proteins by gel filtration, by comparison with standard purified protein preparations. *Vo:* void volume; *Ve:* elution volume of the proteins; *M. W.:* molecular weight on a log scale. *S-1:* Sephadex G-100 column (2.5 by 95 cm., 25 ml. per hour, 5 ml. per tube); *S-2:* Sephadex G-200 (2.5 by 90 cm., 30 ml per hour, 5 ml. per tube); *S-6:* Sepharose 6B (2.5 by 83 cm., 13 ml. per hour, 5 ml per tube). *Rnc:* ribonuclease (bovine pancreas); *CtA:* chymotrypsinogen A (bovine pancreas); *Ova:* ovalbumin; *HSA:* human serum albumin; *Ads:* aldolase; *HIG:* human serum immunoglobulin G; *HIM:* human serum immunoglobulin M; *Trf:* human transferrin; *Thy:* porcine thyroglobulin; *Cat:* catalase; *Gal:* β-galactosidase *(E. coli)*; *αMg:* α2-macroglobulin. Arrows show pregnancy-associated proteins A, C, and D.

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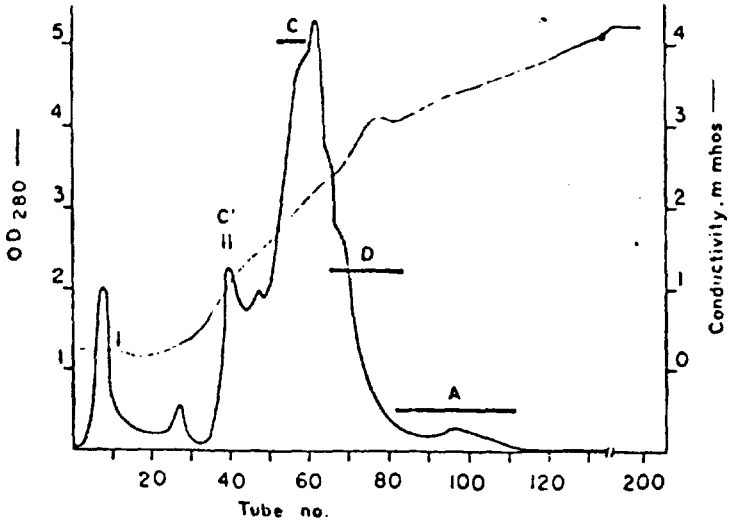

Fig. 7. Chromatography of human pregnancy plasma on a DEAE-cellulose column. A 53 ml. quantity of plasma was applied into a 2.5 by 21 cm. column. After flushing with 120 ml. of starting buffer, it was eluted with 2.7 L. of a concentration gradient from 0.005 to 0.35M Tris-phosphate, pH 8.6, at a flow rate of 90 ml. per hour, and finally flushed with 200 ml. of limiting buffer. The single arrow shows the start of gradient. Eluates were collected 15 ml. per tube. The pregnancy-associated proteins are indicated by the horizontal bars. The double arrows show the minor peak of PAPP-C activity.

PAPP-A was found to be 4.4 ± 0.1 with 2 per cent ampholyte in a pH 3 to 6 gradient (Fig. 10). The pI of partially purified PAPP-C was estimated in a 1 per cent ampholyte of similar pH gradient. The major component showed a pI of 3.8 ± 0.2; a minor component which was found had a pI of 6.0 (PAPP-C″) (Fig. 11). These two components were shown to be immunologically identical in immunodiffusion, the former giving a much stronger precipitation line. PAPP-D revealed a pI of 5.7 using 1 per cent ampholyte between pH 3 and 10, as shown in Fig. 12.

Lipid and iron staining. The precipitin line of the PAPP-C′ system occasionally showed very faint staining of uncertain significance with Sudan Black; the other two never did.

The immunoprecipitate line of the PAPP-C system was found to react with two distinct reagents designed to stain specifically for iron: Bathophenanthroline and ferrocyanide. The other PAPP systems did not react at all with these reagents.

Attempted identification with human tissue antigens and enzymes. The above indications that PAPP-C contained detectable iron prompted tests to further confirm that this protein was not related to known plasma proteins capable of carrying this metal. Comparative immunodiffusion tests performed

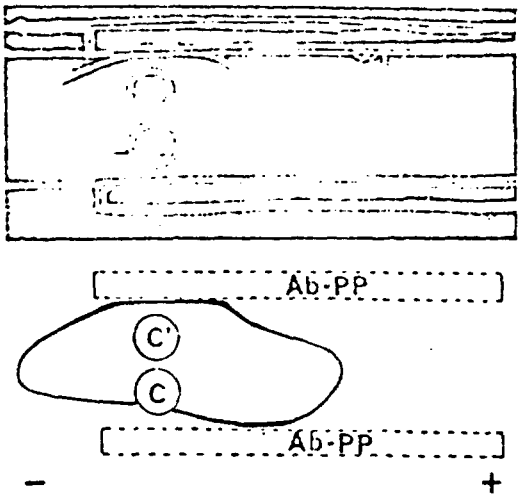

Fig. 8. Immunoelectrophoresis of major and minor PAPP-C fractions from DEAE-cellulose chromatography. *C*: the major PAPP-C; *C′*: the minor PAPP-C; *Ab-PP*: antibody concentrate of rabbit anti-human pregnancy plasma antisera absorbed with nonpregnant normal human plasma.

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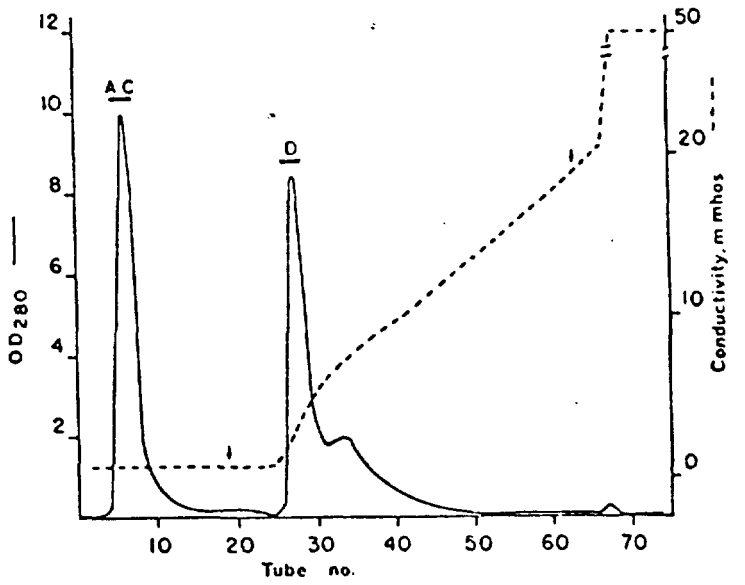

Fig. 9. Chromatography of human pregnancy plasma on a hydroxyl apatite column. A 20 ml. quantity of plasma was applied onto a 2.2 by 7 cm. column equilibrated with 0.005M potassium phosphate, pH 6.8. After flushing with 200 ml. of the same buffer, it was eluted with 500 ml. of linear concentration gradient toward 0.5M potassium phosphate, pH 6.8 (the first arrow), and finally flushed with 0.5M potassium phosphate plus 1M ammonium sulfate (the second arrow) at a flow rate of 150 ml. per hour. Eluates were collected (10 ml. per tube). The presence of pregnancy-associated proteins is indicated by the thick horizontal bars.

with specific antisera to human transferrin, human hemopexin, and human haptoglobin revealed that these proteins were completely unrelated immunologically to any of the PAPP's. In addition, tests with specific anti-human lactoferrin, the iron transport protein found in milk and various tissues, showed that this was unrelated to any of the PAPP's. The embryonic protein synthesized in liver tissue (α-1-fetoprotein) was also shown by immunodiffusion to be unrelated to the pregnancy proteins. None of the pregnancy-associated proteins were detected in buffered saline extracts of normal male or female human heart, liver, kidney, or skeletal muscle.

Previous tests of the pregnancy-associated plasma protein precipitin patterns had shown that none of them revealed acid or alkaline phosphatase, oxytocinase, catalase, glucosaminidase, or esterase activities.[13] Further assays for enzymatic properties of the PAPP systems were carried out and none was found to possess detectable glucuronidase or arylsulfatase activities. Positive control systems were satisfactory (human liver anti-liver patterns.[22]

Comment

The pregnancy-associated antigens dealt with in the present study were shown to be protein in nature. They were considerably different from each other in a number of properties, including molecular weights, isoelectric points, sedimentation rates, and elution characteristics from certain adsorbents. Gel filtration, ion exchange column chromatography, and sucrose gradient centrifugation methods could readily separate them from each other. Their resistance to pH extremes and high temperature also varied, the larger molecules being somewhat more labile.

PAPP-C showed heterogeneity in several aspects. In addition to being precipitated over a wide range of ammonium sulfate concentrations, it split into two overlapping populations in DEAE-cellulose column chro-

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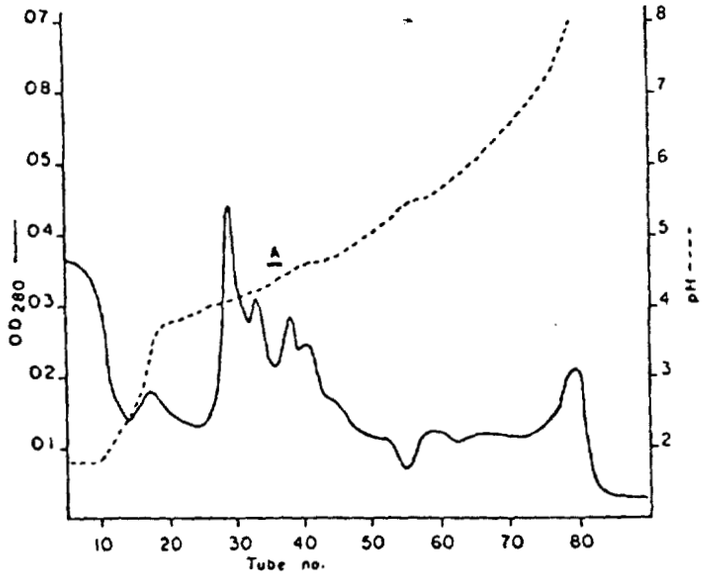

Fig. 10. Isoelectric focusing of partially purified PAPP-A. A 5 mg. quantity of sample was applied into a column containing 2 per cent ampholine, pH 3 to 6, in sucrose gradient. PAPP-A activity is indicated by the thick horizontal bar. Eluates were collected (5 ml. per tube).

matography. The major one had the same electrophoretic mobility as that readily seen in whole pregnancy plasma. The minor peak (PAPP-C′) showed no or little migration under the same conditions. The major population, however, when subjected to isoelectric focusing, again showed two subcomponents --the principal one had a pI of 3.8 and the other (PAPP-C″) had a pI of 6.0. All of these three components showed "reactions of identity" in immunodiffusion.

With the antisera used, the PAPP-C systems always appeared thick or split in immunodiffusion, suggesting two overlapping immune systems. Although it has not yet been feasible to substantiate the latter possibility, the reagents used in this study showed all four PAPP immune systems by crossed immunoelectrophoresis. The PAPP-B "hill" was relatively faint even with this technique, but its electrophoretic mobility appeared to be similar to that of PAPP-C.

Bohn[11, 21] detected four pregnancy-associated plasma proteins using rabbit anti-human placenta serum absorbed with male serum. Of these, only one was considered to be specific for pregnancy, and it was shown to be a β-1 glycoprotein. Its reported electrophoretic mobility and molecular weight were very similar to those found for PAPP-C in the present study. However, PAPP-C sedimented somewhat more slowly than human serum albumin (4.6S), whereas Bohn found his pregnancy-specific β-1-glycoprotein to have a sedimentation coefficient of 4.6. The β-1-glycoprotein was reported to have a high carbohydrate content (28 per cent) and lost electrophoretic mobility when sialic acid was removed from the molecule. It is conceivable that these derivatives could be represented by the PAPP-C′ and/or PAPP-C″ seen in the present study. Other investigations,[6, 7, 11, 21] using either immunoelectrophoresis or starch gel electrophoresis, have also found pregnancy-specific or pregnancy-associated proteins of β mobility in plasma, but lack of more detailed information makes comparisons with PAPP-C difficult.

PAPP-A has a larger molecular weight, a higher pI, and a greater electrophoretic mobility than PAPP-C. Stimson and Eubank-Scott[7] have purified a pregnancy-specific human plasma protein with similar properties: i.e., a pI of 4.6 vs. a pI of 4.4 for PAPP-A,

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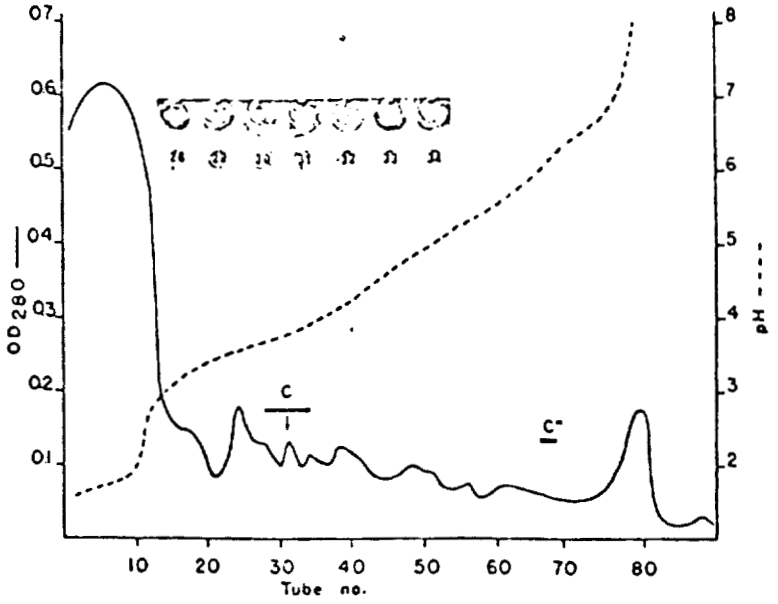

Fig. 11. Isoelectric focusing of PAPP-C. An 8 mg. quantity of partially purified protein was applied into a column containing 1 per cent ampholine (pH 3 to 6) in sucrose gradient. Antigen activity was shown by the horizontal bars. Inset shows CEP of tubes Nos. 28 to 34. Eluates were collected (5 ml. per tube).

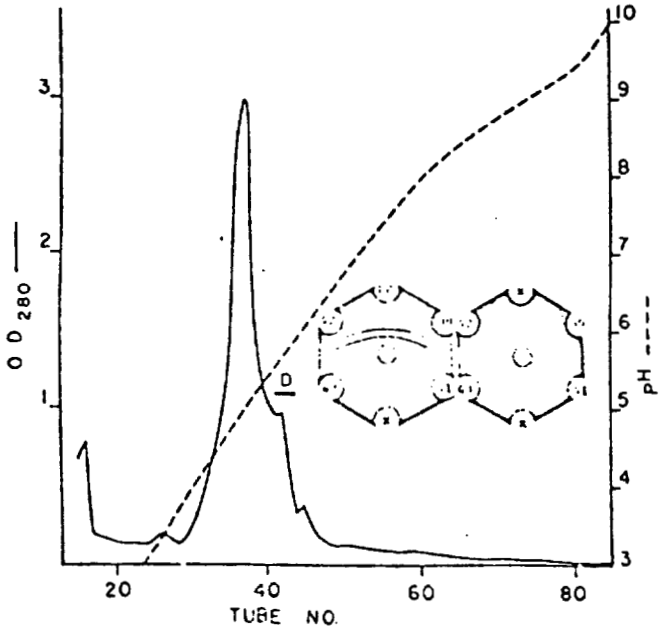

Fig. 12. Isoelectric focusing of PAPP-D fractions from DEAE-cellulose column chromatography. A 50 mg. quantity of protein was applied into the column containing 1 per cent ampholine (pH 3 to 10) in a sucrose gradient. Eluates were collected (5 ml. per tube). PAPP-D activity is indicated by the thick horizontal bar. Inset shows immunodiffusion patterns with samples from the designated tubes. The central wells contained the reference antibody to the PAPP's. *PP*: pregnancy plasma; *X*: empty.

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a molecular weight of 507,000 vs. an estimated 750,000 for PAPP-A, and similar electrophoretic mobilities. Stimson[5] has reported that this pregnancy-associated plasma protein interferes with cell metabolism in tissue culture and could be detected in the plasma of some patients with malignant tumors. Since PAPP-D showed the same electrophoretic mobility as the much heavier PAPP-A, it is possible that either of these two proteins represents the $\alpha 2$ pregnancy-associated protein, or zone, reported by other investigators.[1, 6, 7, 10, 11, 26-28] Margolis and Kenrick[29] reported a pregnancy-specific protein with a molecular weight similar to that of PAPP-A, using polyacrylamide gel electrophoresis techniques. With antiplacental serum, Bohn[24] found that the pregnancy zone or pregnancy-associated $\alpha 2$-glycoprotein of molecular weight greater than 300,000 was not specific for pregnancy, but was found in the serum of an appreciable proportion of nonpregnant women. Clarification of the relationships between the various reported pregnancy-associated plasma proteins awaits an exchange of samples.

It is not clear whether the pregnancy-associated proteins appear *de novo* during pregnancy or whether they strikingly increase in concentration from very low "nondetectable" levels. The latter possibility seems more likely, although concentrates of nonpregnancy plasma or of chromatographic fractions from them have not revealed any of the PAPP's in immunodiffusion.

Nothing is definitively known of the biologic significance of the PAPP's. The small molecular size of PAPP-D suggests the possibility that it may be hormonal in nature. The histochemical detection of iron in PAPP-C suggests that it may play a specialized role in transport of this essential metal. In a preliminary trial to see if the iron in PAPP-C is exchangeable, the radioautographic technique of Clausen and associates[30] was used and it was found that the radioisotopic iron ($^{59}FeCl_3$) was incorporated into PAPP-C after treatment of pregnancy plasma with EDTA. This test was controlled with the transferrin-immune precipitate. Bohn[24] has suggested that the pregnancy-specific β-1-glycoprotein, which is similar to the above PAPP-C in many respects, may play a role in the normal functions of the placenta.

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PUBLICATIONS LTD

Beschreibung 2240327

zur Anmeldung von Dr. W. Jankowsky aus Pfungstadt vom 14.8.1972 :

Verfahren und Anordnung für Schwangerschaftstest.

Bekannt ist die Agglutination zwischen Antikörper und Antigen und ihre Verwendung für Schwangerschaftstests sowie ihre Sichtbarmachung durch Latex- oder Blutkörperchen. Heute ist ein Fortschritt notwendig, um die grundlegende Methode empfindlicher zu machen, da die bekannten Tests meist erst nach dem Ausbleiben der Periode verwendbar sind.

Um Antikörper mit hohem Titer herzustellen, soll gemäß der Erfindung neben dem Human-Chorion - Gonadotropin auch Extrakt aus menschlicher oder tierischer Placenta der ersten Schwangerschafts-Monate zur Immunisierung verwendet werden. Dies geschieht weitere Schwangerschaftshormone oder Metabolite zu erfassen. Zur Ausschaltung störender lytischer Komponenten durch Komplement nach erreichtem Titer soll das Serum oder sein Globulin Anteil für 30 Minuten auf 56 Grad Celsius erwärmt und danach zur Haltbarmachung lyophilisiert (gefriergetrocknet) werden.

Zur Durchführung der Reaction werden mit diesem Antikörper nicht nur wie üblich als Antigen der Urin, sondern auch einige Tropfen Blut der zu testenden Frau auf einem Objectträger durch Verrühren mit einem Glasstab vermischt. Das Blutserum enthält bei vorliegender Schwangerschaft zusätzlich Schwangerschaftshormone, die oft im Urin zu gering sind, und die Blutkörperchen dienen bei positivem Ausfall zur Sichtbarmachung der Agglutination. Bei schwachem Ausfall derselben kann auf dem Objectträger eine zweite Mischung nur mit Urin und Blut angesetzt werden, wobei keine Reaction eintritt.

Verfahren und Anordnung sind bereits practisch erprobt worden sowie ihre größere Empfindlichkeit. Die Antrocknung des Antikörpers auf Objectträgern stellt einen weiteren Fortschritt für die Praxis dar.

Patentansprüche :

Verfahren und Anordnung für Schwangerschaftstest dadurch gekennzeichnet

1. daß zur Herstellung des Antikörper- Serums nicht nur Chorion-Gonadotropin, sondern auch Extracte aus Placenta der ersten Monate verwendet wird,
2. daß das Serum zur Ausschaltung von Komplement für 30 Minuten auf 56 Grad Celsius erwärmt wird, und zur Haltbarmachung gefriergetrocknet (lyophilisiert) wird.
3. daß dies Serum nicht nur in Ampullen verpackt, sondern auf Objectträgern aus Glas oder Plastik angetrocknet wird, die einzeln in Röhrchen gepackt sind.
4. Daß zur Reaction nicht nur wie üblich Urin, sondern zugleich einige Tropfen Blut der zu testenden Frau verwendet werden.

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Immunochemical Investigations on the Problem of the "Pregnancy Zone"

II. Antiplacenta Immune Serum

by

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Submitted May 27, 1969

Translated from the German by
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Oak Ridge National Laboratory
July 1972

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Immuno-Chemical Investigations on the Problem of the "Pregnancy Zone"

II. Anti-Placenta Immune Serum

Summary. The placenta is suitable for obtaining antigens induced by pregnancy. With use of an absorbed antiplacenta immune serum from a placental extract at least 7 lines of precipitation can be obtained by means of immunoelectrophoresis.

The placenta immune serum precipitates altogether three pregnancy-specific antigens when sera of pregnant women, amniotic fluid, sera from the umbilical cord and from puerperas are used.

The principal usefulness of absorbed antiplacenta immune serum for the quantitative radial immunodiffusion of pregnancy-induced antigens is being studied.

Introduction

Pregnancy can be considered as a special case of homologous transplantation (14, 23). For elucidation of the immunological significance of the placenta many authors have dealt with the analysis and detection of antigens of the organ (3, 4, 6, 8, 9, 10, 19, 22). According to more recent investigations, the marginal layer of the placental trophoblast assumes the significance of an immunologically neutral buffer zone between the mother and the fetus (24). Investigations of the placenta in the normal functioning state involve the danger of injury to the child. Also, pregnancy serum is not available in sufficient quantity for production of immune serum, while placentas after birth can easily be processed for obtaining antigens. Since in the preceding studies we found a relationship of the "pregnancy zone" to antigens of the placenta, the purpose of the present work is to

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test the suitability of absorbed antiplacenta immune serum for detecting and correlating pr[illegible]y-induced serum antigens.

Methods

I. Processing of Organs

a) Extraction of protein. Placentas from women with a pregnancy of normal term and spontaneous birth at the end of the carrying period are examined and then immediately freed of the ovular membrane and the umbilical cord. For extraction of the cytoplasmic proteins of the organ, the organ is first rinsed for 120 min. in cold running tapwater. Then as much as possible the fat and connective tissue which is not associated with the parenchyma of the organ is removed. The placentas are then cut up into pieces the size of beans and the pieces of the organs rinsed in 0.14 M NaCl solution at 4°C. the rinse solution appears clear and colorless, whereby up to 50 renewals of the rinse solution are required. After each washing step the moist pieces of the organs are allowed to drain on a Perlon sieve before they are again stirred in fresh washing solution. Contact with base metals must be avoided under all conditions during the whole process in order to prevent inactivation of enzymes and labile proteins by metal ions. Once the washing solution is clear, the pieces of the organ are mixed with twice their volume of pH 7.4 M/15 phosphate buffer and homogenized for 1 min at 0° C in the Ultra-Turrax (Janke and Kunkel KG, Staufen). EXTRACTION

Formula of buffer: Sörensen M/15 phosphate, pH 7.4 (2).

Solution 1: $NaH_2PO_4 \cdot H_2O$ 9.199 g per 1000 ml dist. water

Solution 2: $NaH_2PO_4 \cdot 2\ H_2O$ 11.876 g per 1000 ml dist. water

Mixing 18.2 parts of solution 1 with 81.8 parts of solution 2 results in a pH of 7.4.

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EXTRACTED

Instead of the potassium dihydrogen phosphate of the original Sörensen buffer, sodium salt was used, as potassium ions interfered in animal immunizations.

The thinly fluid slurry of the organs is placed in a graduated cylinder, the total volume determined and the mixture then immediately centrifuged for 20 min at 20,000 g and 0° C in the preparative untracentrifuge (M. Christ, Osterode, Type Omega, red rotor 6 x 100 ml). The supernatants (solution A) are combined in a graduated cylinder and after determination of the total volume of solution A, the determination of hemoglobin is made (0.29 Hb/100 ml). From solution A the proteins are precipitated with ammonium sulfate, p.a. For this, solid $(NA_4)_2(SO)_4$ is added to solution A to full saturation, and the solution then allowed to stand 24 hours at 4° C. The precipitate is filtered off and the filtrate discarded; the residue is dissolved in M/15 phosphate buffer, pH 7.4. In order to eliminate the $(NH_4)_2SO_4$, dialysis is then carried out against the same phosphate buffer for 48 hours at 4° C (buffer renewed after 25 hours). A solution B results (2.1 g protein per 100 ml) in which are contained only $(NH_4)_2SO_4$ - precipitable proteins from the organs. In order to increase the protein concentration of solution B, it is concentrated to 4 g protein/100 ml in a dialyzing tube against a 20% (w/v) solution of polyethylene glycol (Fluka, Buchs, Switzerland) for 48 hours at 4° C. This solution C serves as inoculating material for preparation of the polyvalent antiplacenta sera (4.0 g protein/100 ml). For the electrophoretic and immunochemical analyses the protein content is raised to about 8-10 protein /100 ml solution by vacuum ultrafiltration (for details see article I). The highly concentrated protein solution is then centrifuged in the preparative ultracentrifuge (M. Christ, Osterode, Type Omega, green rotor 8 x 10 ml) for 60 min at 100,000 g and 0° C, and the clear supernatant (solution D) used for electrophoretic and immunochemical analyses.

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b) Determination of protein. The determination of protein in solutions A to D from the organs was made by the biuret method (12). The calibration curve was plotted with beef serum albumin as a protein standard (VEB Serum- and Impstoffwerk Dessau).

c) Determination of hemoglobin. The determination of the hemoglobin concentration in placenta solutions A to D was used as a relative measure for determining the admixture of blood in the extracts. The determinations were carried out according to the procedures of Ruckpaul et al. (18) by measurement of the extinction of cyanohemoglobin.

II. Preparation of Antiplacenta Immune Serum

For the immunization 10 rabbits (Chinchilla ♀ ♂, 6-12 months old) were used. 1 ml of placenta extract C emulsified in the proportion of 1 : 1 in incomplete Freund adjuvant was injected subcutaneously weekly over a period of 10 months. After checking the antibody titer (every two weeks by the Ouchterlony test and immunophoresis) the animals were bled and the sera stored at -20° C for later absorption.

III. Absorption of Antiplacenta Immune Serum

After the equivalent proportions had been determined in a preliminary experiment, the polyvalent antiplacenta immune sera were absorbed and lyophilized according to the procedure given in article I.

IV. Agar Gel Diffusion, Immunoelectrophoresis, Mancini Technique

The procedures have been described in detail in the part on methods in the first paper of this series.

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Results and Discussion

After immunization of the rabbits, a precipitating immune serum is obtained which reacts both with pregnancy serum and placenta extract. After absorption of the anti-placenta immune serum with human serum, the antibodies which are directed against serum proteins are removed. In immunoelectrophoresis at least 7 precipitation lines were obtained against placenta extract 20 D with this absorbed antiplacenta immune serum which are distributed over the whole range of electrophoretic mobility of 0 to 7 x 10^{-5} $cm^2V^{-1}sec^{-1}$. In the reaction between absorbed antiplacenta immune serum and pooled pregnancy serum immunoelectrophoresis shows at least one precipitation line with the medium electrophoretic mobility of 3.2 x $10^{-5}cm^2V^{-1}sec^{-1}$ (fig. 1). It is known that during pregnancy components of the placenta may pass over into the organism of the mother (10). It is probable that the protein found in pregnancy serum with absorbed antiplacenta immune serum is of placental origin. This finding is contradictory to earlier results of other authors (22)

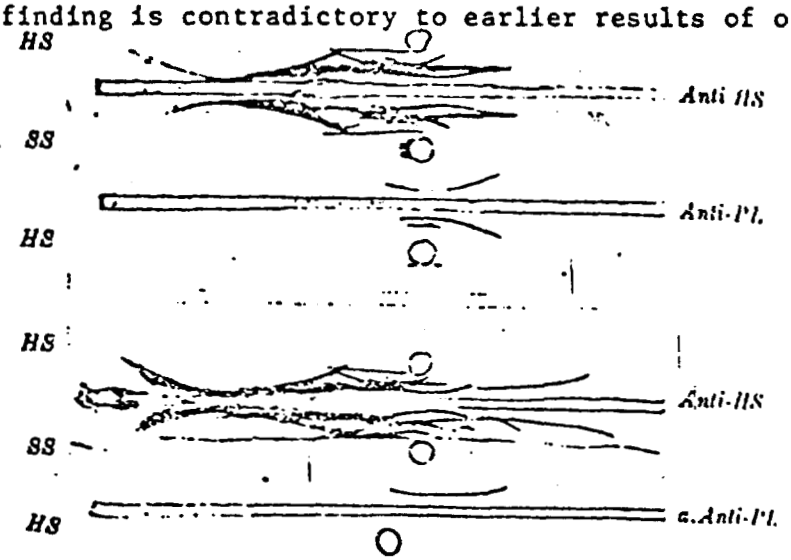

Fig. 1 Immunoelectrophoretic analysis of human (HS) and pregnancy serum (SS) using anti-human serum (anti-HS), antiplacenta serum (Anti-PL) and absorbed antiplacenta serum (a. Anti-PL) at pH 8.0 (veronal acetate buffer).

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and may be explained by an inadequate method for placenta extraction or variable quality of the immune sera. Bayer (3) found in immunoelectrophoretic analysis of pregnancy serum against "neutralized" antiplacenta serum a precipitation line in the alpha-2 range which is similar to "pregnancy-induced" protein and may be a metabolic product of the mother. It is possible that Bayer's antigen is identical with the placental protein which we found.

In the diffusion test of Ouchterlony the suitability of absorbed antiplacenta immune serum for precipitation of antigens in umbilical cord serum and puerperal serum as well as in amniotic fluid and placenta extract was also investigated. The result may be seen in Fig. 2. Human serum is

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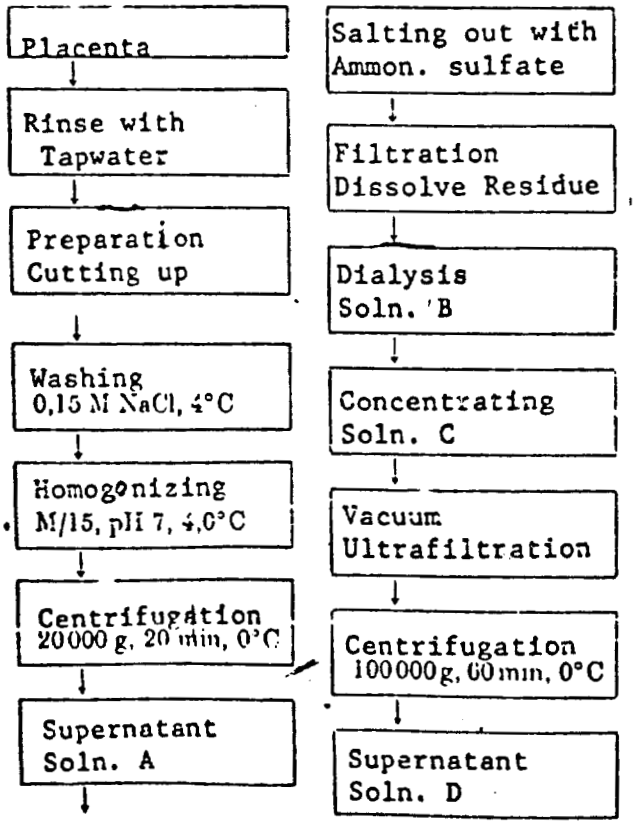

not precipitated by absorbed antiplacenta immune serum; the absorption is therefore to be regarded as complete. In the reaction of pregnancy serum at least 2 precipitation lines are obtained which are immunologically identical with 2 of the 5 precipitation lines of the placenta extract. Between umbilical cord serum and aborbed antiplacenta immune serum at least 1 precipitation line can be demonstrated which shows a crossing phenomenon with the neighboring antigens of the pregnancy serum. From this finding it may not be concluded that the umbilical cord antigen is not contained in pregnancy serum; the detection of precipitation lines by immunodiffusion is decisively influenced by the concentration of the reaction partners (2). In the case of the reaction between amniotic fluid and absorbed antiplacenta immune serum at least 2 precipitation lines may be detected which likewise show immunological identity with antigens of the placenta extract. On the contrary, a phenomenon of crossing occurs with 1 precipitation sickle, which is visible between puerperal serum and absorbed antiplacenta immune serum.

From the literature it is known that with sufficient concentrating of the amniotic fluid nearly the whole spectrum of the blood serum proteins may be obtained immunoelectrophoretically. The fact that the high-molecular serum proteins IgA and IgM were not found in the amniotic fluid is explained by a sieve effect (17). Bevis (4) reports an immunoelectrophoretic studies of amniotic fluid and placenta extract with antihuman serum. He found two

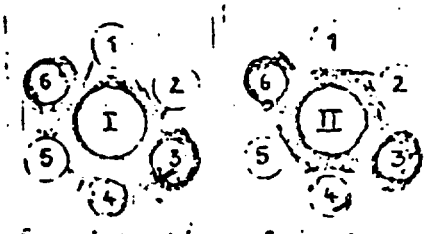

Fig. 2. Diffusion test for detection of antigens in umbilical cord serum (2), pregnancy serum (3), placenta extract (4), amniotic fluid (5) and puerperal serum (6). Control: Human serum (1). I absorbed antiplacenta serum. II Non-absorbed antiplacenta serum.

precipitation lines which, however, were identical with antigens of human serum and placenta extract. Lambotte and Salmon (13) in electrophoresis with human aminiotic fluid and antiaminiotic fluid immune serum of rabbits which had been absorbed by human serum found 2 precipitation lines in the α_1 and α_2 range, the latter showing lipoprotein character as evidenced by staining with Sudan black. In some cases the authors could detect the α_1-globulin post partum in the serum of the mother (13a).

Fig. 3 shows immunopherograms of pregnancy serum, aminiotic fluid, placenta extract, umbilical cord serum and puerperal serum which were developed with absorbed antiplacenta immune serum. In the placenta extract at least 7 precipitation lines may be seen. Since absorbed antiplacenta immune serum was used as antiserum, it is highly probable that the antigens of pregnancy serum, aminiotic fluid, umbilical cord and puerperal sera demonstrated with this antiserum are contained in placenta extract. Just as with absorbed antipregnancy immune serum, here also the precipitation lines were formed mainly in the α_2/β, range, so that possibly the same antigens are involved that were also demonstrated with absorbed antipregnancy immune serum (see article 1 in this journal). Work is being done on the description of the preparation and on the characterization of the placenta antigens.

These findings prove the existence of several pregnancy-specific antigens. A pregnancy-specific enzyme is oxytocinase (11) which is separable into 3 zones (20) by means of starch gelelectrophoresis. The HCG formed in the placenta shows a partial immunological identity with LH (5, 11, 15). A further factor which is typical of pregnancy, the HPL, is presently the subject of many studies (7, 16, 21).

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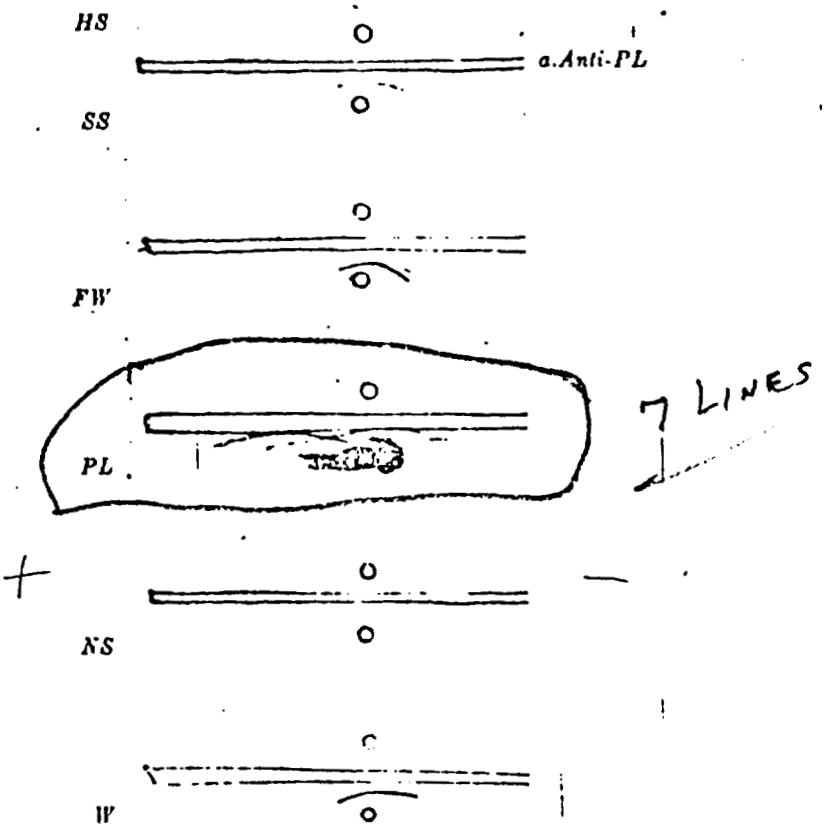

Fig. 3. Immunoelectrophoretic detection of the antigens in pregnancy serum (SS), aminiotic fluid (FW), placenta extract (PL), umbilical cord serum (NS) and puerperal serum (W) by means of absorbed antiplacenta serum (a Anti-PL).

During pregnancy the placenta assumes central importance. For this reason absorbed antiplacenta immune serum should be used to examine the problem as to whether the pregnancy-induced serum antigens of the mother are detectable with his immune serum. Moreover, a quantitative determination of these serum antigens can indicate new possibilities of diagnosis for the clinic in the recognition of pathological courses of pregnancy. Fig. 4 shows the suitability in principle of absorbed antiplacenta immune serum for the Mancini method of quantitative radial immunodiffusion. The preparation of monospecific precipitating immune sera from placental extracts off

the possibility of determining pregnancy-specific antigens quantitatively by immunological methods.

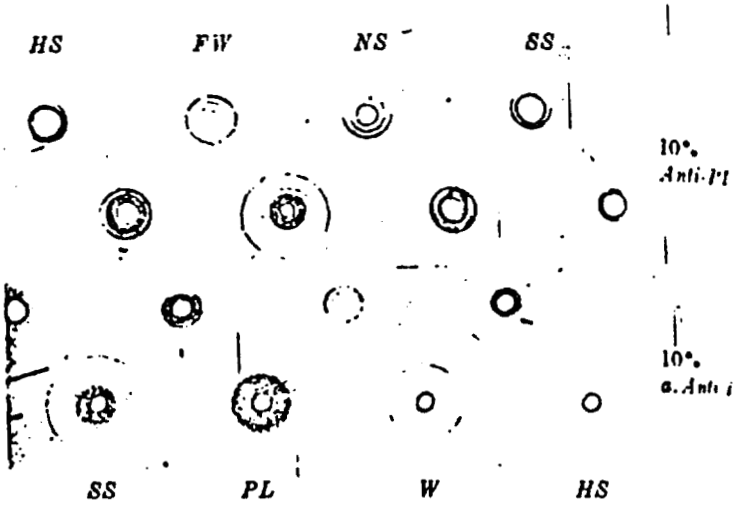

Fig. 4. Mancini radial immunodifussion of human serum (HS), pregnancy serum (SS), aminiotic fluid (FW), placenta extract (PL), umbilical cord (NS) and puerperal serum (W) against antiplacenta serum (Anti-PL) and against absorbed antiplacenta serum (a. Anti-PL).

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United States Patent [19]

Bohn et al.

[11] **4,065,445**

[45] **Dec. 27, 1977**

[54] **PREGNANCY-SPECIFIC β_1-GLYCOPROTEIN AND PROCESS FOR ISOLATING IT**

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[21] Appl. No.: **569,476**

[22] Filed: **Apr. 18, 1975**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 292,735, Sept. 27, 1972, abandoned.

[30] **Foreign Application Priority Data**

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[51] **Int. Cl.**[2] **A23J 1/06**
[52] **U.S. Cl.** ..... **260/112 B**; 424/12
[58] **Field of Search** ..... 260/112 R, 112 B; 424/12

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[57] **ABSTRACT**

A pregnancy-specific β_1-glycoprotein, characterized by
- an electrophoretic migration speed in agar gel in the range of the β_1-globulins,
- a sedimentation constant of 4.6 S ± 0.5 S,
- a molecular weight of 100 000 ± 15 000,
- an extinction coefficient $E_{1cm}^{1\%}$ of 11.6 ± 0.5 (278 mμ; 1/15-molar phosphate buffer, pH 7.0),
- a carbohydrate content of 28.05 ± 1.55%, comprising 10.7 ± 1.0% of hexoses, 10.0 ± 0.5% of hexosamine, 0.55 ± 0.05% of fucose and 7.0 ± 0.5% of neuraminic acid,

and process for isolating it from the placenta or blood or urine of pregnant women. This protein may be used as a reagent and for the preparation of antisera as reagents for the detection of pregnancy and for pre-natal supervision.

12 Claims, No Drawings

1075663

PREGNANCY-SPECIFIC β_1-GLYCOPROTEIN AND PROCESS FOR ISOLATING IT

This application is a continuation-in-part application of application Ser. No. 292,735 filed Sept. 27, 1972 and now abandoned.

This invention relates to a pregnancy-specific β_1-glycoprotein and to a process for isolating it.

It has not been known hitherto that the placenta and the urine of pregnant women contain a pregnancy-specific β_1-glycoprotein. Although a protein in the β-range has already been proved immunologically in the serum of pregnant women, a process for isolating it has not yet been described.

The object of the invention is a process for isolating the pregnancy-specific β_1-glycoprotein (abbreviated SP_1) by fractionation from the placenta and the blood or urine of pregnant women. Another object of the invention is the pregnancy-specific β_1-glycoprotein which is characterized by

- an electrophorectic migration speed in agar gel in the range of the β_1-globulins,
- a sedimentation constant of 4.6 S ± 0.5 S,
- a molecular weight of 100 000 ± 15 000,
- an extinction coefficient $E_{1\ cm}^{1\%}$ of 11.6 ± 0.5 (278 mμ; 1/15-molar phosphate buffer, pH 7.0),
- a carbohydrate content of 28.05 ± 1.55 %, comprising 10.7 ± 1.0% of hexoses, 10.0 ±0.5 of hexosamine, 0.55 ± 0.05% of fucose and 7.0 ± 0.5% of neuraminic acid.

An analytical amino acid assay of the pregnancy specific β_1-glycoprotein was carried out according to the method of Moore et al.

(Anal.Chem. 30 (1958) 1185) using the liquid chromatograph Multichrom B (Fa. Beckmann) and the ion exchange resins M 71 and M 82. Cystine (half) was determined as cysteic acid after oxidation of the proteins with performic acid (S. Moore; J.Biol.Chem. 238 (1963) 235) and subsequent chromatography as mentioned before. Tryptophane was determined with the direct photometric method according to H. Edelhoch (Biochemistry 6 (1967) 1948.

The analytical amino acid assay of the pregnancy specific β_1-glycoprotein shows the following composition:

Amino acid	Amino acid residues per 100 amino acids	
	mean	double standard deviation (± 2S)
Lysine	4.37	± 0.28
Histidine	1.77	± 0.07
Arginine	5.07	± 0.22
Aspartic acid	9.85	± 0.17
Threonine	8.24	± 0.39
Serine	9.07	± 0.55
Glutamic acid	8.97	± 0.22
Proline	7.96	± 0.31
Glycine	7.07	± 0.83
Alanine	3.74	± 0.33
Cystine (half)	1.57	± 0.14
Valine	5.84	± 0.32
Methionine	0.88	± 0.19
Isoleucine	5.79	± 0.40
Leucine	9.60	± 0.28
Tyrosine	5.75	± 0.20
Phenylalanine	2.12	± 0.21
Tryptophane	2.39	± 0.70

The ranges given for the amino acid contents in the above table are mainly due to the imprecision of the

analytical method and not to a non-uniformity or contamination of the product submitted to analysis.

The pregnancy-specific β_1-glycoprotein of the invention serves for the preparation of antisera. With these, a pregnancy can be proved by immunological methods (hemagglutination - inhibition test, complement binding reaction, radio-immuno-assay, latex agglutination). The proof can be carried out with the serum or urine of the pregnant woman. A quantitative determination of SP_1, for example by radial immuno-diffusion, may become important for pre-natal supervision.

For preparing the pregnancy-specific β_1-glycoprotein, comminuted placentas are extracted with water or a weak salt solution. A weak salt solution in the sense of the invention means an aqueous solution of one or more physiologically compatible salts having an overall concentration not essentially below or above that of a so-called physiological saline solution. Accordingly, suitable concentrations range between 0.1 and 5% by weight of solution. Suitable salts are those which are usually used in the aqueous extraction of tissue material, preferably sodium chloride, sodium phosphate, sodium citrate, tris-(oxymethyl)-aminomethane/hydrochloric acid, glycine/hydrochloric acid.

The aqueous extract thus obtained or the blood serum or urine of pregnant women is then precipitated at an acid, neutral or weakly alkaline pH by addition of acridine or quinoline or of a derivative, thereof.

The most preferred pH for this precipitation is pH 7-8 with acridine derivatives and 5-6 with quinoline derivatives. However, higher or lower pH values can be used with almost the same success so long as they are in the range between pH 5 and pH 9.

As acridine or quinoline compounds used in the present invention there are suitable, in addition to the basic compounds per se, the derivatives obtained from them by substitution. The substituents may be alkyl, alkoxy and/or amino groups in various positions. Since the acridine and quinoline bases are sparingly soluble in an aqueous medium it is preferred to use slightly water-soluble salts. E.g. 2-ethoxy-6,9-diamino-acridine, preferably in the form of its lactate, and bis-(2-methyl-4-amino-quinolyl-6)-carbamide suitably in the form of its hydrochloride, have proved especially suitable.

The acridine or quinoline compound is added to the placental extract or body fluid in form of an aqueous solution. The concentration of the acridine or quinoline compounde in the final stage shall be between 0.2 and 1.0% by weight calculated on the volume of the solution.

The acridine or quinoline compound should act on the extract or the body fluid for a period of time which gives the components sufficient opportunity to react and to form a precipitate. In general, a contact time of about 10 to 30 minutes is sufficient. Longer times may be used without hesitation. A shorter contact time is not advisable, although under certain circumstances it may also give good results.

The preferred temperature is room temperature i.e. 20° - 25° C, but higher or lower temperatures in the range between 0° and 40° C will be suitable, too. The contact of the liquid with the acridine or quinoline compound can be improved by intensive mixing, for example by stirring.

The precipitate that forms, i.e. the first precipitate in this process is then separated from the solution. This can be done in known manner, for example by decantation, filtration or, preferably, by centrifugation. The

precipitate is discarded. In the remaining liquid containing the SP_1, the precipitating agent which is not bound in the precipitate can be removed, if desired, according to known methods, for example by adsorption on suitable adsorbing agents such as silica gel, bentonite, active charcoal or by precipitation with alkali metal halides. After separation of the adsorbing agent or of the precipitate formed by precipitation of the excess acridine or quinoline compound, which separation is carried out according to the above-described known methods, or even directly for separating the first precipitate, the SP_1 remaining in the supernatant is precipitated together with gammaglobulin by salt addition. For this purpose an inorganic physiologically acceptable salt e.g. ammonium sulfate, sodium sulfate, potassium phosphate is added as a solid to the liquid up to a salt concentration of 50% calculated on the volume of solution. After standing for a while a precipitate (the second precipitate in this process) is formed. It contains the SP_1 together with the main quantity of gamma-globulin. This second precipitate is collected by a suitable method, e.g. decantation, filtration or centrifugation and is dissolved in an suitable aqueous solvent for further separation of the SP_1. This solvent may be pure water. More suitably, however, the solvent is one of the known saline or buffer solutions used in the isolating procedures for proteins. Especially suitable solvents in this stage of the process are tris-(oxymethyl)-aminomethane/hydrochloric acid buffer solutions or sodium phosphate buffer solutions.

The solvent is used in an amount sufficient to dissolve the whole second precipitate. A slight excess of the solvent is not deleterious. However, a large excess should be avoided. The solution thus obtained, if desired, is dialyzed against a saline or buffer solution for further purification.

Subsequently the SP_1 is adsorbed from this dialyzed solution on an ionexchanger, preferably an anion exchanger such as diethylaminoethyl (DEAE)-cellulose. the adsorption may be carried out in known manner in a batch process or continuously in a column. After adsorption, the SP_1 is eluted from the exchanger with a weakly acid buffer solution, e.g. a tris-oxymethylaminomethyl-hydrochloric acid buffer, preferably of pH 6.5. Generally, all buffer solutions having pH values between 2.0 and 9.0 are suitable for elution. The buffer solution may contain as additional ingredients physiologically compatible neutral salts such as sodium chloride in concentrations between and 0.1-10% and/or as stabilizers e.g. alkali metal azides in amounts ranging from 0.01 to 0.2%. The eluate may be precipitated again by salt addition, e.g. by ammonium sulfate, whereby the SP_1 is obtained in enriched form. It can be further purified by a molecular sieving method like ultrafiltration or gel filtration for example with the aid of Sephadex ® G-150. For further purification, preparative zone electrophoresis with PVC as a carrier in a suitable buffer solution, preferable in a solution containing volatile buffer salts for example 0.075% ammonium-bicarbonate may be used. After the electrophoretic separation, the SP_1-containing β-zone of the carrier is cut out, eluted with ammonium-bicarbonate and lyophilized. Other suitable fine purification methods comprise chromatographing on DEAE-Sephadex and subsequent elution with a sodium chloride gradient, and fractionation with alcohol, preferably with ethanol. The pregnancy-specific β_1-glycoprotein is isolated in similar manner from the blood or urine of pregnant women.

In addition to the fractionation steps that take advantage of its precipitation behavior and its electric charge, the pregnancy-specific β_1-glycoprotein may be obtained by a process which is based on the interaction between the pregnancy-specific β_1-glycoprotein and the antibodies obtainable therefrom. In this process, the pregnancy-specific β_1-glycoprotein is removed from its solutions by specific immune adsorption to a carrier containing an insoluble antipregnancy-specific β_1-glycoprotein, and eluted thereafter.

An antibody that can be bound according to known processes to a solid carrier can be prepared by immunization of animls with the SP_1, for example the product obtained according to Example 3, recovering blood from the so-immunized animals and isolating in known manner the immunoglobulin fraction from the serum of this blood.

The thus obtained anti-SP_1 serum is bound to a suitable carrier according to known methods as described e.g. in Cuatrecasas, P., Ann. Rev. Biochim 40, 259 (1971): Feinstein, G., Naturwissenschaften 58, 389 (1971) or Axen, R. et al., Nature 214, 1302 (1967). Suitable carriers are high polymer carbohydrates such as cellulose or agar, synthetic resins, for example polyacrylamide or ethylene-maleic acid anhydride-copolymers, or even glass particles.

A preferred carrier material is purified agarose in the form of small spheres of a diameter of about 20-200 μm to which antibodies against pregnancy-specific β_1-glycoprotein may be bound covalently according to the method of Axen, inter alia.

For the isolation of the pregnancy-specific μ_1-glycoprotein a solution which contains still other proteins or carbohydrate impurities in addition to pregnancy-specific β_1-glyoprotein, for example a placental extract or serum or urine of pregnant women, is brought into contact with the specific anti-pregnancy-specific β_1-glycoprotein adsorbent, the pH of the solution being adjusted to > 4. Because the upper pH value is limited by denaturation phenomena of the protein, it is advantageous to choose the pH within the range from 4 to 9. The salt concentration of this solution is not critical. Conditions that are known to dissolve antigen-antibody-bonds must be avoided. Advantageously, the aqueous solution contains neutral salts and/or buffer substances commonly used in biochemistry, especially sodium chloride, phosphates suitable as buffer substances, trishydroxymethyl-aminomethane or buffer substances that are enumerated by Good et al., Biochemistry 5, 472 (1977), preferably in a concentration range of 0.01-0.2M/1. The ratio of protein solution to adsorbent is advantageously within the range from 1 : 1 to 10 : 1, preferably 2 : 1. After the adsorbent has been in contact with the pregnancy-specific glycoprotein solution for 0.1 to 5 hrs., the pregnancy-specific β_1-glycoprotein is specifically bound by the antibodies. The adsorbent is then separated from the solution by centrifugation or by filtration and washed several times with a neutral salt solution to eliminate excess salts and unspecifically adsorbed impurities.

The elution of the pregnancy-specific β_1-glycoprotein can be carried out using measures which are known for their capability of dissolving antigen-antibodies.

Thus the elution can be carried out using a solution having a pH value of <4, preferably within the range of from 2 to 4. This procedure is effected by contacting the adsorbent bearing the SP_1 with a weakly concentrated

aqueous salt or buffer solution, preferably one containing 0.2 to 8 moles per liter of neutral salts or buffer substances. Most simply a dispersion of the adsorbent in the solution is stirred for 0.1 to 2 hours, whereupon the pregnancy-specific β_1-glycoprotein is desorbed from the immune adsorbent. After separation of the adsorbent the solution containing the pregnancy-specific β_1-glycoprotein is adjusted to a pH near the neutral point, i.e., between 6 and 8. Thereafter further purification, for example with hydroxylapatite may follow, if desired.

Likewise the elution can be carried out by the use of solutions of protein-dissociating agents, for example, urea solutions or solutions of so-called chaotropic salts, for example $NaNO_3$, NaBr, $NaClO_4$, CF_3COONa, NaSCN and CCl_3COONa having a molarity of 0.2 to 8 m/l. To remove the chaotropic salts from the solution after desorption of the pregnancy-specific β_1-glycoprotein and separation of the adsorbent, the solution is dialysed against a neutral salt or buffer solution having a concentration between 0.1 -1 M/1. Further purification may follow also in this case, for example with hydroxyl apatite as described below.

Generally, the pregnancy-specific glycoprotein obtained according to the process steps described has a degree of purity within the range of from 90 to 95%.

An especially adequate method of highly purifying the product is an adsorption step using a hydroxylapatite suspension as adsorbent and choosing the adsorption conditions such that the pregnancy-specific $\beta_1$glycoprotein is not adsorbed, but only the impurities still present are bound by the hydroxylapatite.

It is essential that the adsorption takes place in an aqueous medium containing phosphate ions, preferably in a buffer solution containing phosphate ions, for example a solution of potassium phosphate or sodium phosphate in a pH range of 6 to 8 and with 0.001 to 0.01 mols phosphate ions. For the adsorption of the impurities, batch processes as well as column chromatography are suitable. According to these processes, the pregnancy-specific β_1-glycoprotein is obtained in a purity of above 99%.

The following Examples illustrate the invention:

EXAMPLE 1

Isolation from placentas

150 kg of deep-frozen placentas were comminuted and extracted with 150 liters of a 0.5% aqueous solution of sodium chloride. The extract was adjusted to pH 8 by means of 2N-sodium hydroxide and combined with 50 liters of a 3% aqueous solution of diaminoethoxyacridine lactate (Rivanol®). After 1 hour the supernatant, which contained the SP_1 together with gamma-globulin, was siphoned off from a first precipitate which had formed, combined with 5 % of solid sodium chloride (11kg) in order to separate the Rivanol which still had remained in solution, filtered and combined with 26.5%, referred to the weight of the liquid, of solid ammonium sulfate and well stirred. After 1 hour the precipitate which formed was filtered off.

500 g of the precipitate that had deposited on the filter were dissolved in 500 ml of distilled water and dialyzed against a 0.01 molar tris-(oxymethyl)-aminomethane (hereinafter referred to as "tris")/hydrochloric acid buffer solution having a pH-value of 7.0 and containing 0.05% of sodium azide. The dialyzed solution was centrifuged and the supernatant was filled up with the same buffer solution to a volume of 2.000 ml, the pH-value was adjusted to 8.0 by means of 0.1 N sodium hydroxide solution and stirred with 250 g of wet DEAE-cellulose for 1 hour.

The DEAE cellulose was then separated by filtration from the solution, washed twice with 1 liter portions of 0.01-molar tris/hydrochloric acid buffer having a pH-value of 8.0 and then eluted thrice with 500 ml portions of a 0.02-molar tris/hydrochloric acid buffer having a pH-value of 6.5 and containing 0.85% of sodium chloride and 0.05% of sodium azide. The combined eluates were combined with 30% of ammonium sulfate, referred to the weight of the liquid, and the whole was stirred. The precipitate which contained the SP_1 was dissolved in 300 ml of distilled water and subjected to gel filtration by means of Sephadex G-150 and using a 0.1-molar tris/hydrochloric acid buffer having a pH-value of 8.0 and containing, per liter, 1.0 mol of sodium chloride (100 : 20 cm column).

The eluates were then tested with specific SP_1-antiserum, the SP_1-containing fractions were collected and the proteins were precipitated again therefrom as described above with the aid of 30% solid ammonium sulfate.

A further purification was effected by preparative zone electrophoresis with polyvinyl chloride (PVC) as carrier. As buffer, a 0.075-molar ammonium-bicarbonate solution (pH 8.0) was used. After the electrophoretic separation, the SP_1-containing β-zone was cut out, eluted with the ammonium-bicarbonate buffer and the eluates were lyophilized.

The dry substance was dissolved in a 0.01-molar tris/-hydrochloric acid buffer having a pH-value of 7.0 and containing 0.2% of sodium chloride, chromatograhed on DEAE-Sephadex (column 5 × 20 cm) and eluted with a linear gradient of 0.2 to 2.0% of sodium chloride in a 0.01-molar tris/hydrochloric acid buffer having a pH-value of 7.0. Then, precipitation with 40% ethanol was carried out at −5° C. After the precipitate was separated by centrifugation, the supernatant was dialysed against distilled water until it was free from salt and lyophilized. Purity 90 -95%. This preparation could be used for the manufacture of antisera.

EXAMPLE 2

Isolation from blood

5 Liters of the serum of pregnant women were diluted with distilled water in a ratio of 1 : 1 and the main quantity of the plasma proteins was precipitated therefrom with the aid of 2.5 liters of 3% of Rivanol at a pH-value of 8.0. For removing the Rivanol, the supernatant obtained by centrifugation was combined with 5% of sodium chloride. The resulting precipitate was removed by centrifugation and the supernatant was separated from the sediment and combined with 30% of solid ammonium sulfate. The precipitate contained the gamma-globulin and the pregnancy-specific β_1-glycoprotein. The precipitate was dissolved in a 0.01-molar tris/hydrochloric acid buffer having a pH-value of 7.0, dialyzed against a 0.01-molar tris/hydrochloric acid buffer having a pH-value of 7.0 and then purified with DEAE-cellulose. The treatment on DEAE-cellulose was carried out in a 0.01-molar tris/hydrochloric acid buffer having a pH-value of 8.0.

Elution from the DEAE-cellulose, gel filtration with Sephadex, preparative zone electrophoresis, purification on DEAE-Sephadex and precipitation with alcohol were carried out as described in Example 1.

EXAMPLE 3

Isolation of pregnancy-specific β_1-glycoprotein from its solutions by immune adsorption

An immune adsorbent suitable for specifically binding pregnancy-specific β_1-glycoprotein was prepared by binding 1.75 g of protein of an immunoglobulin fraction obtained from anti-pregnancy-specific β_1-glycoprotein rabbit serum to 175 g of Sepharose (registered trade mark) 4 B, (cf. Handbook of Biochemistry, selected data for molecular Biology, Edited by Herbert A. Sober, 2nd Edition, 1970, page K 73) that was activated with 21, 8 g of BrCN (cf. Axen R., Porath, J., Ernbach, S.: Nature 214, 1302 (1967)). By means of this immunoadsorbent, pregnancy-specific β_1-glycoprotein was isloted from the extract of placentae according to the following process:

1 liter of a placental extract containing pregnancy-specific-β_1-glycoprotein the protein content of which is 5%, is dialysed against 10 liters of 0.1 molar trishydroxymethyl-aminomethanehydrochloric buffer (pH 8) which contains 1 mol/1 of NaCl and 1g/1 of sodium azide. Thereafter the adsorbent is obtained above is added to this solution, the solution is stirred at 4° C for 2 hours and the adsorbent is suction-filtered together with the bound pregnancy-specific β_1-glycoprotein. The adsorbent is washed with 2 × 250 ml of 0.1 molar trishydroxymethylaminomethane-hydrochloric acid-buffer solution and then with 2 × 250 ml of a physiological sodium chloride solution (0.9%); then the absorbent is treated six times for 20 minutes each with 400 ml portions of a 0.5 molar glycine-HCl buffer of pH 2.5. The combined eluates are adjusted to pH 7 with 1 N NaOH and concentrated to 10 ml on an ultrafilter. The concentrate is dialysed against a 0.005 molar sodium phosphate buffer of pH 6.8 and further purified by chromatography on hydroxylapatite as described in Example 4.

The same process can be used when the pregnancy-specific β_1-glycoprotein is to be obtained from the serum of the urine of pregnant women.

EXAMPLE 4

For highly purifying pregnancy-specific β_1-glycoprotein, 50 mg of the product obtained according to Examples 1 or 2, having a purity of 90–95%, were dissolved in 10 ml of 0.005 molar sodium phosphate buffer (pH 6) and applied to a column of a dimension of 2 × 8 cm filled with hydroxylapatite (Messrs. Serva, Heidelberg) which had been equilibrated before with 0.005 molar phosphate buffer. Upon eluting the substance with 0.005 molar phosphate buffer, the pregnancy-specific β_1-glycoprotein migrates through the column, whereas the impurities remain adsorbed on the hydroxylapatite. The protein-containing eluate was dialysed against 10 times its volume of water to eliminate the salts and then lyophilized. Yield of pregnancy-specific β_1-glycoprotein about 40 mg; degree of purity: > 99%. That is according to immunological processes, none of the admixtures ascertained before purification could be reascertained.

We claim:

1. A purified, isolated pregnancy-specific β_1-glycoprotein characterized by an electrophoretic migration speed in agar gel in the range of the β_1-globulins,
   a sedimentation constant of 4.6 ± 0.5 S,
   a molecular weight of 100,000 ± 15,000,
   an extinction coefficient $E_{1cm}^{1\%}$ of 11.6 ± 0.5 (278 mμ; 1/15-molar phosphate buffer, pH 7.0),
   a carbohydrate content of 28.05 ± 1.55%, comprising 10.7 ± 1.0% of hexoses, 10.0 ± 0.5% of hexosamine,
   0.55 ± 0.05% of focose and 7.0 ± 0.5% of neuraminic acid.

2. A process for isolating a pregnancy-specific β_1-glycoprotein, which comprises
   a. combining the blood serum, urine or an extract in a weak salt solution of the comminuted placentas of pregnant women with an acridine or quinoline compound at a weakly alkaline pH whereby a first precipitate is formed;
   b. discarding said first precipitate and combining the remaining supernatant liquid with an inorganic physiologically compatible salt, whereby a second precipitate is formed;
   c. collecting said second precipitate, dissolving it in an aqueous medium and dialysing the resulting solution against a saline or buffer solution;
   d. filtering the dialyzed solution on anion-exchanger, whereby the pregnancy-specific β_1-glycoprotein is absorbed on said ion-exchanger and
   e. eluting the β_1-glycoprotein from said ion-exchanger.

3. A process as in claim 2 wherein said eluted β_1-glycoprotein is further purified by precipitation with ammonium sulfate, by gel filtration, or by preparative zone electrophoresis.

4. A process as in claim 2, wherein as an acridine compound diaminoethoxyacridine lactate is added as a 3 percent solution in an amount from one-third to one-fourth of the volume of the blood serum, urine, or extract to which it is added.

5. A process as in claim 2, wherein as an inorganic salt solid ammonium sulfate is added to said supernatant liquid in an amount from 26.5 to 30 percent by weight of the supernatant liquid.

6. A process as in claim 2, wherein said buffer solution is a 0.01 to 0.1 molar tris-oxymethylaminomethane/hydrochloric acid solution.

7. A process as in claim 2, wherein said β-glycoprotein is eluted from said ion exchanger with a buffer solution.

8. A process for isolating a pregnancy-specific β_1-glycoprotein which comprises contacting the blood serum, urine or an extract, in a weak salt solution. of the comminuted placentas of pregnant women with an antibody of said pregnancy specific β_1-glycoprotein bound to a solid carrier at a pH between 4 and 9 and subsequently eluting the glycoprotein from the carrier at a pH between 2 and 4 or by treatment with a protein dissociating compound.

9. Process of claim 8, wherein the antibody is obtained by administering the pregnancy-specific β_1-glycoprotein to rabbits and isolating the antibody therefrom.

10. Process of claim 8, wherein the carrier is a polymeric carbohydrate.

11. Process of claim 8, wherein the protein dissociating compound is urea.

12. Process for the purification of an isolated pregnancy-specific β_1-glycoprotein, wherein said glycoprotein in an aqueous solution containing phosphate is contacted with hydroxylapatite and subsequently the hydroxylapatite is separated from the aqueous medium containing the glycoprotein.

* * * * *

BIOCHEMISTRY

4

TRYPSIN [illegible] INHIBITORS IN MOUSE UTERI DURING [illegible]. Martha Somerville* and Danica Dabich. University School of Medicine, Detroit, Mich.

[illegible] activity of inhibitors of trypsin-like activity [illegible] in 27,000 x g supernatant solutions prepared from non-pregnant, pregnant, and delayed implantation uteri. Activity measurements were based on a fluorometric kinetic assay utilizing benzoyl-arginine-naphthylamide. Specific activities of day 4 thru day 8 delayed implantation and day 4 normal pregnant uteri were indistinguishable from non-pregnant uteri. By day 5, the day of implantation, an increase in specific activities were found in normal pregnant uteri, and became statistically significant by day 6. The elevated values returned to normal by day 12. Qualitative changes in inhibitor activity were not found by disc gel electrophoresis after analysis of the various samples. The increase in uterine inhibitor activity on the day after implantation may counterbalance the increase in blastocyst cytoplasmic tryptic-like activity which occurred on the previous day. The relationship between uterine proteinase inhibitor activities and the corresponding enzymes of the blastocyst may represent a link in the regulation of a biochemical system involved in implantation.
Investigations supported by NICHD grant 06234.

444

DETERMINATION OF PITUITARY AND PLASMA OXYTOCIN IN [illegible] LEVELS IN RATS DURING PARTURITION AS DETERMINED BY RADIOIMMUNOASSAY (RIA). [illegible] Subramanian, P. R. Anandarangam, and [illegible] (SPON: Ralph R. Anderson) Hormonal Labs. [illegible]/CYN, Coney Island Hospital, Brooklyn, NY 11235

This laboratory previously reported a RIA for pituitary and plasma oxytocin levels in rats during various stages of pregnancy. (Kumaresan et al. Prog. of the Fifty-[illegible] Annual Meeting of the Endo. Soc. 1973 Abst. 238). The present report has concerned about the study during parturition and the experiment was repeated simultaneously during pregnancy and was compared with the previously reported study. The data indicate that the mean OT levels increased in plasma significantly on day 19, 20 and 21 compared with day 18. Further demonstrates that just before delivery the mean OT level reached in plasma to its basal level and continuously [illegible] up to delivery of first pup and gradually increased during third and fifth pup's delivery and significantly increased [illegible] ing completion of delivery. Maternal pituitary oxytocin level was significantly increased on day 21 of pregnancy compared with day 18 of pregnancy and gradually the level was reduced during subsequent delivery. (Supported by NIMHS Grant HD-04984-02 and the Population Council of NY).

PHYSIOLOGY

442

THE CONTRACTILE RESPONSE OF BOVINE UTERUS TO $PGF_{2\alpha}$ AND NOREPINEPHRINE. O.P. Verma. Tuskegee Institute, AL 36088 (SPON: W.C. Bowie)

Isolated preparation of the uteri obtained from pregnant and non-pregnant cows were placed in a muscle chamber containing Krebs's-Ringer solution, tied to the force transducer (Myograph A) by a silk thread and the contractions were recorded on a four channel physiograph. After the contractions were stabilized, controlled tracings of 20-30 period were recorded before the addition of drugs. Prostaglandin ($PGF_{2\alpha}$) was added at dose levels in 1 μg/ml, 2 μg/ml and 4 μg/m. and followed by norepinephrine at the same dose levels. The results from prepubertal heifers having small developing follicles (group 1), heifers having well developed follicles (group 2) and cows in their first trimester of pregnancy (group 3) indicate that the uterine activity is highest in group 1 and lowest in pregnant group. Addition of $PGF_{2\alpha}$ produced a stimulatory response in the pregnant uteri, ceased contractions in animals with developed follicles but did not influence the uteri from prepubertal heifers. Norepinephrine produced a response similar to $PGF_{2\alpha}$ in all the animals investigated. The results suggest that the hormonal status of the animal influences the response of $PGF_{2\alpha}$ and norepinephrine. (Supported by a grant #316-15-134 from CSRS, USDA)

445

PHYSIOLOGY

EFFECT OF INTRAUTERINE COPPER WIRE ON THE [illegible] IMPLANTATION RAT MORULAE AND BLASTOCYSTS IN VITRO. C. C. [illegible]. Biomedical Division, The Population Council, The Rockefeller University, New York, N.Y. 10020

Pregnancy in rats and hamsters can be [illegible] when a small copper ring is transfixed [illegible] the uterine lumen prior to implantation. The effect [illegible] the viability of rat morulae and blastocysts was [illegible] in vitro. Morulae or blastocysts were cultured in [illegible] medium containing 10% inactivated fetal [illegible] serum [illegible] 5% CO_2 in air for 24 hrs. A 99.9% pure [illegible] length, 0.1 mm in diameter was placed in [illegible] culture [illegible]. The zona pellucida remained intact over the exposure [illegible]. Normal implantation did occur when [illegible] morulae [illegible] cysts were transferred into the uterine [illegible] of [illegible] pseudopregnant recipient rats. There [illegible] no significant difference in the percentage of implantation between [illegible] "influenced" and control embryos (57.3 vs 25.9 of b[illegible] 42.0 vs 41.4 of morulae). It is concluded [illegible] system Cu wire under the experimental conditions [illegible] any deleterious effect on the zona pellucida and is [illegible] to the preimplantation embryos. They remain viable [illegible] transferred into a normal recipient uterus where they [illegible] implant and develop normally. (Supported in part by [illegible] No. 1 P01 HD 0567-01)

443

IMMUNOLOGY

[illegible] OF PREGNANCY-ASSOCIATED PLASMA PROTEINS (PAPP'S) DURING GESTATION AND THEIR IMMUNOLOGICAL IDENTIFICATION. T.N. Lin, [illegible] Palmbert, W.N. Spellacy, and S. Gall*. Univ. Miami Med. Sch. (Pediatrics and Obstetrics & Gynecology) Miami, FL 33152.

Further studies on the four PAPP's described in our laboratory (Fed. Proc. 32:623,1973) revealed that by gel diffusion methods, none of them was detectable in women taking oral contraceptives, in persons with various malignancies, and in plasma from the umbilical vein and artery. None was related to placental alkaline phosphatase. One of them (PAPP-D) was identified as [illegible] while another (PAPP-C) was identical to the pregnancy-[illegible] glycoprotein (SP_1) described by Bohn. The third one (PAPP-A, m.w. 750,000) was distinct from α_2-pregnoglobulin, pregnancy zone, pregnancy-associated glycoproteins SP_2 and SP_3, pregnancy-associated globulin (PAG or PA1) and pregnancy α-macroglobulin. All the latter (except SP_2) were shown to be immunologically [illegible] other. PAPP-B was also unrelated to any of the known pregnancy-associated proteins. By crossed-immunoelectrophoresis, PAPP-A was found to increase steadily during pregnancy till the time of delivery, while PAPP-C and PAPP-D rose to a plateau in the late 3rd trimester. PAPP-B was detected in at least half of the specimens in the last two months of gestation, but was not seen during the 2nd trimester. PAPP-D dropped sharply on the day of delivery and was not detectable thereafter. Both PAPP-A and PAPP-C decreased markedly in the [illegible] week postpartum and slowly after that. Several obstetric parameters appeared to be related to the levels of some of these PAPP's. (Supported by B.Phs-NIH grant HD-5736).

446

CORRELATION BETWEEN FERTILITY AND THE SERUM CORTICOSTERONE RHYTHM IN MICE. Judith A. Ramaley, Department of Physiology & Biophysics, Univ. of Nebr. Med. Center, Omaha, Nebr.

Female C 57/Bl 6 mice in different reproductive [illegible] subjected to two daily 5 minute periods of immobilization stress at 8 am and 7 pm (lights on 6 am to 8 pm) for [illegible] We have previously reported (Fertil. and Steril. 24:540, 19[illegible]) that stress delays puberty in immature mice and increases litter size and percentage of conceptions in older mice. Groups of 7 mice each were decapitated [illegible] hour intervals before stress and 1 and 7 days after [illegible] stress period. In 32 day old non-stressed mice the peak serum corticosterone (B) concentration was at 8 pm (32.1±1.2 μg/100 ml serum) and trough at 8 am (12.1±0.7 μg/100 ml). In 60 day old mice before stress the peak and trough of B were at 8 pm and 8 am respectively but the values were slightly higher (36.5±1.1 μg% and 16.5±0.9 μg%, p = 0.05, student's t). There was no significant daily variation in serum B in persistent estrous females (200 days old) before stress (F test, p [illegible]). A week of stress shifted the B peak to 4 am in the young [illegible] but the peak had returned to 8 pm 7 days after stress [illegible] in cycling adults caused an inversion of the normal daily rhythm both 1 and 7 days after stress. In old, non-cycling adults, stress elicited a daily rhythm of B with a peak [illegible] pm (30.1±3.4 μg%) 1 and 7 days after stress. This study demonstrates that changes in fertility induced by stress are accompanied by a change in the daily pattern of serum corticosterone. Supported by NSF Grant GB 35730.

1075668

[illegible] OF A TRANSGLUTAMINASE FROM THE COAGU- [illegible] AND OF THE GUINEA PIG PROSTATE. D. Wimer, [illegible] L. Lorand and H.G. Williams-Ashman (SPON: [illegible] Jr.) Biochem. Div., Dept. Chem. Northwestern [illegible], Evanston, IL 60201 and Ben May Laboratory, [illegible] of Chicago, Chicago, IL 60637.

[illegible] pig coagulating gland contains an enzyme similar in [illegible] respects to fibrinoligase and liver transglutaminase. It [illegible] calcium dependent transamidations such as the in- [illegible] of putrescine into N, N-dimethylcasein (Anal. Bio- [illegible] 53, 633, 1973) and dansylcadaverine into benzyloxycar- [illegible] (ibid. 44, 207, 1971). Purified pre- [illegible] of the enzyme show essentially a single band on both [illegible] and SDS gel electrophoresis. Molecular weight as [illegible] by the latter method was 70,000 ± 10,000. Reac- [illegible] iodoacetamide occurs in the presence and absence [illegible] calcium. However, alkylation in the presence of calcium [illegible] to be limited to a single site at the active center. Specific alkylation is accompanied by a proportional decrease in enzyme activity.

Supported by NIH HL-02212-5T1-GM-626.

A COMPARISON OF THE METABOLISM OF ANDROGENS IN THE ANTERIOR PITUITARY (AP) AND THE TESTIS OF THE MATURE RAT. Lidia W. Liu Kan*, A. Perez Lloret* and Judith Weisz* (SPON: C. W. Lloyd). Div. of Pepr. Biol., Dept. of Ob-Gyn, The M. S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pa. 17033.

Seminiferous tubules (ST), testicular interstitial tissue (IT) or AP were incubated with tritiated testosterone (T), dihydrotestosterone (DHT), 5α-androstane-3α,17β-diol (3α-Adiol), or its 3β isomer (3β-Adiol). The T, DHT, 3α-Adiol, 3β-Adiol, and androstenedione (A) were isolated and quantified by double isotope dilution technique. In all 3 tissues, most of the DHT was metabolized (residual DHT: 6-17%), whereas a large portion of T remained unconverted (58-83%). A higher percentage of 3α-Adiol was formed from T in AP (13.9%) than in ST (3.9%) or IT (0.1%), while more A was formed in IT (20.7%) and ST (11.2%) than in AP (1.4%). The amounts of DHT formed by ST and AP from T was 1.5 and 1.9% respectively, but only 0.2% by IT. DHT was converted in both AP and ST to 3α-Adiol (57-69%). In IT, in addition to 3α-Adiol (25.8%), 3β-Adiol (8.7%) was formed. Conversion of 3α-Adiol to DHT occurred in incubations of all 3 tissues, whereas conversion of 3β-Adiol to DHT occurred only in those of ST or IT. 3β-Adiol was converted to polar metabolites by AP (75.6%). Thus, the previously demonstrated effect of 3α-Adiol on the responsiveness of AP must be due to 3β-Adiol or its metabolites. On the other hand, the action of 3α-Adiol on any of the 3 tissues and of 3β-Adiol on ST could be due to DHT, 3α-Adiol or 3β-Adiol.

A [illegible] AND HIGHLY SENSITIVE METHOD TO QUANTITATE INDIVIDU- [illegible] 11-DEOXYANDROGENS UTILIZING THE TESTOSTERONE-BINDING [illegible] (TeBG) OF RABBIT PLASMA AND THE RESPECTIVE ^{3}H-LIGANDS. [illegible]* (SPON: M. Reynolds). Univ. of Pennsylvania, [illegible] Med., Kennett Square, PA, 19348.

Plasma TeBG has recently been demonstrated to have [illegible] physico-chemical properties as that of human TeBG. [illegible] access to rabbit plasma and the availability of [illegible] testosterone (T), dihydrotestosterone (DHT) and [illegible] diol (Ad) prompted this study. Following ethereal [illegible] of samples T, DHT and Ad are individually fraction- [illegible] Sephadex LH-20 column chromatography. TeBG-isotope [illegible] employed is prepared by spiking 0.5% rabbit plasma [illegible] of the desired ligand (T, DHT or Ad) in phosphate [illegible] 7.4) and Florisil is used to separate the bound [illegible] at 4°C. Sensitivity for T was 100 pg and [illegible] T and Ad. As an index of precision the [illegible] variability for all three ligands the coefficient [illegible] was 10%. Since both specific activities as well [illegible] ion constants of these ligands to TeBG are differ- [illegible] methods using only H^3-T and human TeBG to [illegible] and Ad levels are inaccurate. The present study [illegible] the above problems and thus is a more reliable [illegible] simultaneous quantitation of T, DHT and Ad from [illegible] samples.

FETOPLACENTAL CONTRIBUTION TO MATERNAL STEROID POOL IN RATS. Evangelos A. Petropoulos and Rafael Lopez-Manzanares.* Dept. of Anatomy, University of California, San Francisco, Ca. 94143.

Plasma estrogen and progesterone were determined by radioimmunoassay on day 15, 17, 19 and 21 of pregnancy in the following groups of Long-Evans rats: normal pregnant (N), fetectomized ($\bar{F}$), ovariectomized ($\bar{O}$), and $\bar{F}\bar{O}$adrenalectomized ($\bar{F}\bar{O}\bar{A}$) on day 13 of pregnancy; in all instances placenta was left in situ. Although progesterone decreased drastically in N rats on day 21, estrogen did not change throughout gestation. No change was observed in $\bar{F}$ rats. Progesterone decreased dramatically, but estrogen moderately, in $\bar{O}$ and $\bar{F}\bar{O}$ rats, although $\bar{F}\bar{O}$ rats showed a slight increase in progesterone over $\bar{O}$ rats. Although both progesterone and estrogen were further decreased in $\bar{F}\bar{O}\bar{A}$ rats, they remained in appreciable levels, throughout gestation. These results indicate that during pregnancy in the rat the main source of progesterone is the ovary; however, the maternal adrenals and the placenta also contribute both estrogen and progesterone into the maternal steroid pool. The cause for the higher progesterone observed in $\bar{F}\bar{O}$ than in $\bar{O}$ rats is obscure; however, we may speculate that fetectomy+ovariectomy had, perhaps, stimulated the secretion of progesterone by the maternal adrenals. (USPHS grant #HD-40491).

ALPHA-2 PREGNOGLOBULIN LEVELS - A TEST OF PLACENTAL FUNCTION IN EARLY PREGNANCY. Bernard H. Berne, Med. Sci. Prog., Indiana Univ., Bloomington, IN 47401

Alpha-2 pregnoglobulin (α_2P) is a unique protein (mol.wt. 326,000) greatly elevated in serum in pregnancy and in estrogen and oral contraceptive users (Berne, B.H., IRCS Med.Sci.(73-10) 10-26-4, 1973). We measure levels by radial immunodiffusion.

In controls, 45% of college women and 5% of men have measurable levels up to 5 units. At term in pregnancy, 247 women's levels distributed widely on an asymmetric curve: mode 20 units at the 30th percentile; median (M) 31; mean 32; range (R) 2-150. Some 5% have only non-pregnant levels. The literature suggests the mean to be 90 mg/100 ml.

To evaluate levels during spontaneous abortion:

	Normal Preg.			Spont. Abort.			
Weeks	n	M	R	n	M	R	P (Mann-Whitney)
4-6	66	4	0-18	10	2	0-5	<0.06
7-9	129	5	0-56	17	3	0-10	<0.002
10-12	67	9	0-49	20	4	0-20	<0.001

The only two aborts above 7 units had a febrile respiratory illness and an IUD *in utero*, respectively. Later abortions were not significantly different from normal, nor were 6 preeclamptics at term. There was no correlation with fetal sex, prematurity, congenital anomalies or late fetal death. A level of 53 in a choriocarinoma patient was matched only by 2 of 50 pregnancies of the same duration (12-14 weeks). Levels rise in the second trimester, but remain stable during the third, unlike estrogens. α_2P may be useful in evaluating threatened abortion in early pregnancy. (USPHS SO5 RR 7031).

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30 A 51
30 D 3
JAPAN GROUP CLASS 125 424

特許庁
特許公報
特許出願公告 昭42－10369
公告 昭42.6.5
（全3頁）

Application copy

10369/67 Human chorionic gonadotrophin (H.C.G.) is obtained by treating the impure HCG which is extracted from the urine of pregnant women or human placenta with human urine (except that of pregnant women or certain tumour patients) and adsorbing the impurities serum-scientifically. The H.C.G. is useful as an antigen for preparing a highly selective antibody. 25.6.64(Non-Con) TEIKOKU HORMONE MFG. CO. LTD. 5.6.67 (30A)

高純度の胎盤性性腺刺激ホルモンの製

特　　願　昭 39－35811
出 願 日　昭 39. 6. 25
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発明の詳細な説明

本発明は血清学的に高純度の胎盤性性腺激戟ホルモンの製法に関する。

血液、尿その他の体液中に存在する抗原性のある物質を、血清学的手段によつて検出または検定することが近年盛んに行われるようになつた。例えば尿中の胎盤性性腺刺激ホルモン（以下HCGとして示す）を兎に免疫して得られた抗HCG兎血清を用い、血球凝集阻止反応その他の血清学的方法によつて検出することが、妊娠診断の一手段として利用されている。その他体液中の生長ホルモンやインシユリンその他の蛋白性ホルモンの血清学的検定も、臨床的診断法として利用できる。

このような血清学的検定においては、その特異性および精度を高める上に力価の高い特異的な抗体を含む抗血清が必要であり、それを可能にするためには高純度の抗原を必要とする。しかし一般に化学的手段によつて微量の不純物を除去するためには種々の困難を伴い、ある程度以上に純化することは容易ではない。例えばHCGの抗血清を得るためには、抗原として通常妊婦尿から得られるHCGを用いるが、この抗原HCGを化学的な方法で6000IU～8000IU/mg以上の純度に精製することは困難である。これに含まれる不純物もまた抗原性を有するため、HCGを抗原として用いる場合、それらは抗原性をもつことによつて障害となる。この不純物は尿成分であるが、抽出物中のHCG以外の不純抗原物質を血清学的に吸着することによつて、不純物質を除去し得ることを見出した。この精製純化したHCGは、特異性の極めて高い抗体の製造その他の場合に、抗原として有利に使用できる。

本発明は、動物の体液または胎盤から抽出した不純な胎盤性性腺刺激ホルモンを、該ホルモンを含有しないかもしくは極めて少量含有する動物の体液またはそれら抽出した物質によつて免疫した別種の動物の抗血清で処理することを特徴とする、免疫血清学的に高純度の胎盤性性腺刺激ホルモンの製法である。

本発明において原料の体液または胎盤としては、例えば人、牛、馬等の血清、尿等または人の胎盤が有利に使用される。

本発明を実施するに際しては、まず被吸収検体HCGを例えば妊婦尿から分離精製する。その方法としては例えば妊娠尿から安息香酸に吸着して分離するグリンまたはクリーソンの方法（J. Biol.Chem. 128 525（1939）、Acta. Endocrinol. 11（1948））、あるいはイオン交換樹脂に吸着して分離する松島の方法（特許第266190号参照）が用いられる。また一方では中和、吸収用抗血清製造のため、これと同じ処理によつて健康な男子または妊娠していない婦人の尿から乾燥粉末を製造し、この粉末を例えば生理食塩水に溶解し、あるいはこの溶液にアジユバント（免疫補強剤）を添加したものを用いて、人以外の動物例えば兎に注射して免疫する。この場合体液そのものも使用可能であるが、通常は抽出物を使用する方が優れている。この免液血清を用いて好ましくは羊の赤血球を常法により感作する。こうして得られた感作血球を前記HCGの水溶液に加え、2～3分よく振り混ぜ、室温で2時間放置し、さらに4℃で一夜充分に反応させ、その反応生成物を遠心分離して除去することによつてHCGを免疫血清学的に純化することができる。

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after filtration, it is desirable to remove further impurities with the addition of active carbon for injection use. 14.1.64(Non-Con) as 1319/64 TAKEYAMA, F. 5.6.67 (30A)
10365/67 Isovaleric acid ester having a calmative function is obtained from the root of valeriana officinalis using an oleophilic ... (26.1.63 CE

Y44-100

30 A 51
(30 D 31)
JAPAN CLASS 125
CLASS 424

特　許　庁
特　許　公　報
特許出願公告
昭42－10365
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高純度の胎盤性性腺刺激ホルモンの製法

特　　願　昭　39－35811
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発明の詳細な説明

本発明は血清学的に高純度の胎盤性性腺激戟ホルモンの製法に関する。

血液、尿その他の体液中に存在する抗原性のある物質を、血清学的手段によつて検出または検定することが近年盛んに行われるようになつた。例えば尿中の胎盤性性腺刺激ホルモン（以下HCGとして示す）を兎に免疫して得られた抗HCG兎血清を用い、血球凝集阻止反応その他の血清学的方法によつて検出することが、妊娠診断の一手段として利用されている。その他体液中の生長ホルモンやインシュリンその他の蛋白性ホルモンの血清学的検定も、臨床的診断法として利用できる。

このような血清学的検定においては、その特異性および精度を高める上に力価の高い特異的な抗体を含む抗血清が必要であり、それを可能にするためには高純度の抗原を必要とする。しかし一般に化学的手段によつて微量の不純物を除去するためには種々の困難を伴い、ある程度以上に純化することは容易ではない。例えばHCGの抗血清を得るためには、抗原として通常妊婦尿から得られるHCGを用いるが、この抗原HCGを化学的な方法で6000IU～8000IU/mg以上の純度に精製することは困難である。これに含まれる不純物もまた抗原性を有するため、HCGを抗原として用いる場合、それらは抗原性をもつことによつて障害となる。この不純物は尿成分であるが、それは血清成分が尿中に排泄されたもので、従つて血清成分と共通性を有しているのである。

本発明者等は研究の結果、HCGを含まない尿または血清、即ち妊娠婦人およびHCGを分泌するある種の腫瘍患者以外の者の尿または血清に対する抗体を製造し、この抗体を用いて粗製HCG抽出物中のHCG以外の不純抗原物質を血清学的に吸着することによつて、不純物質を除去し得ることを見出した。この精製純化したHCGは、特異性の極めて高い抗体の製造その他の場合に、抗原として有利に使用できる。

本発明は、動物の体液または胎盤から抽出した不純な胎盤性性腺刺激ホルモンを、該ホルモンを含有しないかもしくは極めて少量含有する動物の体液またはそれら抽出した物質によつて免疫した別種の動物の抗血清で処理することを特徴とする、免疫血清学的に高純度の胎盤性性腺刺激ホルモンの製法である。

本発明において原料の体液または胎盤としては、例えば人、牛、馬等の血清、尿等または人の胎盤が有利に使用される。

本発明を実施するに際しては、まず被吸収検体HCGを例えば妊婦尿から分離精製する。その方法としては例えば妊娠尿から安息香酸に吸着して分離するグリンまたはクリーソンの方法（J. Biol. Chem. 128 525（1939）、Acta. Endocrinol. 11（1948））、あるいはイオン交換樹脂に吸着して分離する松島の方法（特許第266190号参照）が用いられる。また一方では中和、吸収用抗血清製造のため、これと同じ処理によつて健康な男子または妊娠していない婦人の尿から乾燥粉末を製造し、この粉末を例えば生理食塩水に溶解し、あるいはこの溶液にアジュバント（免疫補強剤）を添加したものを用いて、人以外の動物例えば兎に注射して免疫する。この場合体液そのものも使用可能であるが、通常は抽出物を使用する方が優れている。この免疫血清を用いて好ましくは羊の赤血球を常法により感作する。こうして得られた感作血球を前記HCGの水溶液に加え、2～3分よく振り混ぜ、室温で2時間放置し、さらに4℃で一夜充分に反応させ、その反応生成物を遠心分離して除去することによつてHCGを免疫血清学的に純化することができる。

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前記の感作血球を用いないで、HCG水溶液と前記の健康男子または非妊娠婦人の尿よりの抽出物で免疫して得られた免疫血清とを直接反応させることによつても、HCGを純化することができる。このとき例えば兎の免疫血清でHCGの純化を行なつた場合は、そのHCG溶液中には兎の血清成分が混入するが、純化されたHCGで兎を免疫するためにはこの兎の血清成分は支障とはならない。いずれの場合も不純物を完全に除去するためには、予め予備試験を行つて、感作血球を用いる場合はその必要にして充分な量を、また抗血清のまま使用する場合は混合の最適比を求めて置く必要がある。

実施例　1

妊娠3～4カ月の婦人尿を濾過し、そのPHを5.0としたのち濾過し、濾過尿1ℓに対して安息香酸で飽和したアセトン溶液を75mℓ滴下して加える。しばらく放置後、吸引濾過して沈殿を集め、約4倍重量のアセトンを加え、不溶物を遠心分離して集め乾燥する。乾燥末をアンモニアでPH6.0とした50％エタノールで抽出し、抽出液に2倍容のエタノールを加える。沈殿を遠心分離して集め、これをPH4.8の0.3M酢酸－酢酸ナトリウム緩衝液とエタノールの等量混合物で3～4回抽出する。抽出液にこれと等量の無水エタノールを加え、沈殿を遠心分離して集め、乾燥する。こうして1mg当り2000～6000Iじの力価を有する粉末HCGが得られる。

一方HCGを含有しない正常男子尿から、妊婦尿と全く同様の処理によつて正常人尿末が得られる。

前記の正常人尿末10～20mgを1mℓの滅菌生生理食塩水に溶解し、これを1mℓのアジュバントに加えてよく混合し、兎（体重約3kgの健康雄または非妊雌）の4足にそれぞれ0.25mℓずつ皮下注射する。その後1週間の間隔で、各1mgの正常人尿末を1mℓの生理食塩水に溶かしたものを静脈より9回注射し、最終投与日から5～7日目に兎の耳静脈より約3mℓの血液を採取し、正常人尿末感作羊血球を使つてその抗血清の凝集力価を測定する。1：2000以上の抗体価が認められないときはさらに継続免疫を行う。抗体価が1：2000以上に達したとき全採血し、常法に従つて血清を分離し、56℃に30分間加温し非動化して、抗正常人尿末血清が得られる。

正常人尿末感作羊血球は次のようにして製造された。すなわち羊の頸静脈より血液を無菌的に採取し、等量のアルサバー氏液（デキストロース2.05g、クエン酸ナトリウム0.8gおよび塩化ナトリウム0.42gに蒸溜水を加えて全量を100mℓとし、PHを10％クエン酸で6.1に調整する）に加え、4℃に保存する。約1週後に生理食塩水で3回遠心洗浄し、8％に生理食塩水に浮遊させ、等量の3％中性ホルマリン－生理食食水と混合し、37℃で30時間固定する。これを10容の蒸溜水および50容の燐酸緩衝液（PH6.4）でそれぞれ3回洗浄し、4％にリン酸緩衝液に浮遊させ、その1～2万分の1量のタンニン酸を加え、56℃で30分反応させる。次いで50容の燐酸緩衝液で2回遠心洗浄したのち8％の濃度に燐酸緩衝液に浮遊させ、正常人尿末を25mg/mℓの濃度に溶解した燐酸緩衝液の等量と混合し、56℃で2時間反応させる。50容の燐酸緩衝液で3回遠心洗浄したのち、この血球を0.2％の正常兎血清を含む燐酸緩衝液に3％の濃度に浮遊させる。翌日遠心分離して上澄液を除き、沈殿に0.2％の正常兎血清を含む燐酸緩衝液を加え、血球濃度が再び3％になるように懸濁する。

ボイデン法（Boyden, S. V. ：J. Exp. Med. 93107,（1951））またはワイド法（Wide：Acta Endocr. Suppl. 70,（1962）に従い、羊血球をタンニン酸で処理したのち、2単位以上の前記抗正常人尿末穴清により感作し、血球濃度が3％になるように燐酸緩衝液に懸濁させて、抗正常人尿末血清による感作血球を製造する。

被吸収HCGを1mℓ当り10mgの濃度に燐酸緩衝液PH6.4に溶解し、前記の抗正常人尿末血清による感作血球浮遊液をHCG溶液1mℓに対して0.5mℓ加え、2～3分間よく振りまぜ、室温で2時間放置し、次いで4℃で一夜静置したのち、遠心分離し、上澄液を凍結乾燥する。本品は高純度のHCG製品である。

後記の方法によりHCG末によつて免疫した兎の抗HCG血清に対して、免疫電気泳動法（Scheidegger 法）または免疫拡散法（Ouchterlony 法）により、免疫に用いたHCG末と抗正常人尿血清による感作血球で処理した純化HCG（以下純化HCGと略称する）とを反応させた場合、処理前のHCGとその抗血清との間では、HCGとその抗血清との間では、HCGによると思われる沈降反応線の他に、1～2本の沈降線が認められる。これに対して純化HCGでは後者の沈降線の消失を認めた。また補体結合反応でもその

特異性が認められた。
成分の除去が行なわれ
なおこの処理によつて
たHCG検定法による
生物学的効力低下はな
て製造した純化HCG
らかにHCGに対する
つHCGに特異的な
た。

抗HCG血清の製
末1000-01Uを
解し、以下抗正常人
赤血球凝集反応法に
価を有する抗HCG
HCG純度測定法：
1）免疫拡散法：Ou
ergy 5, 1（19
Geol. 26B, 1
/V）寒天、ベロナ
強度0.1）を使用する
2）免疫電気泳動法
h. Allergy 7.
法により、ベロナ
スに流し、6 volt

氏液（デキストロース2.05
0.8g および塩化ナトリウ
えて全量を100mℓと
後で6.1に調整する）に加
1週後に生理食塩水で3
理食塩水に浮遊させ、等
一生理食食水と混合し、
る。これを10容の蒸溜
液（PH 6.4）でそれぞ
酸緩衝液に浮遊させ、
ンニン酸を加え、56
いで50容の燐酸緩衝
8％の濃度に燐酸緩衝
を25mg/mℓの濃度
量と混合し、56℃で
の燐酸緩衝液で3回遠
を0.2％の正常兎血清を
度に浮遊させる。翌日
沈殿に0.2％の正常
加え、血球濃度が再び

. V. ：J. Exp. Med
またはワイド法（Wi）
l. 70, (1962)
後で処理したのち、2
次抗血清により感作し、
燐酸緩衝液に懸濁さ
る感作血球を製造す

10mgの濃度に燐酸
記の抗正常人尿末血
CG溶液1mℓに対
く振りまぜ、室温
で一夜静置したのち、
燥する。本品は高純

によつて免疫した兎
電気泳動法（Sc-
散法（Ouchterl-
たHCG末と抗正常
理した純化HCG
とを反応させた場
清との間では、H
HCGによると
2本の沈降線が
CGでは後者の
結合反応でもそ

特異性が認められた。これはHCG以外の不純尿成分の除去が行なわれたことを示すものである。なおこの処理によつては、日本薬局方に定められたHCG検定法による検定の結果、認むべきHCG生物学的効力低下はなかつた。また実施例によつて製造した純化HCGで兎を免疫した場合は、明らかにHCGに対する抗体の産生が認められ、かつHCGに特異的な単一な抗HCG抗体を確認した。

抗HCG血清の製法は次のとおりである。HCG末10000IUを1mℓの滅菌生理食塩水に溶解し、以下抗正常人尿血清の製法と同様に処理し、赤血球凝集反応法により1：2000以上の抗体価を有する抗HCG血清を得る。

HCG純度測定法：

1）免疫拡散法：Ouchterlony 法（Progr. Allergy 5, 1(1958)、Ark. Kemi. Min. Geol. 26B, 1(1949)）により、1％（W/V）寒天、ベロナール緩衝液（PH 8.2, イオン強度0.1）を使用する。

2）免疫電気泳動法：Scheidegger（Int. Arch. Allergy 7. 103(1955)のマイクロ法により、ベロナール緩衝液寒天をスライドグラスに流し、6volt/cmで40分作用させる。

3）補体結合反応による特異性の検定：Svedmyr等の方法（Proc. Soc. Exp. Biol. N. Y. 79, 296(1952)）によるミクロ補体結合反応、または厚生省衛生検査指針Ⅰに記載の補体結合反応による。（この場合上記実施例によつて得られた純化したHCGで兎を免疫して得た抗血清に対して検体として乳汁分泌刺激ホルモン、生長ホルモン、下垂体性性腺刺戟ホルモン等の蛋白性ホルモンおよび上記HCGをベロナール緩衝液に溶解したものを用いた。）検査方式は2次元測定である。抗血清、抗原、補体2単位の混液を氷室（10～0℃）に3～4時間放置し、次いで37℃に15分間加温後、溶血素で感作し、羊血球浮遊液を加え振盪後37℃に30分加温して判定する。

特許請求の範囲

1　動物の体液または胎盤から抽出した不純な胎盤性性腺刺激ホルモンを、該ホルモンを含有しないかもしくは極めて少量含有する動物の体液またはそれから抽出した物質によつて免疫した別種の動物の抗血清で処理することを特徴とする、免疫血清学的に高純度の胎盤性性腺刺激ホルモンの製法。

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SERUM CONCENTRATION OF HUMAN PLACENTAL LACTOGENIC HORMONE (HPL) IN PREGNANCY COMPLICATIONS

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Abstract. HPL concentration was determined by an immunochemical method from 48 serum samples of 41 women with a complication of pregnancy. While no correlation was found between HPL and pregnancy week, a statistically significant correlation was observed between HPL and oestriol. Placental and infant birth weights had a highly significant correlation with HPL. No significant deviation from the standard was observed in the serum HPL of toxaemia patients, or in HPL of those with prolonged pregnancies or variations in foetal heart rate. Because of great individual variation no general limits could be calculated for maternal serum HPL concentration at 30–42 weeks of pregnancy.

The concentration of human placental lactogenic hormone (HPL) in the serum of a normal pregnant woman rises throughout pregnancy and reaches its highest level at the time of birth. After removal of the placenta HPL disappears from maternal blood within a few hours (Beck et al., 1965). At term the level of HPL is related neither to placental weight nor to the infant's birth weight. The blood content of HPL does not change with the time of day, the activity of the subject or the blood glucose level (Spellacy et al., 1966). It has been established that HPL is produced by the trophoblast. By immunofluorescent studies HPL has been localized in the syncytio-trophoblast (Sciarra et al., 1963).

In a search for indicators of placental function we compared the serum HPL levels measured immunochemically, with urinary oestriol excretion, placental and infant birth weights, and duration of gestation at different stages of pathological pregnancies.

MATERIAL AND METHODS

Specific anti-HPL serum, prepared in a rabbit by immunizing it with a purified HPL preparation (Tallberg et al., 1965), was used. The immunochemical measurement of HPL was performed by the radial immunodiffusion technique of Mancini et al. (1960). A mean HPL level of 20 sera from normal healthy pregnant women at term served as a standard and was designated by the number 100. The HPL content of the sera examined was expressed according to this level, as a percentage of the standard. Oestriol was determined by a modified method of Brown (1960). Serum and urine samples for HPL and oestriol determinations were collected over the same two day period in each case.

Forty-eight serum and urinary specimens from 41 pregnant women were examined. All these were patients who required admission to hospital because of pregnancy complications such as variation in foetal heart rate (15 cases), toxaemia (13), or postmaturity (7), diabetes mellitus (2), anaemia (1), thyrotoxicosis (1), placenta praevia (1) and jaundice of pregnancy (1). Correlation analyses were performed between HPL and oestriol, HPL and placental weight, HPL and infant birth weight, and HPL and duration of gestation.

RESULTS

The overall results are presented in Table I, and the results of the correlation analyses in Table II. The mean HPL value in the series was 103.8, which is quite close to the standard. In the three major complication groups the following mean values for HPL were obtained: variation in foetal heart rate 113.2, toxaemia 98.6, and postmaturity 93.0. None of these represent a significant difference from the standard.

On the basis of 30 specimens from 23 patients HPL and daily output or urinary concentration of oestriol showed a correlation ($r = 0.40 \pm 0.17$ and 0.46 ± 0.17, respectively (Figs. 1 and 2), which is statistically significant $t_{0.975} = 2.05 < t = 2.3$ and $t_{0.99} = 2.47 < t = 2.9$, respectively). At the time of birth maternal serum HPL concentration was re-

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Table I. *Maternal serum HPL, urinary oestriol excretion, infant and placental weights, and duration of gestation in 41 complicated pregnancies*

T = toxaemia, D = diabetes, A = anaemia, Thyr = thyrotoxicosis, HR = variation in foetal heart rate, J = Jaundice of pregnancy PP = placenta praevia, Prol = duration of pregnancy more than 41 weeks

No.	HPL (%)	Oestriol (mg/24 h)	Oestriol (mg/l)	Infant's weight (g)	Placental weight (g)	Duration of gestation (weeks)	Complication of pregnancy
1	154	14	23	3870	770	39	T
2	107	16	12	—	—	34	T
2	110	16	11	2400	540	37	"
3	138	41	26	3450	720	37	D
4	46	22	12	—	—	37	T
4	36	13	7	2230	430	39	"
5	71	32	22	3570	610	39	T
6	42	9	8	—	—	—	PP
6	36	9	6	1770	350	—	"
7	93	14	11	—	—	32	T
8	107	23	17	3550	780	32	A
9	110	13	7	3450	600	30	D
10	110	—	—	3360	670	38	HR
11	93	8	5	—	—	35	HR
12	88	11	10	2270	650	32	T
13	73	29	13	3570	490	39	T
14	110	—	—	2800	560	39	J
15	93	41	48	3500	540	39	T
16	154	19	18	3640	680	38	T
17	147	44	59	4270	650	39	T
18	119	30	38	3130	630	38	HR
19	165	18	15	—	—	37	HR
19	141	6	5	3840	650	41	"
20	119	20	20	3630	680	37	Thyr
21	165	39	29	3940	780	40	HR
22	110	28	22	—	—	35	T
22	122	22	14	—	—	37	"
22	128	33	31	3470	610	38	"
23	49	9	6	—	—	31	T
23	48	9	8	—	—	32	"
24	105	—	—	3190	500	41	Prol
25	90	—	—	3870	530	42	Prol
26	108	23	31	3110	650	39	HR
27	109	—	—	3350	600	40	HR
28	113	—	—	3780	650	41	Prol
29	108	—	—	3750	700	39	HR
30	76	—	—	3550	820	41	Prol
31	97	—	—	3600	670	41	Prol
32	88	—	—	3300	580	41	Prol
33	171	—	—	3650	690	38	HR
34	77	—	—	3070	470	38	HR
35	77	—	—	3580	630	40	HR
36	66	—	—	3270	500	40	HR
37	116	—	—	3170	560	38	HR
38	82	—	—	3430	500	41	Prol
39	87	5	4	3740	680	38	T
40	110	—	—	4300	680	39	HR
41	128	13	9	3660	700	37	HR

lated to the weight of the child ($r = 0.54 \pm 0.14$, $t_{0.005} = 2.76 < t = 4.28$) on the basis of 38 deliveries (Fig. 3). A positive correlation between HPL and placental weight was also observed ($r = 0.64 \pm 0.13$, $t_{0.005} = 2.76 < t = 5.1$, Fig. 4). There was no significant correlation between HPL and pregnancy week in our series of 46 samples examined between 30 and 42 weeks of gestation (Fig. 5). The Apgar scores of the children ranged from 2 to 10. In the group of mothers having low HPL

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Table II. *Correlation coefficients (r) and the respective standard errors (ε_r) of maternal serum HPL concentration and urinary oestriol excretion, infant and placental weights, and duration of gestation*

n = number of variable pairs

	Oestriol (mg/24 h)	Oestriol (mg/ml)	Infants weight	Placental weight	Pregnancy week
r	0.40	0.46	0.54	0.64	0.14
ε_r	0.17	0.17	0.14	0.13	0.15
n	30	30	38	38	46

concentrations (below 80% from the standard) the lowest Apgar score of the child was 9.

DISCUSSION

The radial immunodiffusion method is a simple and reliable technique for the measurement of serum proteins provided that a specific antiserum is available. For clinical use the weakness of the method is that the results cannot be recorded for 48 hours. Our results agree in general with previous observations (Spellacy et al., 1966), but observed a highly significant correlation betw HPL and placental weight. That no correlat between HPL and pregnancy week was found considered to be due to the nature of the pr nancy complications, where continuing placen and foetal development remained questional Moreover, our findings reflect marked individu variations in serum HPL concentrations betwe different individuals. The fairly poor correlati between HPL and oestriol may be because of d ferent sources of origin of these hormones. Wh HPL is a placental product, oestriol excretion d pends on the function of the foetoplacental un However, for clinical use, the results indicate th no definite conclusions about placental function foetal state are justified on the basis of sing HPL determinations from maternal serum. It possible that a marked reduction in maternal HP concentration might indicate danger to the foetus but this remains to be established. Large series ar necessary to find out the significance of HPL ir different pathological conditions of pregnancy.

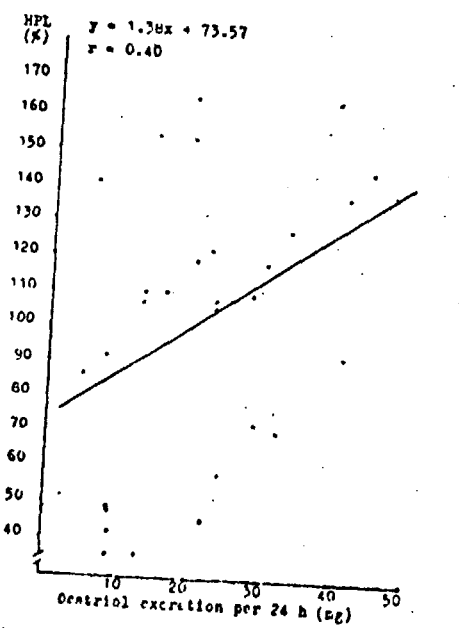

Fig. 1. Comparison of serum HPL and urinary oestriol excretion per 24 h.

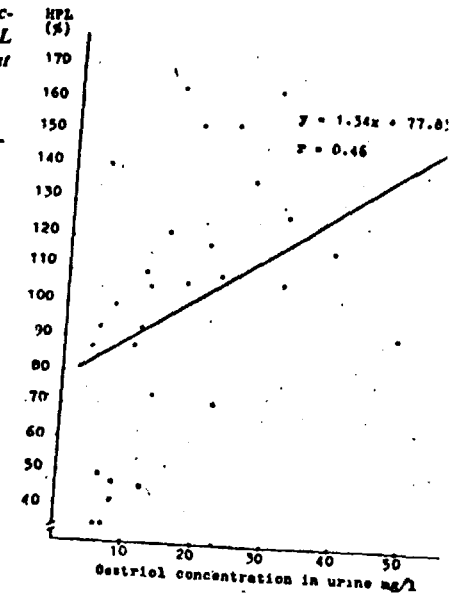

Fig. 2. Comparison of serum HPL and urinary oe concentration.

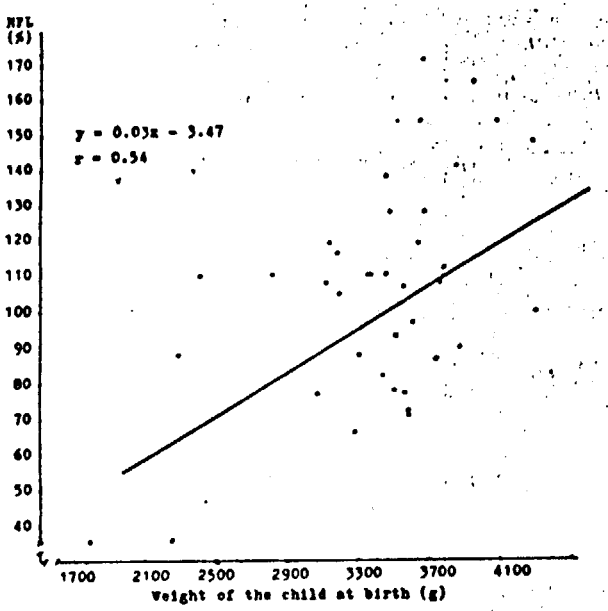

Fig. 3. Comparison of serum HPL and birth weight of the child.

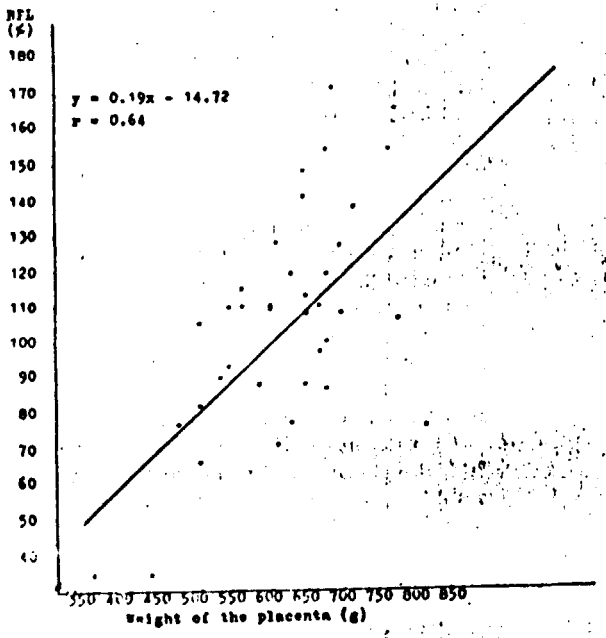

Fig. 4. Comparison of serum HPL and weight of the placenta.

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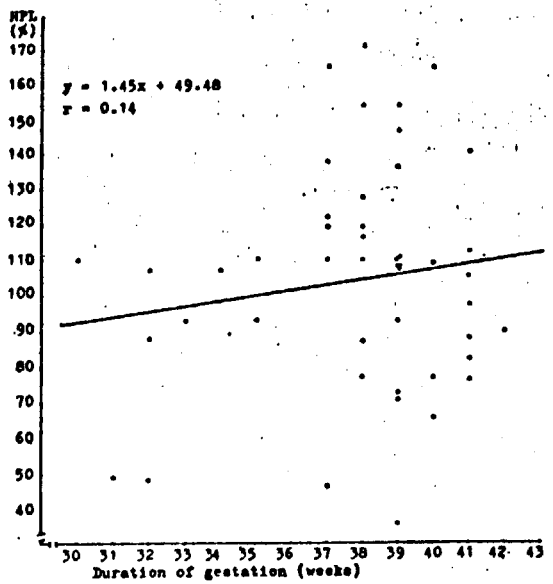

Fig. 5. Comparison of serum HPL duration of gestation.

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3,171,783

DIAGNOSTIC PROCEDURE

Roy Thomas Fisk, Glendale, Calif., assignor to Hyland Laboratories

No Drawing. Filed Aug. 15, 1962, Ser. No. 216,962
1 Claim. (Cl. 167—84.5)

This invention is concerned with an immuno-chemical pregnancy test.

Pregnancy tests, in general, are based on the determination of hormones which are produced by the developing placenta, such as gonadotropic hormones similar to those produced by the anterior pituitary gland and steroid hormones similar to those of the ovary and adrenal gland. The majority of currently employed pregnancy tests are based upon assay for the placental hormone, human chorionic gonadotropin (HCG).

Human chorionic gonadotropin (HCG) is a hormone which is found in body fluids (blood serum and urine) only during pregnancy and when rare hormone-producing tumors are present. An International Unit (I.U.) of HCG was adopted in 1938 and is defined as the specific gonadotropic activity of 0.1 mg. of a dried standard kept at the National Institutes of Health, London, England.

Human chorionic gonadotropin (HCG) is generally considered to be detectable in the urine about the twenty-fourth day of the gestation period. However, one should be mindful that the "detectability" of HCG in urine is influenced by the concentration of the HCG and the sensitivity of the particular test system employed. The twenty-fourth day of the gestation period is usually about the tenth day after ovulation, but is called the twenty-fourth day because gestation is conveniently calculated from the first day of the last normal menstrual period. By the fortieth day, HCG in urine may reach a level of about 5,000 I.U. per twenty-four hours; from day sixty to day seventy there is a very high peak, which may reach 500,000 I.U. per twenty-four hours. While this peak more commonly occurs at sixty to seventy days, it may be present at any time between fifty and ninety days; it rarely exceeds ten days in duration. For the remainder of pregnancy the level normally ranges between 4,000 and 11,000 I.U. per twenty-four hours.

The variety of tests currently used for the diagnosis of pregnancy affords ample evidence that an "ideal" procedure has not yet been achieved. Historically, the most commonly employed test procedures have been based upon the response of various experimental animals to hormonal substances contained in the body fluids (urine and blood serum) during pregnancy. The first of these so-called biologic tests to be developed was designated the Ascheim-Zondek or "A-Z" Test. In this test urine from a suspected pregnancy is injected into immature female mice. There then followed the Friedman Test in which mature, non-pregnant and non-estrus female rabbits replaced mice as the test animal. In both mice and rabbits, the HCG causes ovulation and corpus luteum formation, a response which is observed upon the sacrifice and examination of the animals to determine the presence of corpora lutea in mice and hemorrhagic follicles in rabbits, both of which indicate a positive test.

In recent years, various species of frogs and toads of both sexes have been used for pregnancy testing. Although these animals are relatively insensitive as compared to mice and rabbits, they are at this time possibly the most commonly used animals for biologic tests since they pose fewer problems in storing and handling.

The A-Z mouse and Friedman tests are capable of detecting levels of HCG of about 750 I.U. of urinary HCG per twenty-four hours. HCG levels of this order can be

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detected with toads and frogs only if adequate volumes of urine are grossly concentrated.

The sensitivity of various animals to HCG is dependent upon a number of factors, including species, maturity, weight, season, environment, reuse, health and route of administration. Perhaps the most serious handicaps incident to the use of biologic pregnancy tests is the propensity of such tests to give false positive reactions. Such false positive reactions in pregnancy test animals are positive responses to substances other than HCG. For example, false positive reactions are noted with mice and rabbits when high levels of follicle stimulating hormone (FSH) are present. Such FSH may be present in large amounts in menopausal patients or patients with primary ovarian failure. It is generally believed that a false positive reaction in a pregnancy test may have serious consequences because of the possibility of considerable psychological trauma to the patient. For this reason and for the other reasons, hereinbefore mentioned, attempts have been made to develop non-biologic pregnancy tests.

One class of non-biologic pregnancy tests involves the use of immunological or immunochemical techniques. Since HCG is a protein hormone, it acts antigenically in a heterologous species. That is, when HCG is injected by suitable techniques into a suitable animal, as for example a rabbit, antibody to HCG is produced. This antibody to HCG, under proper conditions, will combine with HCG to form a visible precipitate. In theory, this describes a classic antigen-antibody direct precipitin reaction. However, when related to the practicality of the determination of pregnancy in a human patient, this theoretical perfection becomes imperfect. The direct precipitin reaction is not sufficiently sensitive to detect, in unconcentrated urine, the relatively low levels of HCG that are present in such urine during early stages of pregnancy. When, in order to increase the I.U. of HCG per unit volume of urine, the urine is concentrated to a smaller volume, substances which interfere with the precipitation reaction likewise become more concentrated.

Difficulties attendant to the use of a direct precipitin pregnancy test have led to the evolution of so-called indirect tests. Such indirect tests are based essentially on an inhibition phenomenon. In such tests, antiserum to HCG is added to a patient's urine. A urine specimen from a pregnant woman will contain HCG. The HCG in such a specimen neutralizes the HCG antibodies in the antiserum that has been added to the urine. Thus a carrier (latex or sensitized cells) which has been coated with HCG is not agglutinated following addition of the coated carrier to the urine specimen. Had the urine specimen been from a non-pregnant woman, the added HCG antibodies from the added HCG antiserum would have been present at the time of the addition of the HCG coated carrier, and agglutination would have occurred. Although indirect tests such as these are useful for the diagnosis of pregnancy, they have certain limitations. In general, the urine to be tested must be the first morning urine. It is in such first morning urine that the HCG appears in the highest concentration. Urine passed after the first morning urine generally contains too low a concentration of HCG to be useful for indirect diagnostic tests. For the same reasons as pertained for the direct precipitin test, it is not possible to artificially concentrate low specific gravity urines for use in indirect tests.

It is an object of this invention to provide a direct (as opposed to indirect or inhibition) test for pregnancy.

It is a further object to provide for the diagnosis of pregnancy a direct test that is based upon immunochemical principles and that includes the use of gel diffusion techniques.

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It is another object to provide an immunochemical direct pregnancy test that employs urine as a test specimen, but does not limit the specimen to the first morning urine.

It is yet another object to provide novel equipment and methodology for immunochemical direct pregnancy testing.

It is yet another object to provide a pregnancy test which minimizes the likelihood of false-positive results.

Other objects and advantageous features will become apparent from the following detailed description and illustrative examples.

The instant inventive concept is based upon the immunological reaction between HCG and antiserum to HCG, the reaction taking place in semi-solid media utilizing the technique of immunodiffusion. In the diagnosis of pregnancy employing this inventive concept, urine is employed as the test substrate. The HCG, if present in the urine, is concentrated by adsorption on an appropriate adsorbent and by elution from the adsorbent preferably using a total volume of eluting agent substantially smaller than was the volume of the urine specimen. A small amount of the HCG-containing eluate is then introduced into or onto a defined space in or on a semi-solid medium which contains antibody to HCG. The HCG in the eluate diffuses through the semi-solid medium and thereby contacts the antibody to HCG. This contact between HCG and antibody to HCG results in the formation of a visible precipitate, indicating pregnancy.

Several types of semi-solid media can be used for immunodiffusion. Representative of gelling agents for such media are agar, gelatin, pectin and chemicals which polymerize to gel when catalysts are added to their aqueous solutions. Undoubtedly, many other gelling agents could be used, since precipitin tests can be carried out in or on any substance through which antigen or antibody can diffuse in aqueous solution. The semi-solid medium should not substantially interfere physically or chemically with uniform antigen (HCG) diffusion. Optimally the gelling agent is, therefore, uncharged or negatively charged and the resulting gel is of a cohesive structure, having characteristics such that it will permit the passage of macromolecules such as antigens. Agar is the preferred gelling agent in the practice of the present invention, because it satisfies all of the above criteria. In the description which follows agar is employed as illustrative of satisfactory gelling agents.

In the instant invention, agar which has been made fluid by heating, and antiserum to HCG are admixed. The HCG antiserum is prepared in a conventional way by inoculating an appropriate animal with HCG which can either be relaitvely pure or relatively impure. Rabbits, horses, goats and sheep have been found to be appropriate animals. Perhaps the highest specific activity reported for an HCG preparation is 12,000 I.U. per milligram. Such a preparation is essentially a laboratory curiosity. The nonantigenically specific HCG preparations normally employed for the production of HCG antiserum contain on the order of 2,000 to 9,000 I.U. per milligram. The HCG antiserum produced through the use of nonantigenically specific HCG, of the order of about 2,500 I.U. contains substantial amounts of antibodies to the non-HCG antigens contained in the HCG preparation. These antibodies to non-HCG antigens must be removed from the HCG antiserum since they may give a false positive pregnancy indication by forming a precipitate when brought into contact with their corresponding non-HCG antigens which may be present in nongravid urine. Normally, this removal would be accomplished by conventional means, using a concentrate of male and postmenopausal and nongravid premenopausal female urine to adsorb the non-HCG antibodies. In the instant invention, when an HCG preparation of relatively low IU content (on the order of 2,500 I.U. per milligram) is employed for the production of antiserum, it is preferable to adsorb with nongravid human serum.

The antiserum to HCG thus obtained is then admixed with the agar. HCG of the order of 8,000 I.U. per milligram produces antisera with a sufficiently low non-HCG antibody content that adsorption is not required for the successful application of the antisera to the gel diffusion technique of this invention.

Also admixed with the agar is glycine in an amount sufficient to prevent the formation of precipitates which can result from the interaction of the agar and certain substances that are normally present in the body fluids employed for diagnosis of pregnancy. Such interaction results in the formation of precipitates which can be misinterpreted as evidence of pregnancy, a so-called false-positive. Seventy-five grams of glycine per 1,000 ml. of agar will function to inhibit this interaction.

Additionally, when it is inadvisable to autoclave and thereby heat-sterilize the agar with the admixed glycine and HCG antiserum, microbiological contamination can be avoided by the addition to the agar of a preservative agent such as sodium azide.

In the practice of the invention, melted agar, to which has been added antiserum to HCG, glycine, and a preservative, is added into an appropriate container and is allowed to solidify. To be appropriate, a container preferably will be capable of receiving the molten agar and restraining it in a form such that the agar will have a uniform depth throughout. Also, the vertical walls of this container preferably will be of a height such that when the container is completely filled with agar the depth of the agar does not exceed about ⅛ inch. In a preferred embodiment, small wells of, for example, about 1/16 inch diameter are punched in the agar. For quantitation the wells preferably are circular, but they may assume any conveient form for qualitative purposes. In other embodiments adsorptive disks are placed in or on the agar. It is only necessary that the structure employed be capable of accepting and retaining a test specimen in such a manner that any HCG contained in the specimen will diffuse into the agar upon standing.

If the agar-containing containers are to be covered with a lid and stored for any period of time, as would be the case with a commercial embodiment of the invention, the deposit of water vapor condensate on the surface of the agar or in the wells must be prevented. It has been found that such deposit of condensate will not occur if the surface of the agar is covered with a material such as Saran or laminated cellophane. Additionally, to permit visual observation of the precipitate which indicates pregnancy, the container is constructed of transparent or translucent material, e.g., polystyrene.

The urine specimen employed for diagnosis in the instant invention need not be the conventionally employed first morning urine. The use of urine other than first morning urine is made possible by the application of a rapid semi-micro concentration technique which forms a part of this invention. In this technique, to a moderate volume of urine, e.g., one to 10 ml., in a receptable such as a test tube, is added an adsorbent under acidic conditions. An acidified suspension of kaolin has been found to be a useful adsorbent for this purpose. The HCG in the urine under these conditions is adsorbed on the kaolin. The urine-kaolin mixture is then centrifuged and the suspernatant fluid discarded. To the kaolin in the test tube is then added an amount of alkaline eluting fluid which is of lesser total volume than was the volume of the urine and which, preferably, contains an acid-base indicator to assure that the resulting mixture is alkaline. Under these conditions of alkalinity the HCG is eluted from the kaolin. Although the alkaline kaolin-HCG mixture can at this juncture be applied directly to the agar, as by placement in the wells, it is preferable to first centrifuge the mixture and transfer the supernatant to the agar, as by pipette.

The instant invention will be more clearly illustrated by reference to the following illustrative examples.

EXAMPLE I

Preparation of the antiserum

Twenty mg. of a commercially available (Organon) HCG having 2,275 I.U. per mg. are dissolved in 2 ml. of water. The resulting solution is emulsified by the addition of an equal volume of Freund's Adjuvant. This emulsion is then injected into a horse subcutaneously at multiple injection sites. After three weeks the multiple subcutaneous injections are repeated using a solution prepared by dissolving 10 mg. (22,750 I.U.) of HCG in 2 ml. of water. After two more weeks the HCG antibody concentration is sufficiently high to permit the use of the antiserum in the practice of this invention.

Five liters of blood were obtained from the horse. This volume of blood provided 2,700 ml. of serum. To this 2,700 ml. of horse serum was added 2,700 ml. of pooled nongravid human serum. The mixture was permitted to stand overnight at 4° C. at which time a precipitate had formed. This precipitate was removed by centrifugation. To three parts of the resulting antiserum were added an additional two parts of pooled nongravid human serum and the mixture allowed to stand in the cold (4° C.) for twenty-four hours. The centrifugation was repeated and the resulting centrifugate passed through a Seitz-type filter pad. The resulting filtrate was satisfactory for use in the subsequent gel diffusion technique.

EXAMPLE II

Materials employed in test procedure kaolin adsorbent

Sodium acetate	gm	12.3
Glacial acetic acid	ml	75.0
Water	ml	100.0
Kaolin-Celite[1]	gm	35.0

[1] Kaolin of the Oxford-English type and Celite 545 in a 1 : 1 w./w. mixture are used.

Eluting fluid

One normal ammonium hydroxide containing 10 mg. of bromthymol blue per 100 ml.

Positive control

This control contains 200 I.U/ml. of HCG (a stock preparation, Organon, of 2,200 I.U./mg. purity was used) in glycine-saline buffer (0.1 Molar glycine in 1% sodium chloride) at pH 8.2 with sodium azide 0.1% added as a preservative and with 10 mg. of bromthymol blue per 100 ml. present as an indicator.

Antihuman chorionic gonadotropin precipitin agar

Difco noble agar	gm	20
Glycine	gm	75
Sodium chloride	gm	10
Sodium azide	gm	1
Water	ml	1000

pH adjusted to 7.1 with NaOH.

With 3.5 parts by volume of the molten agar preparation at a temperature of 57° C., there was thoroughly admixed 0.5 part by volume of the antiserum of Example I, and the admixture was used to fill a rectangular transparent plastic plate. This plate was internally 1 inch in width and 3 inches in length wtih vertical sides ⅛ inch in height and with a ¼ inch flange defining the outer upper perimeter of the vertical sides. The agar in the plate was permitted to harden and a series of five circular wells 1/16 inch in diameter and ½ inch apart were made down the center of the long axis of the plate. Each of these wells had a 10 microliter capacity. The surface of the agar was then covered with a sheet of Saran and the plates stored in the refrigerator until needed.

Hereinafter, in these examples, when reference is made to precipitin plates, such plates are the plates of this example.

EXAMPLE III

Test procedure

(1) Place 2 ml. of clear urine in 10 x 75 mm. test tube.
(2) Add two drops of kaolin adsorbent and mix.
(3) Allow tube to stand one minute, re-mix and centrifuge for one minute to pack the kaolin adsorbent.
(4) discard the supernatant and drain the tube against adsorbent tissue.
(5) Add one drop of eluting fluid, flick the tube to break up the kaolin button and centrifuge for one minute.
(6) Use a capillary tube to collect the supernatant eluate and transfer enough to fill a well of a precipitin plate.
(7) Add the positive control to a second well and a negative control (the negative control being the same as the positive control, but without HCG) to a third well.
(8) Incubate at 37° C. for four hours and observe for a precipitin reaction which will be seen as a sharply defined circular zone of precipitation around a well. The zone surrounding the test specimen well may be larger or smaller than that surrounding the positive control well depending on the level of the chorionic gonadotropin in the specimen.

For the most reliable test results, the urine specimen will not be more than one day old and will have been kept under refrigeration until tested or will have been fozen if prolonged storage is necessary. The HCG concentration is usually higher in the first morning specimens and for this reason such specimens are preferred for use in the practice of this invention, although they are in no sense essential to the successful practice of the invention.

The eluting fluid contains an indicator to demonstrate that the elution has been conducted at the proper alkalinity. The eluate, when bromthymol blue is the indicator, must be blue or greensih blue (not yellow) for satisfactory results.

Overfilling a well may result in a surface deposit which might be mistaken for a positive reaction or which may mask a true precipitin zone in the agar below.

While in the foregoing specification, a detailed description of the invention has been set forth for the purpose of illustration, it will be apparent to those skilled in the art that many modifications in the details of these embodiments may be made without departing from the spirit and principles of the invention.

What I claim is:

In an immunochemical test for diagnosis of pregnancy based on the observation of the visible precipitate which develops when a specimen derived from urine and containing human chorionic gonadotropin is combined with an anti-serum containing antibodies to human chorionic gonadotropin, the improvement which consists essentially of the combination of the following steps:

(*a*) preparing crude anti-serum to human chorionic gonadotropin by injecting an appropriate animal with human chorionic gonadotropin and after a period of time bleeding said animal to obtain said crude antiserum,

(*b*) treating said crude anti-serum with human nongravid serum so as to remove from said anti-serum those antibodies which are non-specific for human chorionic gonadotropin and recovering purified antiserum,

(*c*) combining said purified anti-serum with a liquid gel-forming material and a minor amount of glycine and depositing a thin layer of the combined materials in a suitable container,

(*d*) after a gel has formed in said container, providing at least one well therein adapted to receive a test specimen,

(*e*) admixing a urine sample taken from the test subject with acidified kaolin whereby the human

chorionic gonadotropin is absorbed on said kaolin,

(*f*) separating said kaolin from the remainder of the urine sample,

(*g*) desorbing the human chorionic gonadotropin from said kaolin by means of alkaline eluting liquid,

(*h*) depositing said alkaline eluate in said test well in said container and incubating said container so as to form said visible precipitate which results from the combination of the human chorionic gonadotropin and the antibodies thereto contained in said purified anti-serum.

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IMMUNOCHIMIE. — *Antigènes embryonnaires et cancer du foie chez l'Homme. Association de la α_1-fœtoprotéine sérique avec l'hépatome primaire.* Note (*) de M. José Uriel, Mlle Béatrice de Nechaud, MM. Marc Stanislawski Birenzwajg, René Masseyeff, Louis Leblanc, Cyprien Quenum, Félix Lossilier et Pierre Grabar, présentée par M. Étienne Wolff.

Une α_1-fœtoprotéine sérique a été recherchée par des méthodes d'immunodiffusion dans 3 425 échantillons sériques humains.

D'après notre protocole de résultats, l'α_1-fœtoprotéine n'est décelable que chez des individus adultes atteints d'hépatome primaire (81 cas examinés). Cet antigène embryonnaire est absent du sérum des malades atteints aussi bien de cancers d'origine non hépatique (60 cas examinés) que d'hépatopathies diverses (284 cas), et il n'a jamais été retrouvé chez l'adulte cliniquement sain.

La fréquence des résultats positifs parmi les 81 cas de cancer primaire du foie est de 79 %.

Étant donné la spécificité du test immunologique, la possibilité de son utilisation comme moyen diagnostique que de ce type de carcinome est envisagée.

La recherche d'antigènes spécifiques des tumeurs a fait l'objet dans le passé de nombreux travaux, mais ce n'est que récemment que l'existence de substances d'origine embryonnaire associées à des processus de cancérisation a été découverte. Ainsi Abelev et coll. (1) ont les premiers démontré par des méthodes immunologiques qu'une α-globuline plasmatique présente dans le sérum des embryons de souris, mais disparaissant peu de temps après la naissance, réapparaît de nouveau dans le plasma de souris adultes ayant développé des hépatomes induits à l'aide de cancérigènes chimiques.

Une étude similaire portant sur l'hépatome de rat a été réalisée par l'un d'entre nous (2). Il en ressort que parmi les trois antigènes embryonnaires (3) décelables dans le plasma et le liquide amniotique de rat, celui dénommé « antigène LA » semble spécifiquement associé avec l'hépatome expérimental induit dans cet animal [(1), (2)].

Ces travaux ont encouragé la recherche des phénomènes analogues d'association dans le cancer du foie humain. Une α_1-fœtoprotéine a été trouvée par Kithier et coll. (4) dans un sérum et par Tatarinov (5) dans neuf sérums de malades atteints de cancer primaire du foie, les dix échantillons représentant la totalité des cas examinés.

De notre côté nous avons cherché à une plus grande échelle la fréquence de la α_1-fœtoprotéine dans les sérums de ces malades, ainsi que des malades atteints d'autres hépatopathies et des carcinomes d'origine diverse. La plupart des échantillons sériques examinés provenaient du Sénégal. Ils avaient été obtenus chez des malades hospitalisés dans divers hôpitaux de Dakar ou prélevés dans la population autochtone de plusieurs villages de Gambie au cours d'enquêtes sanitaires (6). Un certain nombre de cas provenaient de malades hospitalisés à Paris.

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MATÉRIEL ET MÉTHODES. — *Échantillons sériques.* — Les sérums ont été congelés aussitôt que possible et conservés sous cette forme jusqu'à leur examen immunologique.

Immunsérum anti-α_1-fœtoprotéine. — L'antisérum a été obtenu par immunisation de plusieurs lots de lapins avec un mélange de sérums riches en α_1-fœtoprotéine et épuisement ultérieur des antisérums avec du sérum humain normal. Le mélange de sérums provenait des malades atteints de cancer primaire du foie. Les sérums ont été sélectionnés après électrophorèse en acrylamide-agarose. Les échantillons montrant un taux élevé de α_1-fœtoprotéine ont été mélangés et ont servi pour l'hyperimmunisation des lapins. La spécificité de l'antisérum a été contrôlée par l'analyse immuno-électrophorétique.

TABLEAU I.

	Nombre de cas.	Diagnostic: Clinique.	Diagnostic: Autopsie, Biopsie ou Scintographie.	α_1-fœto-protéine sérique. +	α_1-fœto-protéine sérique. —
Cancers primaires du foie...	45	Cancers primaires du foie	Confirmés	35	10
	34	» » »	Non établis	27	7
	1	Cancer du pancréas avec métastases au foie	Cancer du foie avec métastases au pancréas	1	—
	1	Cirrhose	Cancer primaire du foie	1	—
Hépatopathies...	65	Cirrhoses	—	—	65
	16	Ictères	—	—	16
	49	Hépatites	—	—	49
	8	Hépatomégalies	—	—	8
		Abcès du foie (amibiase)	—	—	21
Cancers divers....	21	Cancers de l'estomac	—	—	11
	11	Épithélioma rénaux	2 cas avec métastases au foie	—	9
	9	Myélomes	—	—	19
	19	Localisations variées excepté le foie	—	—	2 993
Adultes sains.....	2 993	Néant	—	—	17
Nourrissons.....	17	9 cas d'ictère physiologique	—	10	7

Électrophorèse en acrylamide-agarose. — La méthode d'électrophorèse dans des gels mixtes d'acrylamide-agarose a été utilisée (*). Lorsque la concentration en α_1-fœtoprotéine sérique est suffisamment grande la présence de cette globuline peut être décelée après électrophorèse.

Immunodiffusion. — La technique de double diffusion en gels d'agarose a été employée pour détecter la α_1-fœtoprotéine. Six échantillons sériques ont été examinés simultanément autour d'un réservoir central contenant

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l'antisérum anti-α_1-fœtoprotéine. Dans un premier examen, les échantillons ont été placés sans dilution préalable dans les réservoirs respectifs. Les sérums ne montrant pas de réaction positive ont été réexaminés en remplissant jusqu'à trois fois successives le réservoir correspondant ([10]). Un sérum ne donnant pas de réaction dans ces conditions est considéré comme négatif. Les sérums dont le premier examen était positif ont été éprouvés par dilutions progressives dans le but d'avoir une idée approximative sur la teneur en α_1-fœtoprotéine.

Résultats. — Notre étude porte sur l'examen de 3 425 échantillons sériques parmi lesquels 81 cas de cancer primaire du foie, 60 de cancers d'origine non hépatique et 296 cas d'hépatopathies diverses.

L'ensemble des résultats obtenus est résumé dans le tableau I.

En ce qui concerne la spécificité de l'épreuve immunologique et d'après notre protocole de résultats : il semble bien établi que la α_1-fœtoprotéine sérique n'est décelable que chez les individus adultes atteints de cancer primaire du foie. Cet antigène embryonnaire est absent du sérum de malades atteints aussi bien de cancers d'origine non hépatique que d'hépatopathies diverses, et il n'a jamais été retrouvé chez l'adulte cliniquement sain.

L'α_1-fœtoprotéine sérique persiste pendant un certain temps après la naissance ([11]), ce qui explique les cas positifs trouvés chez des nourissons.

La fréquence des résultats positifs parmi les 81 cas de cancer primaire de foie est de 79 %. Le 21 % de cas négatifs malgré l'existence d'un cancer primaire du foie peut être dû soit à l'absence d'α_1-fœtoprotéine, soit au manque de sensibilité de la méthode d'immunodiffusion pour déceler des traces d'α_1-fœtoprotéine.

Un cas intéressant de notre protocole de résultats est celui d'un malade au diagnostic de cirrhose au moment de l'examen de son sérum. L'essai immunologique décelait la présence d'α_1-fœtoprotéine. L'autopsie de ce malade, décédé quelques mois plus tard, montra l'existence d'un cancer primaire du foie. Cet exemple permet d'envisager la recherche de l'α_1-fœtoprotéine sérique comme moyen diagnostique de ce type de carcinome.

(*) Séance du 26 juin 1967.

([1]) G. I. Abelev, B. D. Perova, N. I. Khramkova, Z. A. Postnokova et I. S. Irlin, *Transplantation*, 1, 1963, p. 174.

([2]) M. Stanislawski-Birencwajg, *Comptes rendus*, 260, 1965, p. 364.

([3]) Par antigène embryonnaire nous entendons toute substance humorale ou tissulaire présente dans l'embryon et absente dans l'animal adulte.

([4]) M. Stanislawski-Birencwajg, C. Frayssinet et P. Grabar, *Arch. Immun. Therap. Exp.*, 14, 1966, p. 530.

([5]) M. Stanislawski-Birencwajg, *Association d'antigène embryonnaires avec l'hépatome et d'autres lésions hépatiques chez le Rat* (*Thèse Doctorat ès sciences*, Paris, 1966).

([6]) K. Kitiher, J. Houstek, J. Masopust et J. Radi, *Nature*, 212, 1966, p. 414.

([7]) Y. S. Tatabinov, *Vopr. Med. Chimii.*, 10, 1964, p. 90 et 11, 1965, p. 20.

([8]) Le Docteur Mc Gregor et les Professeurs M. Snkale, J. Chabal et P. Menye ont collaboré.

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(9) J. Uriel, *Bull. Soc. Chim. Biol.*, 48, 1966, p. 960.

(10) Quelques sérums initialement négatifs mais provenant de malades atteints d'hépatome primaire, se sont avérés après triple remplissage des réservoirs d'antigènes, comme étant en réalité positifs mais avec un taux très faible en α-fœtoprotéine.

(11) Une enquête sur la vitesse de disparition de l'α-fœtoprotéine du sérum de nourrisson est en cours.

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interference of aureomycin with penicillin occurs not only *in vitro* but also in mice inoculated with either *S. pyogenes* or *K. pneumoniae*(7). Mice treated with both penicillin and aureomycin showed a higher death rate than did those treated with penicillin alone.

It is of interest that not only chloramphenicol but all 3 of the "newer" antibiotics that are widely used clinically, interfere with the bactericidal action of penicillin. While these agents have certain features in common there is no evidence that they resemble one another chemically. To date no evidence has appeared suggesting that the interference of these drugs with penicillin action is due to a direct physical or chemical interaction. Other possible mechanisms for this "antibiotic antagonism" have been previously suggested(4,6). It might be that the 2 interfering drugs compete with one another for "receptors" on the bacterial cell. No evidence has been produced directly favoring this possibility. One drug might alter the developmental characteristics of the microorganism in such a way as to diminish their susceptibility to other antimicrobial agents. All instances of antibiotic antagonism demonstrated thus far in this laboratory have dealt with an interference of bacteriostatic drugs with the bactericidal action of penicillin. Most of the data are compatible with the hypothesis that the presence of a bacteriostatic concentration of chloramphenicol, aureomycin, or terramycin may interfere with the multiplication of bacteria. Since penicillin principally affects dividing microorganisms such an interference with multiplication might result in interference with penicillin action. Future experiments will have to explore this hypothesis.

While striking manifestations of antibiotic antagonism have been produced experimentally in mice it cannot be inferred that the phenomenon may be of importance in the antibiotic therapy of human patients. It should be noted, however, that the drug levels showing antibiotic antagonism *in vitro* and in experimental animals fall in the range commonly obtained in human body fluids and tissues with ordinary therapeutic doses.

Summary. Aureomycin and terramycin interfered *in vitro* with the bactericidal action of penicillin against *S. pyogenes* and *K. pneumoniae*. The early bactericidal rate was slower with mixtures of aureomycin or terramycin with penicillin than with penicillin by itself. Ultimately, however, the mixtures were more likely to destroy all exposed bacteria than were the single antibiotics. Interference was most marked when bacteriostatic concentrations of aureomycin or terramycin were mixed with actively bactericidal concentrations of penicillin.

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A Serologic Study of Chorionic Gonadotrophin. (18263)

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The antigenicity of chorionic gonadotrophin* has been suggested by Leathem(1), Leathem and Rakoff(2) and others. Chase(3) has demonstrated that the serum of rabbits immunized with gonadotrophic hormones reacted with these hormones in a variety of immunological test procedures. CG in urine

* Hereafter designated as CG.

1. Leathem, J. H., *J. Clin. Endocrinol.*, 1944, v4, 500.

2. Leathem, J. H. and Rakoff, A. E., *Am. J. Obstet. and Gynec.*, 1948, v56, 521.

3. Chase, J., *Yale J. Biol. and Med.*, 1945, v17, 517.

1075685

readily detected by various biological methods. If this hormone be antigenic it is conceivable that it might be demonstrated serologically as are certain soluble bacterial antigens when present in body fluids or excreta(4-6). In order to investigate this possibility, studies were conducted in which the urine of pregnant and nonpregnant women were tested for CG by both biologic and precipitin tests.

Methods. A total of 17 rabbits, each weighing 2½-3½ kg were injected with "APL"†. This biologic preparation is a combined extract of human placental tissue and pregnancy urine which contains 1000 International Units (I.U.) or CG per ml. The animals were approximately equally distributed with respect to sex. After a serological base line was established, the rabbits were injected intravenously with 10 I.U. of CG for 3 successive days, followed by 20 I.U. on the fourth day. After 3 days rest the dosage was increased to 25, 50, 100 and 200 I.U. on successive days, respectively. Blood was obtained 5-6 days after the last injection. Following this, the animals were injected again on the first 4 days of alternate weeks with dosages increasing up to 2000 I.U. weekly. They were bled 5-6 days following the last injection of each series. The sera from the bleedings were tested by the precipitin method according to the following procedure: the serum dilutions used were undilute, 1-2, 1-4, 1-8, and 1-16; 0.1 ml of the serum was placed in 3 mm precipitin tubes, and overlayed with "APL" in concentrations of 62.5, 125, 250, 500 and 1000 I.U. ml. The tubes were incubated at 37°C for 1 hour and then read as a ring test.

Antisera which were positive in a dilution of 1-4 or greater when tested with 62.5 I.U./ml were used for the detection of CG in urine from pregnant and nonpregnant women. These urines were simultaneously assayed by standard biological pregnancy tests. The pH and specific gravity of each urine was determined prior to testing. In a preliminary study of the effect of the pH of urine upon the precipitin reaction, it was found that at or below pH 4.0 and above pH 7.0 it is quite possible to obtain false or interfering reactions. Consequently, the pH of the urines used in the precipitin test was adjusted, where necessary, to fall into the range of pH 4.0 to 7.0.

In order to remove from immune rabbit sera any factors which reacted with urines from normal nonpregnant women, the following absorption procedure was conducted. Each serum showing detectable antibodies was tested with a group of urines from pregnant and nonpregnant women. Known nonpregnancy urines which reacted with the CG antisera were used in absorption. These urines were sterilized by filtration and added to the serum in the ratio of 0.2 cc of urine to 5 cc of serum. Then the mixture was incubated at 37°C for 1 hour and stored overnight in the refrigerator. The mixture was centrifuged, the supernatant restored to the original volume and again tested with known pregnancy and nonpregnancy urines. This procedure was continued until the serum no longer reacted with the test group of nonpregnancy urines, but continued to react with the known pregnancy urines.

Results. Seven of the 17 immunized rabbits showed detectable antibodies when tested with "APL." Six of the 7 were females. The sera from these rabbits then were used for the detection of CG in urine by the precipitin method. A total of 285 urines submitted for pregnancy tests were studied serologically. The results of the precipitin tests in a group of 85 urines as compared with those obtained with the Salmon(7) rat test‡ are shown in Table I.

On the basis of the large number of "false

4. Bacterial and Mycotic Infections of Man, edited by R. J. Dubos, p. 592.

5. Alexander, H. E. and Rake, G., *J. Exp. Med.*, 1937, v65, 317.

6. Dochez, A. R. and Avery, O. T., *J. Exp. Med.*, 1917, v26, 477.

† The material for this study was kindly supplied by Ayerst, McKenna and Harrison.

7. Salmon, U. J., Geist, S. H., Salmon, A. A. and Frank, I. L., *J. Clin. Endocrinol.*, 1942, v2, 167

‡ These urines were assayed in the Endocrine Laboratory of the Department of Obstetrics and Gynecology, Duke Hospital, under the direction of Mr. Austin A. Salmon.

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TABLE I. Comparison of Urines Tested by Salmon Rat Test and Precipitin Tests.

Precipitin test*	Salmon test: Positive urines	Salmon test: Negative urines	Total No. of urines
Positive	42	12	
Negative	6	25	
Totals	48	37	85

* Anti-CG rabbit serum + patient's urine.

TABLE II. Precipitin Test of Chorionic Gonadotrophin Rabbit Serum with Urines from 15 Normal Nonpregnant Women.

Sera	No. of urines giving positive reaction	No. of urines giving negative reaction	"APL" antigen combined
Crude anti-CG #18,5/25	8	7	Positive
Anti-CG absorbed serum #18,5/25	0	15	"
Normal rabbit serum	0	15	Negative

TABLE III. Comparison of Urines Tested by Frog and Precipitin Tests.

Precipitin test	Frog test: Positive urines	Frog test: Negative urines	Total No. of urines
Positive	103	6	
Negative	6	85	
Totals	109	91	200

positives" in this preliminary study, absorbed serum was considered necessary for use. The serum was absorbed as described in the section under "Methods." The result of one such absorption is seen in Table II, where it is shown that although the crude rabbit antiserum reacted with 8 of 15 urines from nonpregnant females, the absorbed serum was significantly more specific. Absorbed serum was then used in the serological testing of an additional 200 urine specimens which had been assayed by the frog test(8)§. The results of this comparison are shown in Table III.

It was desired to determine whether the antibodies which gave a positive reaction with "APL" were the same as those which reacted with the urines from pregnant women. Serum was absorbed with "APL" using the method described above. This absorbed serum no longer reacted with "APL" or with the 10 pregnancy urines tested. Similarly, serum absorbed with pregnancy urine failed to react with the 10 pregnancy urines used or with "APL."

Discussion. The specificity of chorionic gonadotrophin antiserum is of paramount importance if that serum is to be used to detect the presence of the hormone. The first series of 85 urines were tested serologically with unabsorbed antiserum. Positive precipitin tests were obtained with 42 of the 48 urines positive by the rat test; and false positive tests were found with 12 of 37 urines negative by the biologic method. In the next series, 200 urine specimens were tested with absorbed serum and the results were compared with those of the frog test. One hundred and three of the 109 pregnancy urines gave positive precipitin results, while 6 of the 91 urines negative by the frog test reacted serologically, thus constituting 6 false positives. These results indicate that the correlation between the biologic and serologic methods of detecting CG in urine is greater when absorbed antiserum is used. Consequently, before the antiserum is used for test purposes, it is necessary to determine the potency and especially the specificity of the serum. It is not to be implied that the antigen used in preparing this serum was pure. It is conceivable that the antiserum prepared with a more highly purified hormone preparation might give serum of higher potency and greater specificity.

The precipitin test was compared with the Salmon rat test and the frog test. It has been found that the Salmon test as performed at Duke Hospital has an accuracy of 98%. At the present time, it is not possible to state exactly the accuracy of the frog test. For our purposes in this study we have used

8. Cutler, J. N., *J. Lab. and Clin. Med.*, 1949, v34, 554.

§ Veterinary Division, Army Medical Department Research and Graduate School, and by Dr. Cornelius Burns, Department of Pathology, Georgetown University Medical School.

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the biologic test as a basis of comparison; clinical histories can be later used to further assess accuracy.

Included in the series of urines studied were specimens from 3 patients with hydatidiform moles, 1 with chorionepithelioma and 3 with testicular tumors. These specimens were tested either by the rat or frog test, and also by the Aschheim-Zondek method. The serological results with these specimens coincided with the biological assays.

Summary. 1. Seventeen rabbits were immunized with "APL," which is a combined extract of human placental tissue and pregnancy urine. Seven rabbits (6 females) produced detectable antibodies. 2. Two hundred eighty-five urines submitted for pregnancy tests were tested for the presence of CG by biological and serological methods. 3. Native rabbit CG antiserum tends to give many cross-reactions. Therefore, it is necessary to absorb the serum and test it for potency and specificity prior to use in testing urines for the presence of increased amounts of CG. 4. It is suggested that it may be possible to use this serologic method for the detection of pregnancy; the development of an antiserum of somewhat greater specificity would be advantageous.

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Relation of the Adrenal to Glycogen Content and Respiration of Lymphoid Organs. (18264)

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The effects produced by adrenal cortical factors and by epinephrine upon the storage, breakdown and utilization of carbohydrate have been described at length(1-5). Although studies have been made of the influence of the adrenal gland upon the quantities of carbohydrate substances, principally glycogen, in the liver, muscle and certain other organs(6-8), no attention has been paid to the part played by this gland in the carbohydrate metabolism of lymphoid tissue. This is surprising in view of recent work which has shown the importance of the lymphoid organs in a variety of defense reactions within the body and which has stressed the influence of adrenal cortical factors upon this function (9-11).

A knowledge of carbohydrate changes within the lymphoid organs, induced by alterations in adrenal hormone levels, might help to elucidate further the role of this endocrine gland in defense mechanisms. This report describes experiments dealing with the chronic effects of adrenalectomy and cortical extract or epinephrine administration upon the glycogen content of lymphoid organs, the liver glycogen and the respiratory activity of these tissues.

Materials and methods. 1. *Animals.* Female rats of a hardy, closely inbred strain, weighing 140-170 g at the time of operation, were used. The 36 adrenalectomized rats employed were divided into three groups, the first of which consisted of 10 rats, to

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10. Dougherty, T. F., and White, A., *J. Lab. and Clin. Med.*, 1947, v32, 584.
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Schwangerschaftsspezifisches β_1-Glykoprotein und Verfahren zu seiner Isolierung

Es ist bisher nicht bekannt, dass in der Plazenta und im Urin von Schwangeren ein schwangerschaftsspezifisches β_1-Glyoprotein vorkommt. Es ist zwar immunologisch schon ein Protein im β-Bereich von Schwangerenserum nachgewiesen worden, ein Verfahren zu seiner Isolierung jedoch noch nie beschrieben worden.

Gegenstand der Erfindung ist ein Verfahren zur Isolierung des schwangerschaftsspezifischen β_1-Glykoproteins (abgekürzt SP_1), das dadurch gekennzeichnet ist, dass das schwangerschaftsspezifische β_1-Glykoprotein aus Plazenta, sowie dem Blut oder Urin von Schwangeren durch Fraktionieren nach üblichen Methoden isoliert wird. Ein weiterer Gegenstand der Erfindung ist das schwangerschaftsspezifische β_1-Glykoprotein, gekennzeichnet durch eine

elektrophoretische Wanderungsgeschwindigkeit im Agargel im Bereich der β_1-Globuline,

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eine Sedimentationskonstante von 4,6 S,
ein Molekulargewicht von etwa 100 000,
einen Extinktionskoeffizienten $E_{1\,cm}^{1\%}$ = 11,0 (278 mμ; 1/15 molarer Phosphatpuffer pH 7,0)
einen Kohlenhydratgehalt von 26,5 - 29,6 %, davon 10,0 - 11,0 % Hexosen, 9,5 - 10,5 % Hexosamin, 0,5-0,6 % Fucose und 6,5 - 7,5 % Neuraminsäure.

Das erfindungsgemäss hergestellte schwangerschaftsspezifische β_1-Glykoprotein dient zur Gewinnung von Antiseren. Mit diesen wiederum kann durch immunologische Methoden (Haemagglutinations-Hemmungs-Test, Komplement-Bindungs-Reaktion, Radio-Immunassay, Latex-Agglutination) eine Schwangerschaft nachgewiesen werden. Der Nachweis kann mit Serum oder Urin der Schwangeren geführt werden. Eine quantitiative Bestimmung von SP_1, z.B. mit der radialen Immundiffusion, kann für eine Überwachung der Schwangerschaft von Bedeutung werden.

Für die Herstellung des schwangerschaftsspezifschen β_1-Glyoproteins werden zerkleinerte Plazenten mit einer schwachen Salzlösung extrahiert und die mit Diaminodthoxyacridin-lactat fällbaren Proteine bei einem pH-Wert von etwa 8,0 abgetrennt. Das im Überstand verbleibende SP_1 wird zusammen mit Gammaglobulin mit Ammoniumsulfat ausgefällt. Zur Trennung des SP_1 von der Hauptmenge des Gammaglobulins wird die in reinem Wasser wieder aufgelöste Fällung, zweckmässig nach einer Vorbehandlung

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Durch Dialyse gegen eine Pufferlösung, an Diäthylaminoäthyl (DEAE) - Cellulose adsorbiert und von dieser wieder eluiert. Als Elutionsmittel hat sich eine 0,02 molare Tris-(hydroxymethyl)-aminomethyl-Salzsäure-Pufferlösung vom pH-Wert 8,5 bewährt, die noch etwa 0,85 % Natriumchlorid und eine kleine Menge, z.B. 0,05 % Natriumazid enthalten kann. Ausfällen des Eluats mit Ammoniumsulfat liefert das SP_1 in angereicherter Form, das durch Gelfiltration beispielsweise mittels Sephadex G-150 weiter gereinigt werden kann.

Zur weiteren Reinigung eignet sich die präparative Zonenelektrophorese mit PVC als Träger in einer geeigneten Pufferlösung, z.B. 0,075 % Ammoniumhydrogencarbonat. Nach der elektrophoretischen Auftrennung wird die SP_1-haltige Zone des Trägers herausgeschnitten, mit Ammoniumhydrogencarbonat eluiert und lyophilisiert. Weitere geeignete Feinreinigungsverfahren sind eine Chromatographierung an DEAE-Sephadex mit nachfolgender Elution mit einem Natriumchlorid-Gradienten und eine Fraktionierung mit Alkohol, vorzugsweise mit Äthanol. Das schwangerschaftsspezifische β_1-Glykoprotein wird in ähnlicher Weise aus Blut oder Urin von Schwangeren isoliert.

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Beispiel 1 / Isolierung aus Plazenten

150 kg tiefgefrorene Plazenten werden zerkleinert und mit 150 Liter einer 0,5 %igen wässrigen Natriumchlorid-Lösung extrahiert. Der Extrakt wird mit 2 normalem Natriumhydroxid auf pH 8 eingestellt und mit 50 Liter einer 3%igen wässrigen Lösung von Diaminoäthoxyacridinlactat (Rivanol®) versetzt. Nach einer Wartezeit von 1 Stunde wird der Überstand, der das SP_1 zusammen mit Gammaglobulin enthält, abgehebert, mit 9 % festem Natriumchlorid (11 kg) zur Abscheidung des noch in Lösung verbliebenen Rivanols versetzt, filtriert und mit 26,5 %, bezogen auf das Gewicht der Flüssigkeit, festem Ammonsulfat versetzt und gut durchgerührt. Nach 1 Stunde wird der Niederschlag abfiltriert.

500 g des auf dem Filter gesammelten Niederschlags werden in 500 ml destilliertem Wasser gelöst und gegen eine 0,01 molare Tris-(oxymethyl)-aminomethan (im folgenden mit "Tris" abgekürzt)-Salzsäure-Pufferlösung vom pH-Wert 7,0, die 0,05 % Natriumazid enthält, dialysiert. Die dialysierte Lösung wird zentrifugiert und der Überstand wird mit der gleichen Pufferlösung auf 2000 ml aufgefüllt, mit 0,1 normaler Natriumhydroxidlösung auf pH 8,0 eingestellt und mit 250 g feuchter DEAE-Cellulose eine Stunde verrührt.

Sodann wird die DEAE-Cellulose durch Filtrieren von der Lösung abgetrennt, zweimal mit je einem Liter 0,01 molarem Tris-Salzsäure-Puffer vom pH-Wert 8,0 gewaschen und danach dreimal mit je 500 ml 0,02 molarem Tris-Salzsäure-Puffer, pH 6,5, der 0,20 % Natriumchlorid und 0,05 % Natriumazid enthält, eluiert. Den vereinigten Eluaten wird 30 % Ammonsulfat, bezogen auf das Flüssigkeitsgewicht, zugesetzt und das ganze verrührt. Der Niederschlag, der das SP_1 enthält, wird in 500 ml destilliertem Wasser gelöst und der Gelfiltration mittels Sephadex G-150

1075693

unter Verwendung von 0,1 molarem Tris-Salzsäure-Puffer pH 8,0, der 1,0 Mol Natriumchlorid pro Liter enthält, unterzogen (100 x 20 cm-Säule).

Anschließend werden die Eluate mit spezifischem SP_1-Antiserum getestet, die SP_1-haltigen Fraktionen gesammelt und daraus die Proteine nochmals wie oben beschrieben mit 30 % festem Ammonsulfat ausgefällt.

Eine weitere Reinigung erzielt man durch präparative Zonenelektrophorese mit Polyvinylchlorid (PVC) als Träger. Als Puffer wird eine 0,075 molare Ammoniumhydrogencarbonatlösung (pH 8,0) verwendet. Man schneidet nach der elektrophoretischen Auftrennung die SP_1-haltige B-Zone heraus, eluiert mit dem Ammoniumhydrogencarbonat-Puffer und lyophilisiert die Eluate.

Die Trockensubstanz wird in 0,01 molarem Tris-Salzsäure-Puffer pH 7,0, der 0,2 % Natriumchlorid enthält, gelöst, an DEAE-Sephadex (Säule 5 x 20 cm) chromatographiert und mit einem linearen Gradienten von 0,2 bis 2,0 % Natriumchlorid in 0,01 molarem Tris-Salzsäure-Puffer pH 7,0 eluiert. Es folgt eine Fällung mit 40 % Äthanol bei -5°C. Nach Abzentrifugation des Niederschlages wird der Überstand gegen destilliertes Wasser salzfrei dialysiert und lyophilisiert. Reinheit 90 - 95 %. Dieses Präparat kann zur Herstellung von Antiseren Verwendung finden.

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Beispiel 2 / Isolierung aus Blut

1 Liter Schwangerenserum werden mit destilliertem Wasser im Verhältnis 1:1 verdünnt und daraus die Hauptmenge der Plasmaproteine durch 2,5 Liter 3 %igen Rivanols bei pH 8,0 gefällt. Der durch Zentrifugation erhaltene Überstand wird zur Entfernung des Rivanols mit 5 % Natriumchlorid versetzt. Der dabei entstandene Niederschlag wird abzentrifugiert, der Überstand vom Sediment getrennt und mit 30 % festem Ammonsulfat versetzt. Im Niederschlag sind das Gammaglobulin und das schwangerschaftsspezifische B_1-Glykoprotein enthalten. Der Niederschlag wird in 0,01 molarem Tris-Salzsäure-Puffer pH 7,0 gelöst, gegen 0,01 molarem Tris-Salzsäure-Puffer pH 7,0 dialysiert und anschließend mit DEAE-Cellulose gereinigt. Die DEAE-Cellulose-Behandlung wird in 0,01 molarem Tris-Salzsäure-Puffer pH 8,0 ausgeführt.

Die Elution von DEAE-Cellulose, die Gel-Filtration mit Sephadex, die präparative Zonenelektrophorese, die Reinigung an DEAE-Sephadex sowie die Fällung mit Alkohol werden gemäß Beispiel 1 ausgeführt.

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Patentansprüche

1. Verfahren zur Isolierung des schwangerschaftsspezifischen β_1-Glykoproteins, dadurch gekennzeichnet, dass das schwangerschaftsspezifische β_1-Glykoprotein aus Plazenta sowie dem Blut oder Urin von Schwangeren durch Fraktionieren nach üblichen Methoden isoliert wird.

2. Schwangerschaftsspezifisches β_1-Glykoprotein, gekennzeichnet durch

eine elektrophoretische Wanderungsgeschwindigkeit im Agargel im Bereich der β_1-Globuline,
eine Sedimentationskonstante von 4,6 S,
ein Molekulargewicht von etwa 100 000,
einen Extinktionskoeffizienten $E^{1\%}_{1\,cm}$ = 11,0 (278 mμ ; 1/15 molarer Phosphatpuffer, pH 7,0)
einen Kohlenhydratgehalt von 26,5 - 29,0 %, davon 10,0 - 11,0 % Hexosen, 9,5 - 10,5 Hexosamin, 0,5 - 0,6 % Fucose und 6,5 - 7,5 % Neuraminsäure.

3. Verwendung von schwangerschaftsspezifischem β_1-Glykoprotein als Reagenz und zur Gewinnung von Antiseren als Reagenz zum Nachweis und zur Überwachung einer Schwangerschaft.

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of fuel in a fuel control. The 1307 and fuel controls seek to regulate fuel as a function of the mass of air flowing gh the engine. Both controls are re- sive to variations in engine speed to de a signal that is a function of speed n addition, both are responsive to en- operational parameters based on erature and pressure of the air to pro- fuel flow as an ultimate function of air flow. Infringement is clear, as the MFC and 1307 fuel controls per- substantially the same function utiliz- ubstantially the same mechanism in antially the same manner.

ce, in this case, the claims of the patent are not to be narrowly con- d in light of the prior art or the Mock fication and file wrapper, the trial 's original opinion with respect to in- ement will not be disturbed. Inde- nt of that conclusion, the issue has reviewed in the light of the record in se, including the briefs of the parties were before the court. I hold that in- ement has been established.

h respect to the broader claims 1, 3, 3, infringement does not depend resort to the doctrine of equivalents these claims do not contain the s" language. These claims, being er than the claims sought to be nar- l, are infringed by the accused de-

pplemental Conclusion of Law

n the findings of fact and opinion une 19, 1975 and the supplemental n, supplemental and additional gs of fact filed herewith, all of which ade a part of the judgment herein, urt concludes as a matter of law that ms 11, 24, 25, and 28 of Kunz Pat- o. 2,720,751 are invalid, wherefore sition is dismissed as to that patent, claims 1, 3, 4, 5, 7, 8, 11, 12, 13, 14, of Mock Patent No. 2,581,275 are nd infringed, wherefore defendant le for infringement of that patent. dingly, plaintiff is entitled to recover dgment is entered to that effect. The t of recovery is reserved for further edings under Rule 131(c).

dings omitted.]

Patent and Trademark Office Board of Appeals

Ex parte Krenzer

Opinion dated July 31, 1978

PATENTS

1. Patentability — Utility (§51.75)

Significant use for invention claimed must exist in order to satisfy utility requirement of 35 U.S.C. 101; claimed compounds that are described as exhibiting herbicidal activity are disclosed to be "useful."

2. Patentability — Utility (§51.75)

Examiner may require further assurance of usefulness where reasonable doubt exists as to whether invention will function as stated, notwithstanding asserted utility.

3. Patentability — Utility (§51.75)

Pleading and practice in Patent Office — Rejections (§54.7)

Examiner who disavows any finding that asserted utility which, on its face is not contrary to generally accepted scientific principles, is unbelievable, improperly imputed burden of proof to applicant; examiner's unsupported skepticism as to claimed invention's utility does not provide legally acceptable basis for rejecting claims.

4. Specification — Sufficiency of disclosure (§62.7)

Specification that discloses invention in such manner that one skilled in art would be able to practice it without undue amount of experimentation need not contain working example.

5. Pleading and practice in Patent Office — Rejections (§54.7)

Specification — Sufficiency of disclosure (§62.7)

Patent and Trademark Office Board of Appeals does not sustain rejection under 35 U.S.C. 112 on ground that specification fails to set forth best mode contemplated by inventor for carrying out invention, absent cause to suspect inventor of concealing any information as to what he feels is preferred embodiment.

Particular patents — Herbicides

Krenzer, 1-Thiadiazoly L-5-Acylimidazolidinones, claims 1-10 allowed.

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Appeal from Group 122.

Application for patent of John Krenzer, Serial No. 571,466, filed Apr. 25, 1975. From decision rejecting claims 1-10, applicant appeals (Appeal No. 310-20). Reversed.

Robert J. Schwarz, Chicago, Ill., for applicant.

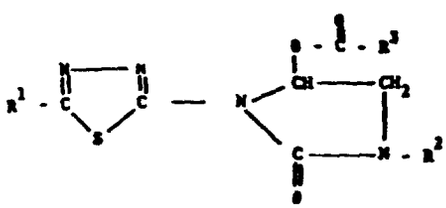
Before Witherspoon and Milestone, Examiners in Chief, and Pellman, Acting Examiner in Chief.

Pellman, Acting Examiner in Chief.

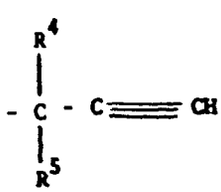
This is an appeal from the examiner's decision finally rejecting claims 1 through 10, all of the claims in the application.

The subject matter on appeal relates to various 1-thiadiazolyl-5-acyloxy-1,3-imidazolidin-2-ones (claims 1-8), a herbicidal composition containing a compound of claim 1 (claim 9), and a method of controlling weeds by contacting them with a herbicidal composition containing a compound of claim 1 (claim 10). To illustrate the subject matter before us, claim 1, the only independent claim, is reproduced as follows:

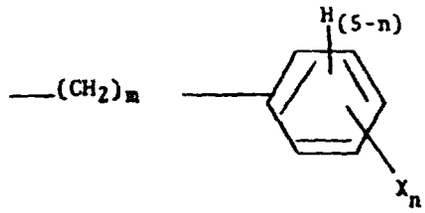
1. A compound of the formula



wherein R^1 is selected from the group consisting of alkyl of up to 3 carbon atoms, cycloalkyl of from 3 to 7 carbon atoms, lower alkenyl, lower chloroalkyl, lower bromoalkyl, lower alkoxy, lower alkylthio, lower alkylsulfonyl and lower alkylsulfinyl; R^2 is selected from the group consisting of lower alkyl, lower alkenyl, lower haloalkyl and



wherein R^4 and R^5 are each selected from the group consisting of hydrogen and alkyl of up to 3 carbon atoms; and R^3 is selected from the group consisting of lower alkenyl, lower haloalkyl, lower alkynyl, lower alkoxyalkyl, cycloalkyl of from 3 to 7 carbon atoms and



wherein X is selected from the group consisting of lower alkyl, lower alkoxy, halogen, lower haloalkyl, nitro, cyano and lower alkylthio; n is an integer from 0 to 3; and m is the integer 0 or 1.

We note, in passing, that in claim 2, which depends upon claim 1, the term "5-acetyloxy" has no proper antecedent basis in the definition of "R^3" in claim 1. See 37 CFR 1.75 (c).

No references have been cited.

All of the claims stand rejected "under 35 USC 101 because the record provides no reasonable assurance that the claimed compounds will function as alleged" (Answer, page 2).

We shall not sustain this rejection.

[1] It is axiomatic that, in order to satisfy the utility requirement of 35 USC 101, a significant use for the invention claimed must exist. Brenner v. Manson, 383 US 519, 86 S. Ct. 1033, 148 USPQ 689; In re Joly et al, 54 CCPA 1162, 376 F.2d 906, 153 USPQ 243. Clearly, the presently claimed compounds are disclosed to be "useful" since they are described as exhibiting herbicidal activity.

[2] However, notwithstanding an *asserted* utility, an examiner may still require further assurance of usefulness where a *reasonable* doubt exists as to whether the invention will function as stated. Such doubt has been held reasonable where, for example, the invention has been characterized as "highly unusual," In re Houghton, 58 CCPA 732, 433 F.2d 820, 167 USPQ 687, as "incredible," In re Citron, 51 CCPA 852, 325 F.2d 248, 139 USPQ 516, or as "too speculative," In re Eltgroth, 57 CCPA 833, 419 F.2d 918, 164 USPQ 221.

In his Answer, at page 4, the examiner explicitly disavows any finding that the utility herein is unbelievable. He states:

> "Here, the Examiner does not take the position that the herbicidal utility is unbelievable (although it is 'unexpected').

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The Examiner's position here is that applicant has failed to provide the record with *any* basis at all for *any* finding, one way or the other, about the believability of the utility."

[3] Regarding the above, the examiner has improperly imputed the burden of proof to appellant. However, the court has already passed upon essentially this same matter, albeit with respect to "enablement," and its holding appears to be uniquely applicable herein. Thus, attention is directed to the decision in In re Marzocchi et al, 58 CCPA 1069, 1073, 439 F.2d 220, 169 USPQ 367, 369, cited by appellant, wherein Judge Baldwin, speaking for the court, stated:

"As a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of §112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support."

Although the Marzocchi et al decision subsequently acknowledges that, in some cases, a statement, *on its face*, may be contrary to generally accepted scientific principles, such exception is not involved herein. Instead, we have before us the type of subject matter and associated circumstances described in In re Gazave, 54 CCPA 1524, 1530-1531, 379 F.2d 973, 154 USPQ 92, 96, as follows:

"Appellant's discovery here does not appear to us to be of such a 'speculative,' abstruse or esoteric nature that it must be considered unbelievable, 'incredible,' or 'factually misleading.' Nor does operativeness appear 'unlikely' or an assertion thereof appear to run counter 'to what would be believed would happen by the ordinary person' in the art. Nor does appellant's field of endeavor appear to be one where 'little of a successful nature has been developed' or one which 'from common knowledge has long been the subject matter of much humbuggery and fraud.' Nor has the examiner presented evidence inconsistent with the assertions and evidence of operativeness presented by appellant" (footnotes omitted).

Consonant with the foregoing, the examiner's unsupported skepticism as to the utility of the claimed invention does not provide a legally acceptable basis for rejecting the claims. It is suggested that, in light of the above, the examiner may better appreciate the pertinency of the holdings in Ex parte Kenaga, 189 USPQ 62 and 190 USPQ 346.

All of the claims also stand rejected "under 35 USC 112 because the specification herein fails to set forth the best mode contemplated by the inventor of carrying out his invention" (Answer, page 4).

[4] From the examiner's explanation of his position, it appears that he is erroneously equating best mode with working examples. However, a specification need not contain a working example if the invention is otherwise disclosed in such a manner that one skilled in the art will be able to practice it without an undue amount of experimentation. In re Borkowski et al, 57 CCPA 946, 950, 422 F.2d 904, 164 USPQ 642, 645. We have no doubt that the invention at issue is disclosed sufficiently to comply with the criteria set forth in Borkowski.

[5] In response to appellant's citation of In re Glass, 492 F.2d 1228, 181 USPQ 31, the examiner points to the statement therein that failure to set forth any mode is equivalent to non-enablement. We invite attention, however, to the very relevant discussion immediately following the sentence to which the examiner refers. Said discussion quotes with approval from In re Gay, 50 CCPA 725, 731, 309 F.2d 769, 772, 135 USPQ 311, 315, which states, in part, that the best mode provision [B]:

"* . . requires an inventor to disclose the best mode *contemplated by him*, as of the time he executes the application, of carrying out his invention. Manifestly, the sole purpose of this latter requirement is to restrain inventors from applying for patents while at the same time concealing from the public preferred embodiments of their inventions which they have in fact conceived."

"As we view portion [B], we think that an inventor is in compliance therewith if he does not conceal what he feels is a preferred embodiment of his invention * * *"

Since, in this case, we have no cause to suspect the inventor of concealing any pertinent information he may possess, we shall not sustain this rejection.

The examiner's decision rejecting claims 1-10 is reversed.

Reversed

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...ture and function in the developing retina of the frog tadpole. ...TRASTRUCT RES 30(1 2): 87-102. Illus. 1970.--The 1st ...retinographic response to light in developing tadpoles (Rana ...) was a purely cornea-negative slow potential which ...ended in time of 1st appearance with the 1st appearance of ...al receptor outer segments as revealed by electron microscopy. ...s stage (6-7 days after fertilization) the receptors possessed ...mal number of developing outer segment disks, which are known ...se the visual pigment molecules. The inner segments, in turn, ...essed all the structural components typical of the adult receptor ... No signs were noted of receptor synaptic structures or of the ...plexiform layer. The visual receptors at this stage of develop... are able to generate a potential in response to a light flash, ...are unable to propagate this excitation inward to the neuronal ...es. The initial negative potential may be, therefore, an isolated ...ptor potential. The appearance of the positive potential somewhat ...er in development was correlated in time with the maturation of ...receptor synaptic apparatus. The correlation in times of appearance ...the positive potential and the mature synaptic apparatus confirms ...ner results with R. catesbeiana, a species characterized by a ...wer rate of development.

79455. HOFMANN, R., H. FRIEMEL, E. BEHM, and J. BROCK. (...ar.-Frauenklin., Rostock, East Ger.) Immunchemische Unter...chungen zum Problem der "pregnancy zone": III. Analytik ...wangerschaftstypischer Antigene. [Immunochemical investigations ...the problem of the pregnancy zone. III. Analysis of pregnancy-...ical antigens.] ARCH GYNAEKOL 208(3): 255-265. Illus. ...). [Engl. sum.]--By means of immuno-diffusion according to ...chterlony and of immunoelectrophoresis after Scheidegger 5 ...egnancy-typical antigens in the sera of pregnant women, in serum ...the unbilical cord, in the serum of puerperas and in amniotic ...id were found using absorbed anti-pregnant women immune serum ...d absorbed anti-placenta immune serum and a mixture of both ...mune sera. Using the mixture of immune sera with immuno...ectrophoresis 3 antigens in the serum of pregnant women, 1 antigen ...the serum of umbilical cord, 2 antigens in the serum of puerperas ...d 2 antigens in the amniotic fluid were precipitated. The electro...retic motilities of the pregnancy-typical antigens and their distribu...ns within the examined substances were tabulated. Sera of 100 ...egnant women were examined immunoelectrophoretically for the ...esence of pregnancy-typical antigens by means of absorbed anti-...egnant women immune serum and absorbed anti-placenta immune ...um. The loss of immunogenic activity in temperatures of 56 C ...some pregnancy-typical antigens from sera of pregnant women ...d sera of the unbilical cord examined.

79456. HOFMANN, R., J. BROCK, and H. FRIEMEL. (Univ.-...rauenklin., Rostock, East Ger.) Immunchemische Untersuchungen ...m Problem der "pregnancy zone". IV. Nachweis schwangerschafts-...ischer Antigene in proteinfraktionen manschlicher placenten. ...mmunochemical investigations on the problem of the pregnancy zone. ... Demonstration of pregnancy-typical antigens in protein fractions ...human placentas.] ARCH GYNAEKOL 208(3): 266-274. Illus. ...--An extract from a human placenta can be fractionated by means ...salt-precipitation, gel-chromatography with Sephadex G-200 and ...e exchange with DEAE-Sephadex A-50. In 4 placental fractions 5 ...tens typical of pregnancy are found. The human placenta is ...table for obtaining antigens and production of mono-specific ...mune sera against pregnancy-typical proteins.

79457. GRAHAM, R. W., and R. J. SCOTHORNE. (Dep. Anat., Univ., ...wcastle upon Tyne, Engl., UK.) Calcium homeostasis in the foetal ...ca pig. QUART J EXP PHYSIOL COG MED SCI 55(1): 44-53. ...1970.--Plasma total Ca levels were studied in pregnant guinea ...and their fetuses from 26 days of gestation to term. Between ...and 33-39 days the fetal plasma Ca falls from nearly the ...ernal level (9.6 mg/100 ml) to 5.5 mg 100 ml. Thereafter the ...plasma Ca rises, reaching the maternal level at term. No trend ...in the maternal levels. Fetal skeletal ossification was studied ...alizarin stain technique. Ossification centers appeared first ...ic, face and skull at 26 days of gestation. After 27 days ...tal plasma protein = 1 g 100 ml) there is a steady rise ...a protein level and at term fetal and maternal levels ...ately equal. Between 41 days gestation and term fetal ...stently more acid than maternal blood and has a higher ...ion] and lower pO_2. There is no evidence of a ...ange during gestation in pH, pO_2 or pCO_2 in either ...al blood. The fall in fetal plasma Ca between 28 days ...gestation may be due to calcification of the fetal ...the full establishment of Ca homeostasis in the fetus. ...plasma protein concentration or fetal parathyroid ...ent for the rise in fetal plasma Ca after 33-39 days.

...O. J. (St. Bartholomew's Hosp. Med. Coll., London, ...velopment of the human wrist joint during the ... REC 166(3): 499-516. Illus. 1970.--Recent ...s have revealed that the hominoid ulna has become ...ed from direct articulation with the carpus by ...an intervening intra-articular meniscus, which in gibbons contains a bony lunula. In man this meniscus has merged with the triangular articular disk, forming a confluent proximal articular surface and constricting off a diverticulum of the synovial radiocarpal cavity, the prestyloid recess, which may lodge the ulnar styloid process. The present study reassesses the development of the human fetal joint in the light of these findings. Initially separate radiocarpal, ulnolunate, radiolunate and ulnotriquetral synovial cavities communicate to form a single continuous wrist joint cavity; the pisotriquetral cavity, at first independent, frequently establishes continuity with the wrist joint cavity, the normal condition in most Primates. After the 60 mm crown rump stage the ulna withdraws from the carpus and an intervening meniscus and prestyloid recess are elaborated. In a number of specimens a cartilaginous nodule, the so-called intermedium antebrachii, was observed adjacent to the ulnar styloid process. There is little doubt that this nodule, which may ossify, is homologous with the gibbon lunula.

† 79459. OLLERICH, DWAYNE A., and EDWARD C. CARLSON. (Univ. N. Dak., Sch. Med., Grand Forks, N. Dak., USA.) Ultrastructure of intranuclear annulate lamellae in giant cells of rat placenta. J ULTRASTRUCT RES 30(3 4): 411-422. Illus. 1970.--The ultrastructure of intranuclear annulate lamellae (IAL) in placental giant cells of the rat was studied. Placentae, taken from Sprague-Dawley rats on day 9 through day 14 of gestation, were fixed in 3% glutaraldehyde, postfixed in 1% osmic acid and embedded in Epon. IAL were rare in 9-day specimens and usually consisted of short lamellae closely associated with the internal nuclear envelope. They increased in frequency and size with time and became quite obvious in thick sections stained with toluidine blue. Electron micrographs revealed curved, linear, or branched profiles of IAL up to 8 μ in length. Continuity of their membranes with the internal nuclear membrane was occasionally evident. The pores of the IAL measured 1100-1600 Å center-to-center and contained central dense granules. The IAL were surrounded by a moderately electron-opaque matrix in which granules 100-150 Å in diameter could occasionally be observed. In 1 specimen IAL were in direct contact with a nucleolus. Here granules 140-160 Å in diameter were arranged around the periphery of the IAL. These granules were separated from the IAL by a sleeve of matrix. The significance of these observations as related to the origin and function of IAL is discussed.

† 79460. VAN LIMBORGH, J. (Anat. Inst., Univ., Amsterdam, Neth.) Die primare Asymmetrie der Gonadenanlagen bei der Ente. [The primary asymmetry of the gonadal primordia in the duck.] Z ANAT ENTWICKLUNGSGESCH 130(1): 37-79. Illus. 1970. [Engl. sum.] --In preceding experimental studies it was shown that the tendencies towards asymmetrical gonadal development are located within the gonadal primordia themselves. This asked for a detailed analysis of the activity of the cortical and medullary territories of the primordia. As an adequate criterion for the activity was chosen the number of primary germ cells present in these areas. A total of 220 embryos were studied. The stages of development varied from stage 16 to stage 27 according to the classification of Hamburger and Hamilton (approximately 75-140 hr of incubation). The 108 embryos of the stages 16-20 were of unknown sex; the genetic sex of the 112 embryos of the stages 21-27 was determined according to the method of Ishizaki and Kosin. The results of the germ cell counts and of the calculations concerning the proportional distribution of the cells are summarized. In the embryos of stages 19-21 the normal germ cells and the degenerating and the dividing germ cells were counted. Up until stage 18 the 2 gonadal primordia develop identically in every respect. During stages 19 and 20 (37-42 somites; approximately 90-97 hr of incubation), however, the right cortical area (germinal epithelium) thins off. The germ cells slightly increase in number on the left side while decreasing on the right. The resulting asymmetrical distribution of the germ cells is due to a process of degeneration which prevails on the right side. From stage 21 until stage 27, when the histological sexual differentiation of the gonads sets in, in all embryos both the right and left cortical areas thicken again and the number of primary germ cells steadily increases. During stages 22-27 (approximately 105-140 hr of incubation) the degree of asymmetry is much more pronounced in the female embryos than in the male ones. This sex difference as to the bilateral germ cell distribution which is mainly due to differences in the proportional numbers of the germ cells in the cortical areas, can be considered the 1st sign of gonadal sexual differentiation. Another feature observed during the entire period under investigation is that the germ cells in the cortical areas continuously increase more rapidly than those in the medullary areas. This phenomenon was encountered in the right and left gonads of both female and male embryos. Finally, no signs were noticed indicative of the existence of a cortico-medullary antagonism. The gonadal territories probably develop independently of one another, at least between the stages studied.

† 79461. SALZER, PETER, and KARL THEILER. (Anat. Inst. Univ., Zurich, Switz.) Uber eine besondere Klappe am Ductus venosus Arantii bei der Maus. [On a special valve in the ductus venosus Arantii of the mouse.] Z ANAT ENTWICKLUNGSGESCH 130(1): 91-94. Illus. 1970. [Engl. sum.]--A valve is present on the right side of the ductus venosus, at the site of its junction with the inferior vena cava. It separates the ductus venosus from the middle and left

uterus in sows and a suitable treatment.] VLAAMS DIERGENEESKD TIDSCHR 40(7/8): 362-370. Illus. 1971. [Engl. summ.]--In the list of 2 bicornual prolapses as incision was made in the prolapsed dorsal vaginal wall through which the total mass was manipulated back into the abdominal cavity. In another similar case, probably of longer duration, the prolapsed uterus was amputated. The 3rd case was a special case of prolapse of the vagina during parturition with an empty coil of the right uterine horn protruding through a vaginal laceration. Here laparatomy was necessary. All 3 sows recovered and raised their litters.--P. L. W.

52053. TOYAMA, KIYOKO and TOMONORI IMAMICHI. (Ec. Vet.-Zootech. Nippon, Nippon, Jap.) [Recherches fondamentales sur le Rat d' "Hydro-testis": I. Defaillance de Virilite chez le Rat d' "Hydro-testis." [Fundamental research on the rat with "hydro-testis:" I. Virility deficiency in rats with hydro-testis.] BULL NIPPON VET ZOOTECH COLL 19. 17-20. 1971. [In Jap. with Fr. summ.]--In a colony of Wistar-Imamichi rats animals with "hydro-testis" were just as virile in appearance as normals, but their testicles showed signs of extreme atrophy and had a brownish color like that observed in hypophysectomized animals. The mating of 2 males with hydro-testis and 30 females did not result in any pregnancies; 25 of these females were then mated with normal males and became pregnant normally. The 5 other females had a genital tract for which dissection revealed no abnormalities. Although male rats with hydro-testis manifest normal copulatory urges, they do not induce pregnancy in the females.
--S. L. R.

52054. TOFFIA, G., J. SCHULZ, I. STUBBE and A. RICHTER. (Margarete-Blank-St. 8, 701 Leipzig, East Ger.) Beziehungen zwischen Nachgeburtsverhaltung und Schwergeburt beim Rind. [Relations between retention of fetal membranes and difficult births in cattle.] MONATSH VETERINAERMED 26(17): 643-646. 1971. [Engl. and Russ. summ.] --The frequency of retained fetal membranes is reported with reference to evaluation of 782 difficult births. A brief introduction concerning the etiology and pathogenesis of retention is followed by an attempt to establish correlations between the percentages of observed cases of retention of fetal membranes in connection with different obstetric diagnoses as well as with reference to various therapies, absence of brucellosis, vitality, freshness, and weight of calves.--P. L. W.

52055. YAMAGAMI, TAKESHI, TOSHIAKI HASHIMURA, MASAKATSU ABE and AKITSU TSUNAWAKI. (Dep. Obstet. Gynecol., Kurume Univ. Sch. Med., Kurume, Jap.) [Abnormal delivery and some tentative curative procedures for it.] J KURUME MED ASSOC 34(6): 583-589. Illus. 1971. [In Jap. with Engl. summ.]--Problems in management of abnormal delivery in clinical practice are discussed.--P. L. W.

52056. RITTENBACH, P. (Sek. Tierprod. Vet., Karl-Marx-Univ., Leipzig, East Ger.) Diphallie beim Hauskaninchen. [Diphallia in the domestic rabbit.] Z VERSUCHTIERK 13(3): 167-169. Illus. 1971. [Engl. summ.]--A rare observation of diphallia totalis s. perfecta in the domestic rabbit is described. This anomaly was accompanied by a subtotal duplication of the urethra and was not followed by functional reduction of reproductive ability or morphological alterations.--P. L. W.

52057. TERZI, IGINO and SERGIO BORDINO. (Osp. Maria Vittoria, Div. Ostet. and Ginecol., Torino, Italy.) Limiti e possibilita nella diagnosi di gravidanza protratta con lo studio della citologia delle membrane ovulari. [Limits and possibilities of the diagnosis of prolonged pregnancy with the cytological study of fetal membranes.] G BATTERIOL VIROL IMMUNOL ANN OSP MARIA VITTORIA TORINO 63(11/12): 987-996. 1970[recd. 1971].--Following a description of the cytological changes in the membranes surrounding the fetus and the amniotic fluid during prolonged pregnancy, the use of amnioscopy in the clinical diagnosis is discussed. Of the 35 patients studied, 21 showed signs of prolonged pregnancy using amnioscopy, and the condition was verified in 17 neonates. The use of this technique can, with other clinical methods, allow for proper therapeutic selection for inducing births thereby preventing pre-natal and neonatal mortality.--G. M. H.

52058. DAVANOV, F. V. K voprosu lecheniya involyutivno-atroficheskikh zabolevanii vul'vy [krauroz, leikoplakiya]. [Treatment of involutional-atrophic diseases of the vulva (kraurosis, leukoplakia).] TR GOR'K MED INST 28. 262-265. 1969. Translated from REF ZH GTD VYP OBSHCH VOP PATOL ONKOL, 1970, No. 4.53.1121.--Twenty-one patients were treated for involutional-atrophic diseases of the vulva, lasting between 3 mo. and 23 yr. The treatment of neuro-hormonal vulval involution included conservative measures and alcohol-novocaine denervation of the vulva. The latter method was used in 11 patients unaffected by conservative treatment. The use of alcohol-novocaine block produced a full effect in 6 patients, manifested by disappearance of pruritic paresthesia, normalization of the well-being of patients, normal consistency and softness of the vulvar tissues, and regression of parakeratosis.
--(Courtesy NSF PL 83-480) 1971. P. L. W.

52059. KOLGANOVA, N. S. Izmeneniya soderzhaniya kaliya i natriya krovi i moche posle massivnoi krovopoteri, nastupivshei vsledstvie akusherско-ginekologicheskoi patologii. [Changes in the content of potassium and sodium in the blood and urine after massive blood loss occurring as the result of obstetric-gynecological pathology.] AKUSH GINEKOL 46(8): 44-47. 1970. [Engl. summ.]--The content of K and Na in the blood plasma and erythrocytes, as well as in the diurnal urine, was studied in 40 women who sustained a massive blood loss. In 18 women the blood loss (30-46 ml/kg) occurred as the result of disturbed tubal pregnancy, in 22 (30-76 ml/kg) — obstetric pathology. The concentration of cations was determined by means of flame photometry. Massive blood loss was accompanied by the development of hypokalemia, hypokaluria and hyponatriuria. Simultaneously the concentration of K decreased in erythrocytes.--P. L. W.

52060. AILAMAZYAN, E. K. (Dep. Obstet. Gynecol., I. P. Pavlov wst Leningr. Med. Inst., Leningrad, USSR.) Nekotorye osobennosti sokratitel'noi deyatel'nosti matki zhenshchin pri tazovom predlezhanii ploda. [Some characteristics of contractile activity of the uterus of women in pelvic presentation of the fetus.] AKUSH GINEKOL 46(4): 76-77. 1970.--A study of 129 pregnant women showed that in pelvic presentation there was no tendency for the frequency, duration and intensity of uterine contractions to increase with the progression of pregnancy. The frequency was considerably less than in parietal presentation, and in some cases of pelvic presentation contractions were not registered during pregnancy. With labor the frequency reached the values registered for parietal presentation. Discoordinated contractile activity occurred in 40%, and myometric insufficiency in the form of weak labor activity was more pronounced in 37.1% compared to parietal presentation. When the fetus was turned from the pelvic to the parietal end the nature of contractile activity did not change. The lack of growth in myometric activity and lack of differentiation between different forms of pelvic presentation and preventive rotation may be associated with incomplete functioning of the nerve receptor apparatus of the uterus, usually due to structural-morphological changes in the tissues of the uterus.--N. L. G.

PHYSIOLOGY AND BIOCHEMISTRY

† 52061. GUPTA, P. K., PARVATI K. MALKANI and KAMAL BHASIN. (All-India Inst. Med. Sci., New Delhi, Delhi, India.) Cellular response in the uterine cavity after IUD insertion and structural changes of the IUD. CONTRACEPTION 4(6): 375-384. Illus. 1971[recd. 1972].--The morphology of the exfoliated endometrial cells was studied with the objective of elucidating the mode of action of an intrauterine contraceptive device [IUD]. The loose cellular material accumulated in the uterine cavity and on the surface of the IUD was studied in 50 women. The device had been in use for periods of time varying from 24 hr to 84 mo. The presumed biologically inert IUD material seems to provoke an acute inflammatory response which is probably the forerunner of macrophage accumulation. The macrophages appear about the third day after the IUD insertion and progressively increase in number in proportion to the duration of usage. Cytomorphological changes were observed in the endocervical smears. Structural changes in the IUD itself also occur.

† 52062. BOHN, H. (Behringwerke AG, Marburg, West Ger.) Nachweis und Charakterisierung von Schwangerschaftsproteinen in der menschlichen Placenta sowie ihre quantitative immunologische Bestimmung im Serum schwangerer Frauen. [Detection and characterization of pregnancy proteins in the human placenta and their quantitative immunochemical determination in sera from pregnant women.] ARCH GYNAEKOL 210(4): 449-457. Illus. 1971. [Engl. summ.]--In sera from pregnant women 4 different pregnancy proteins could be detected by the Ouchterlony gel diffusion technique with antisera which were prepared by immunizing rabbits with protein fractions from human placentas. The proteins are characterized by their electrophoretic mobilities in agar and polyacrylamide gel and by other physico-chemical properties. One of these pregnancy proteins could be identified as human placental lactogen (HPL). A 2nd which also occurred only in sera from pregnant women was termed pregnancy-specific β_1-glycoprotein. The detection of this protein in serum of urine by immuno-chemical method can be used as a pregnancy test. The remaining 2 pregnancy proteins are not only found in sera from pregnant women but can be detected sometimes in sera from nonpregnant female subjects, too, especially in those taking oral contraceptives. These proteins were designated as pregnancy-associated β_1- and α_2-glycoproteins. The pregnancy associated α_2-glycoprotein proved to be identical with the protein of the so-called "pregnancy zone" as well as with the serum factor Xh. The concentration of the pregnancy proteins was quantitatively determined in sera from pregnant women by the single radial immunodiffusion method. The amount of the pregnancy-specific β_1-glycoprotein increases with advancing pregnancy. The possibility of using the quantitation of this pregnancy protein as a diagnostic test for placental insufficiency is discussed.

† 52063. HAMADA, TATSUO. (Natl. Inst. Anim. Ind., Chiba, Jap.) Estimation of lower critical temperatures for dry and lactating dairy cows. J DAIRY SCI 54(11): 1704-1705. 1971.--Based on the schematic diagram of Kleiber, new equations to estimate lower critical tem-

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(54) Bezeichnung: Schwangerschaftsspezifisches β_1-Glykoprotein und Verfahren zu seiner Isolierung

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(71) Anmelder: Behringwerke AG, 3550 Marburg

Vertreter gem. § 16 PatG: —

(72) Als Erfinder benannt: Bohn, Hans, Dipl.-Chem. Dr., 3550 Marbach; Stutzinger, Ferdinand, 3551 Michelbach

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[illegible] AKTIENGESELLSCHAFT, [illegible]burg/Lahn

Aktenzeichen: Hoe 71/B 020 - Ha 141 a

Datum: 19. November 1971

Schwangerschaftsspezifisches β_1-Glykoprotein und Verfahren zu seiner Isolierung

Es ist bisher nicht bekannt, dass in der Plazenta und im Urin von Schwangeren ein schwangerschaftsspezifisches β_1-Glyoprotein vorkommt. Es ist zwar immunologisch schon ein Protein im β-Bereich von Schwangerenserum nachgewiesen worden, ein Verfahren zu seiner Isolierung jedoch noch nie beschrieben worden.

Gegenstand der Erfindung ist ein Verfahren zur Isolierung des schwangerschaftsspezifischen β_1-Glykoproteins (abgekürzt SP_1), das dadurch gekennzeichnet ist, dass das schwangerschaftsspezifische β_1-Glykoprotein aus Plazenta, sowie dem Blut oder Urin von Schwangeren durch Fraktionieren nach üblichen Methoden isoliert wird. Ein weiterer Gegenstand der Erfindung ist das schwangerschaftsspezifische β_1-Glykoprotein, gekennzeichnet durch eine

elektrophoretische Wanderungsgeschwindigkeit im Agargel im Bereich der β_1-Globuline,

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eine Sedimentationskonstante von 4,6 S,
ein Molekulargewicht von etwa 100 000,
einen Extinktionskoeffizienten $E^{1\%}_{1\,cm}$ = 11,0 (279 mμ; 1/15 molarer Phosphatpuffer pH 7,0)
einen Kohlenhydratgehalt von 25,5 - 29,6 %, davon 10,0 - 11,0 % Hexosen, 9,5 - 10,5 % Hexosamin, 0,5 - 0,6 % Fucose und 6,5 - 7,5 % Neuraminsäure.

Das erfindungsgemäss hergestellte schwangerschaftsspezifische β_1-Glykoprotein dient zur Gewinnung von Antiseren. Mit diesen wiederum kann durch immunologische Methoden (Haemagglutinations-Hemmungs-Test, Komplement-Bindungs-Reaktion, Radio-Immunassay, Latex-Agglutination) eine Schwangerschaft nachgewiesen werden. Der Nachweis kann mit Serum oder Urin der Schwangeren geführt werden. Eine quantitiative Bestimmung von SP_1, z.B. mit der radialen Immundiffusion, kann für eine Überwachung der Schwangerschaft von Bedeutung werden.

Für die Herstellung des schwangerschaftsspezifischen β_1-Glyoproteins werden zerkleinerte Plazenten mit einer schwachen Salzlösung extrahiert und die mit Diäthylaminoäthylcellulose-Lactat fällbaren Proteine bei einem pH-Wert von etwa 3,0 abgetrennt. Das im Überstand verbleibende SP_1 wird zusammen mit Gamma-globulin mit Ammoniumsulfat ausgefällt. Zur Trennung des SP_1 von der Hauptmenge des Gammaglobulins wird die in reinem Wasser wieder aufgelöste Fällung, zweckmässig nach einer Vorbehandlung

durch Dialyse gegen eine Pufferlösung, an Diäthylaminoäthyl (DEAE) - Cellulose adsorbiert und von dieser wieder eluiert. Als Elutionsmittel hat sich eine 0,02 molare Tris-oxymethyl-aminomethyl-Salzsäure-Pufferlösung vom pH-Wert 6,5 besonders bewährt, die noch etwa 0,85 % Natriumchlorid und eine kleine Menge, z.B. 0,05 % Natriumazid enthalten kann. Ausfällen des Eluats mit Ammoniumsulfat liefert das SP_1 in angereicherter Form, das durch Gelfiltration beispielsweise mittels Sephadex® G-150 weiter gereinigt werden kann.

Zur weiteren Reinigung eignet sich die präparative Zonenelektrophorese mit PVC als Träger in einer geeigneten Pufferlösung, z.B. 0,075 % Ammoniumhydrogencarbonat. Nach der elektrophoretischen Auftrennung wird die SP_1-haltige β_1-Zone des Trägers herausgeschnitten, mit Ammoniumhydrogencarbonat eluiert und lyophilisiert. Weitere geeignete Feinreinigungsverfahren sind eine Chromatographierung an DEAE Sephadex mit nachfolgender Elution mit einem Natriumchlorid-Gradienten und eine Fraktionierung mit Alkohol, vorzugsweise mit Äthanol. Das schwangerschaftsspezifische β_1-Glykoprotein wird in ähnlicher Weise aus Blut oder Urin von Schwangeren isoliert.

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Beispiel 1 / Isolierung aus Plasmonen

150 kg tiefgefrorene Plasmonen [illegible] und mit 150 Liter einer 0,5 Mol[illegible] [illegible]-Lösung extrahiert. Der Extrakt [illegible] mit 2 [illegible] Natriumhydroxid auf pH 8 eingestellt und mit 30 [illegible] einer 10gen wässrigen Lösung von Diäthylaminoäthylcellulose (Cellulosol®) versetzt. Nach einer Wartezeit von 1 Stunde [illegible] der Überstand, der das SP_1 zusammen mit Begleitstoffen enthält, abgehebert, mit 5 % festem Natriumchlorid (31 kg) zur Abtrennung des noch in Lösung verbliebenen Cellulosols versetzt, filtriert und mit 23,5 %, bezogen auf das Gesamtgewicht Filtrat, festem Ammonsulfat versetzt und zur Aussalzung gerührt. Nach 1 Stunde wird der Niederschlag abfiltriert.

500 g des auf dem Filter gesammelten Niederschlags werden in 500 ml destilliertem Wasser gelöst und [illegible] eine 0,01 molare Tris-(oxymethyl)-aminomethan (im folgenden mit "Tris" abgekürzt)-Salzsäure-Pufferlösung von pH-Wert 7,0, die 0,05 % Natriumazid enthält, dialysiert. Die dialysierte Lösung wird zentrifugiert und der Überstand [illegible] der gleichen Pufferlösung auf 2000 ml aufgefüllt, [illegible] normaler Natriumhydroxidlösung auf pH 8,0 eingestellt und mit 150 g feuchter DEAE-Cellulose eine Stunde verrührt.

Sodann wird die DEAE-Cellulose durch Filtrieren von der Lösung abgetrennt, zweimal mit je einem Liter 0,01 molarem Tris-Salzsäure-Puffer von pH-Wert 8,0 gewaschen und danach dreimal mit je 500 ml 0,02 molarem Tris-Salzsäure-Puffer, pH 6,5, der 0,05 % Natriumchlorid und 0,05 % Natriumazid enthält, eluiert. Dem vereinigten Eluaten wird 30 % Ammonsulfat, bezogen auf das Flüssigkeitsgewicht, zugesetzt und das Ganze verrührt. Der Niederschlag, der das SP_1 enthält, wird in 300 ml destilliertem Wasser gelöst und der Gelfiltration mittels Sephadex G-150

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unter Verwendung von 0,1 molarem Tris-Salzsäure-Puffer pH 8,0, der 1,0 Mol Natriumchlorid pro Liter enthält, unterzogen (100 : 20 cm-Säule).

Anschließend werden die Eluate mit spezifischem SP_1-Antiserum getestet, die SP_1-haltigen Fraktionen gesammelt und daraus die Proteine nochmals wie oben beschrieben mit 90 % festem Ammonsulfat ausgefällt.

Eine weitere Reinigung erzielt man durch präparative Zonenelektrophorese mit Polyvinylchlorid (PVC) als Träger. Als Puffer wird eine 0,075 molare Ammoniumhydrogencarbonatlösung (pH 8,0) verwendet. Man schneidet nach der elektrophoretischen Auftrennung die SP_1-haltige ß-Zone heraus, eluiert mit dem Ammoniumhydrogencarbonat-Puffer und lyophilisiert die Eluate.

Die Trockensubstanz wird in 0,01 molarem Tris-Salzsäure-Puffer pH 7,0, der 0,2 % Natriumchlorid enthält, gelöst, an DEAE-Sephadex (Säule 5 x 20 cm) chromatographiert und mit einem linearen Gradienten von 0,2 bis 2,0 % Natriumchlorid in 0,01 molarem Tris-Salzsäure-Puffer pH 7,0 eluiert. Es folgt eine Fällung mit 40 % Äthanol bei -5°C. Nach Abzentrifugation des Niederschlages wird der Überstand gegen destilliertes Wasser salzfrei dialysiert und lyophilisiert. Reinheit 90 - 95 %. Dieses Präparat kann zur Herstellung von Antiseren Verwendung finden.

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Beispiel 2 / Isolierung aus Blut

5 Liter Schwangerenserum werden mit destilliertem Wasser im Verhältnis 1:1 verdünnt und darauf die Hauptmenge der Plasmaproteine durch 2,5 Liter 2 %igen Rivanol bei pH 8,0 gefällt. Der durch Zentrifugation erhaltene Überstand wird zur Entfernung des Rivanols mit 5 % Natriumchlorid versetzt. Der dabei entstandene Niederschlag wird abzentrifugiert, der Überstand vom Sediment getrennt und mit 50 % festem Ammonsulfat versetzt. Im Niederschlag sind das Gammaglobulin und das schwangerschaftsspezifische β_1-Glykoprotein enthalten. Der Niederschlag wird in 0,01 molarem Tris-Salzsäure-Puffer bei pH 7,9 gelöst, gegen 0,01 molarem Tris-Salzsäure-Puffer [illegible] dialysiert und anschließend mit DEAE-Cellulose gereinigt. Die DEAE-Cellulose-Behandlung wird in 0,01 molarem Tris-Salzsäure-Puffer pH 8,0 ausgeführt.

Die Elution von DEAE-Cellulose, die Gel-Filtration mit Sephadex, die präparative Zonenelektrophorese, die Reinigung an DEAE-Sephadex sowie die Fällung mit [illegible] werden gemäß Beispiel 1 ausgeführt.

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Patentansprüche

1. Verfahren zur Isolierung des schwangerschaftsspezifischen β_1-Glykoproteins, dadurch gekennzeichnet, dass das schwangerschaftsspezifische β_1-Glykoprotein aus Plazenta sowie dem Blut oder Urin von Schwangeren durch Fraktionieren nach üblichen Methoden isoliert wird.

2. Schwangerschaftsspezifisches β_1-Glykoprotein, gekennzeichnet durch

eine elektrophoretische Änderungsgeschwindigkeit im Agargel im Bereich der β_1-Globulin,
eine Sedimentationskonstante von [illegible] S,
ein Molekulargewicht von etwa [illegible],
einen Extinktionskoeffizienten $E^{1\%}_{1\,cm}$ = 11,0 (273 mμ ; 1/15 molarer Phosphatpuffer, pH 7,0)
einen Kohlenhydratgehalt von 26,5 - [illegible] davon 10,0 - 11,0 % Hexosen, [illegible] - 10,5 % Hexosamin, 0,5 - 0,6 % Fucose und [illegible] - 7,9 % Neuraminsäure.

3. Verwendung von schwangerschaftsspezifischem β_1-Glykoprotein als Reagenz und zur Gewinnung von Antikörpern als Reagenz zum Nachweis und zur Überwachung einer Schwangerschaft.

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(61) Zusatz zu: —

(62) Ausscheidung aus: —

(71) Anmelder: Behringwerke AG, 3550 Marburg

Vertreter gem. § 16 PatG: —

(72) Als Erfinder benannt: Bohn, Hans, Dipl.-Chem. Dr., 3550 Marbach; Stutzinger, Ferdinand, 3551 Michelbach

DEUTSCHE AUSLANDSPATENTANMELDUNG, Frankfurt/Main

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Datum: 19. November 1971

Schwangerschaftsspezifisches β_1-Glykoprotein und Verfahren zu seiner Isolierung

Es ist bisher nicht bekannt, dass in der Plazenta und im Urin von Schwangeren ein schwangerschaftsspezifisches β_1-Glyoprotein vorkommt. Es ist zwar immunologisch schon ein Protein im β-Bereich von Schwangerenserum nachgewiesen worden, ein Verfahren zu seiner Isolierung jedoch noch nie beschrieben worden.

Gegenstand der Erfindung ist ein Verfahren zur Isolierung des schwangerschaftsspezifischen β_1-Glykoproteins (abgekürzt SP_1), das dadurch gekennzeichnet ist, dass das schwangerschaftsspezifische β_1-Glykoprotein aus Plazenta sowie dem Blut oder Urin von Schwangeren durch Fraktionieren nach üblichen Methoden isoliert wird. Ein weiterer Gegenstand der Erfindung ist das schwangerschaftsspezifische β_1-Glykoprotein, gekennzeichnet durch eine

elektrophoretische Wanderungsgeschwindigkeit im Agargel im Bereich der β_1-Globuline,

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eine Sedimentationskonstante von 4,6 S,
ein Molekulargewicht von etwa 100 000,
einen Extinktionskoeffizienten $E^{1\%}_{1\,cm}$ = 11,0 (278 mμ; 1/15 molarer Phosphatpuffer pH 7,0)
einen Kohlenhydratgehalt von 26,5 - 29,6 %, davon 10,0 - 11,0 % Hexosen, 9,5 - 10,5 % Hexosamin, 0,5 - 0,6 % Fucose und 6,5 - 7,5 % Neuraminsäure.

Das erfindungsgemäss hergestellte schwangerschaftsspezifische β_1-Glykoprotein dient zur Gewinnung von Antiseren. Mit diesen wiederum kann durch immunologische Methoden (Haemagglutinations-Hemmungs-Test, Komplement-Bindungs-Reaktion, Radio-Immunassay, Latex-Agglutination) eine Schwangerschaft nachgewiesen werden. Der Nachweis kann mit Serum oder Urin der Schwangeren geführt werden. Eine quantitiative Bestimmung von SP_1, z.B. mit der radialen Immundiffusion, kann für eine Überwachung der Schwangerschaft von Bedeutung werden.

Für die Herstellung des schwangerschaftsspezifischen β_1-Glyoproteins werden zerkleinerte Plazenten mit einer schwachen Salzlösung extrahiert und die mit Dimethylhexadecylamin-lactat fällbaren Proteine bei einem pH-Wert von etwa 5,0 abgetrennt. Das im Überstand verbleibende SP_1 wird gemeinsam mit Gamma-globulin mit Ammoniumsulfat ausgefällt. Zur Trennung des SP_1 von der Hauptmenge des Gammaglobulins wird die in reinem Wasser wieder aufgelöste Fällung, zweckmässig nach einer Vorbehandlung

durch Dialyse gegen eine Pufferlösung, an Diäthylaminoäthyl (DEAE) - Cellulose adsorbiert und von dieser wieder eluiert. Als Elutionsmittel hat sich eine 0,02 molare Tris-oxymethyl-aminomethyl-Salzsäure-Pufferlösung vom pH-Wert 6,5 besonders bewährt, die noch etwa 0,85 % Natriumchlorid und eine kleine Menge, z.B. 0,05 % Natriumazid enthalten kann. Ausfällen des Eluats mit Ammoniumsulfat liefert das SP_1 in angereicherter Form, das durch Gelfiltration beispielsweise mittels Sephadex G-150 weiter gereinigt werden kann.

Zur weiteren Reinigung eignet sich die präparative Zonenelektrophorese mit PVC als Träger in einer geeigneten Pufferlösung, z.B. 0,075 % Ammoniumhydrogencarbonat. Nach der elektrophoretischen Auftrennung wird die SP_1-haltige β-Zone des Trägers herausgeschnitten, mit Ammoniumhydrogencarbonat eluiert und lyophilisiert. Weitere geeignete Feinreinigungsverfahren sind eine Chromatographierung an DEAE Sephadex mit nachfolgender Elution mit einem Natriumchlorid-Gradienten und eine Fraktionierung mit Alkohol, vorzugsweise mit Äthanol. Das schwangerschaftsspezifische β_1-Glyxoprotein wird in ähnlicher Weise aus Blut oder Urin von Schwangeren isoliert.

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Beispiel 1 / Isolierung aus Plazenten

150 kg tiefgefrorene Plazenten werden [illegible] und mit 150 Liter einer 0,5 [illegible]gen wässrigen [illegible]chlorid-Lösung extrahiert. Der Extrakt wird mit 2 normalen Natriumhydroxid auf pH 8 eingestellt und mit 50 Liter einer [illegible]gen wässrigen Lösung von Diaminoäthoxyacridinlactat (Rivanol®) versetzt. Nach einer Wartezeit von 1 Stunde wird der Überstand, der das SP_1 zusammen mit Gammaglobulin enthält, abgehebert, mit 5 % festem Natriumchlorid (11 kg) zur Auschneidung des noch in Lösung verbliebenen Rivanols versetzt, filtriert und mit 26,5 %, bezogen auf das Gewicht der Flüssigkeit, festem Ammonsulfat versetzt und gut durchgerührt. Nach 1 Stunde wird der Niederschlag abfiltriert.

500 g des auf dem Filter gesammelten Niederschlags werden in 500 ml destilliertem Wasser gelöst und gegen eine 0,01 molare Tris-(oxymethyl)-aminomethan (im folgenden mit "Tris" abgekürzt)-Salzsäure-Pufferlösung vom pH-Wert 7,0, die 0,05 % Natriumazid enthält, dialysiert. Die dialysierte Lösung wird zentrifugiert und der Überstand mit der gleichen Pufferlösung auf 2000 ml aufgefüllt, mit [illegible] normaler Natriumhydroxidlösung auf pH 8,0 eingestellt und mit 150 g feuchter DEAE-Cellulose eine Stunde verrührt.

Sodann wird die DEAE-Cellulose durch Filtrieren von der Lösung abgetrennt, zweimal mit je einem Liter 0,01 molarem Tris-Salzsäure-Puffer vom pH-Wert 8,0 gewaschen und danach dreimal mit je 500 ml 0,02 molarem Tris-Salzsäure-Puffer, pH 6,5, der 0,85 % Natriumchlorid und 0,05 % Natriumazid enthält, eluiert. Dem vereinigten Eluaten wird 50 % Ammonsulfat, bezogen auf das Flüssigkeitsgewicht, zugesetzt und das ganze verrührt. Der Niederschlag, der das SP_1 enthält, wird in 300 ml destilliertem Wasser gelöst und der Gelfiltration mittels Sephadex G-150

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unter Verwendung von 0,1 molarem Tris-Salzsäure-Puffer pH 8,0, der 1,0 Mol Natriumchlorid pro Liter enthält, unterzogen (100 : 20 cm-Säule).

Anschließend werden die Eluate mit spezifischem SP_1-Antiserum getestet, die SP_1-haltigen Fraktionen gesammelt und daraus die Proteine nochmals wie oben beschrieben mit 30 % festem Ammonsulfat ausgefällt.

Eine weitere Reinigung erzielt man durch präparative Zonenelektrophorese mit Polyvinylchlorid (PVC) als Träger. Als Puffer wird eine 0,075 molare Ammoniumhydrogencarbonatlösung (pH 8,0) verwendet. Man schneidet nach der elektrophoretischen Auftrennung die SP_1-haltige ß-Zone heraus, eluiert mit dem Ammoniumhydrogencarbonat-Puffer und lyophilisiert die Eluate.

Die Trockensubstanz wird in 0,01 molarem Tris-Salzsäure-Puffer pH 7,0, der 0,2 % Natriumchlorid enthält, gelöst, an DEAE-Sephadex (Säule 5 x 20 cm) chromatographiert und mit einem linearen Gradienten von 0,2 bis 2,0 % Natriumchlorid in 0,01 molarem Tris-Salzsäure-Puffer pH 7,0 eluiert. Es folgt eine Fällung mit 40 % Äthanol bei -5°C. Nach Abzentrifugation des Niederschlages wird der Überstand gegen destilliertes Wasser salzfrei dialysiert und lyophilisiert. Reinheit 90 - 95 %. Dieses Präparat kann zur Herstellung von Antiseren Verwendung finden.

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Beispiel 2 / Isolierung aus Blut

5 Liter Schwangerenserum werden mit destilliertem Wasser im Verhältnis 1:1 verdünnt und darauf die Hauptmenge der Plasmaproteine durch 2,5 Liter 3 %igen Rivanols bei pH 8,0 gefällt. Der durch Zentrifugation erhaltene Überstand wird zur Entfernung des Rivanols mit 5 % Natriumchlorid versetzt. Der dabei entstandene Niederschlag wird abzentrifugiert, der Überstand vom Sediment getrennt und mit 30 % festem Ammonsulfat versetzt. Im Niederschlag sind das Gammaglobulin und das schwangerschaftsspezifische β_1-Glykoprotein enthalten. Der Niederschlag wird in 0,01 molarem Tris-Salzsäure-Puffer pH 7,0 gelöst, gegen 0,01 molarem Tris-Salzsäure-Puffer pH 7,0 dialysiert und anschließend mit DEAE-Cellulose gereinigt. Die DEAE-Cellulose-Behandlung wird in 0,01 molarem Tris-Salzsäure-Puffer pH 8,0 ausgeführt.

Die Elution von DEAE-Cellulose, die Gel-Filtration mit Sephadex, die präparative Zonenelektrophorese, die Reinigung an DEAE-Sephadex sowie die Fällung mit Alkohol werden gemäß Beispiel 1 ausgeführt.

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Patentansprüche

1. Verfahren zur Isolierung des schwangerschaftsspezifischen β_1-Glykoproteins, dadurch gekennzeichnet, dass das schwangerschaftsspezifische β_1-Glykoprotein aus Plazenta sowie dem Blut oder Urin von Schwangeren durch Fraktionieren nach Üblichen Methoden isoliert wird.

2. Schwangerschaftsspezifisches β_1-Glykoprotein, gekennzeichnet durch

eine elektrophoretische Wanderungsgeschwindigkeit im Agargel im Bereich der β_1-Globuline,
eine Sedimentationskonstante von [illegible],
ein Molekulargewicht von etwa 180 [illegible],
einen Extinktionskoeffizienten $E^{1\%}_{1\,cm}$ = 11,0 (278 mμ ; 1/15 molarer Phosphatpuffer, pH 7,0)
einen Kohlenhydratgehalt von 26,5 - [illegible], davon 10,0 - 11,0 % Hexosen, [illegible] - 10,5 % Hexosamin, 0,5 - 0,6 % Fucose und 5,5 - 7,5 % Neuraminsäure.

3. Verwendung von schwangerschaftsspezifischem β_1-Glykoprotein als Reagenz und zur Gewinnung von Antikörpern als Reagenz zum Nachweis und zur Überwachung einer Schwangerschaft.

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IMMUNOCHEMICAL INVESTIGATIONS ON THE PROBLEM OF THE "PREGNANCY ZONE" IV

by

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See Abstract

Translated from the German by

R. Gregg Mansfield
Office of Language Services
Information Division
Oak Ridge National Laboratory
July 1972

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IMMUNOCHEMICAL INVESTIGATIONS ON THE PROBLEM OF THE "PREGNANCY ZONE"

IV. Demonstration of Pregnancy-typical Antigens in Protein Fractions of Human Placentas

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Abstract

An extract from a human placenta can be fractionated by means of salt-precipitation, gel-chromatography with Sephadex G-200 and ion exchange with DEAE-Sephadex A-50. Thereby, in 4 placental fractions altogether 5 proteins typical of pregnancy have been found. For obtaining antigens and production of mono-specific immune sera against pregnancy-typical proteins the human placenta is suitable.

Introduction

In the preceding studies a total of 5 pregnancy-typical antigens were obtained in pregnancy serum, puerperal serum, serum from the umbilical cord and in the amniotic fluid by means of Ouchterlony's method of immunodiffusion and Scheidegger's technique of immunoelectrophoresis, using absorbed antipregnancy and absorbed antiplacenta immune serum and a mixture of both immune sera (2c).

antisera: From pregnancy serum + placenta used to find pregnancy typical antigens

Wilkin (4) found no identity of the "pregnancy zone" (pregnancy-typical antigen no. 2) with antigens of certain placental extracts. Bayer (1) agrees only with reservation with the statement that protein produced by pregnancy is present in the placenta, since, according to his opinion, in addition to interferences by serum components the exact

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position of a line is difficult to objectify when reference lines are lacking. The purpose of the present work is the detection of pregnancy-typical antigens in protein fractions of human placentas by comparative immunochemical experiments. At the same time the problem is investigated as to the extent to which the separation of placental proteins by gel chromatography and ion exchange can meet the requirement for the preparation of pure antigen and hence for the preparation of monospecific antisera against pregnancy-typical antigens from placentas.

Purpose to detect preg-typ antigens in human placenta

Materials and Methods

Data on the preparation of immune sera, their absorption and mixing, on the technique of immunodiffusion and immunoelectrophoresis, on concentrating of solutions of antigens, determination of the electrophoretic mobilities of the pregnancy-typical antigens and preparation of the placental extract may be found in the parts concerning methods of papers I, II, and III (2, 2b, 2c).

1. Precipitation with ammonium sulfate. To placental extract C at a pH of 7.0 and at 4°C ammonium sulfate (analytical grade) is added to a degree of saturation of 0.4 (3). After 12 hours the precipitate is centrifuged off in a cooled centrifuge at 20,000 g (K24, Janetzki, Engelsdorf), dissolved in pH 7.4 Sörensen buffer and again salted out with ammonium sulfate (degree of saturation 0.4, pH 7.0). The precipitate is centrifuged off at 20,000 g and 4°C, after a further 12 hours, then dialyzed against pH 7.4 Sörensen buffer and concentrated in Sörensen buffer with polyethylene glycol 20,000 (Fluka, Buchs).

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2. Gel chromatography. Sephadex G-200 served as carrier material. Chromatography was performed by an ascending method using Sörensen buffer, pH 7.4 at 4°C. The gel column had a height of 870 mm and a diameter of 25 mm. The flowrate amounted to 10.1 ml per hour and was maintained constant with a peristaltic pump (4912 A-2, LKB). 5-ml fractions were collected. The photometer for the discharge -- Uvicord II with recorder (LKB) -- served to record the extinction at 280 nm (discharge cuvette 3 mm).

Fractionation of placental protein

3. Chromatography on DEAE-Sephadex A-50. Fraction 91, after chromatography on Sephadex G-200, was concentrated in a dialyzing tube against polyethylene glycol 20,000 (Fluka, Buchs) and then dialyzed against 0.05 M tris (hydroxymethyl) aminomethane/HCl pH 8.0 for 48 hours, the buffer being replaced after 24 hours.

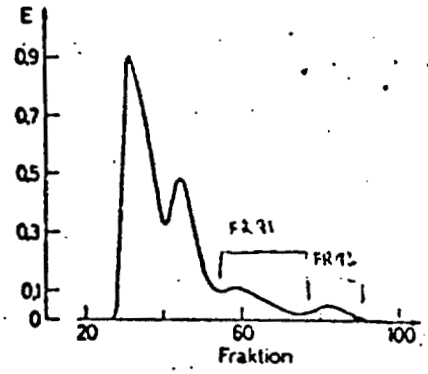
The chromatography on DEAE-Sephadex A-50 was carried out with a linear gradient 0.05 M tris-(hydroxymethyl) aminomethane/HCl pH 8.0 - 0.5 M tris-(hydroxymethyl) aminomethane/HCl pH 8.0 at 4°C. An automatic measuring apparatus served to produce the gradient (3). The bed of gel had a diameter of 24 mm, a height of 370 mm at the beginning of the chromatography. The flowrate was 13 ml per hour. Fractions of 6.5 ml were collected.

Results and Discussion

The 5 pregnancy-typical antigens (2c) were obtained from placental extracts with ammonium sulfate (degree of saturation 0.4, pH 7.0). If these proteins are separated by gel chromatography on Sephadex G-200, then 4 peaks are obtained (fig. 1). Immunochemical analysis of the 4

fractions showed in the diffusion test the presence of pregnancy-typical proteins in fractions 89, 90, and 91. Fraction 92 contained only blood serum proteins.

Fraction 91 was further pruified on DEAE-Sephadex A-50 (fig. 2). Hereby pregnancy-typical proteins were contained in fractions 106 and 108.



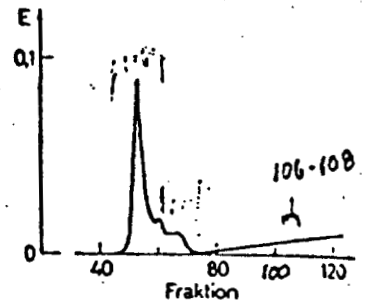


Fig. 1. Elution diagram after chromatography on Sephadex G-200 of placental-extract proteins separable by salting out with ammonium sulfate at a degree of saturation of 0.4. Fractions: 28—40 (FR.89), 41—53 (FR.90), 54—74 (FR.91), 78—91 (FR.92).

Fig. 2. Chromatography of placenta fraction 91 on DEAE-Sephadex A-50. Elution profile. Fractions: 50—58 (FR. 106), 65—70 (FR.108)

The 4 placental fractions (89, 90; 106, 108) which contained pregnancy-typical proteins were tested for immunological identity against serum of pregnant women and puerperal serum, umbilical-cord serum and amniotic fluid by means of immunodiffusion and the immune serium mixture a. ASP.

Figure 4 shows the result of an immunodiffusion of the 4 placental fractions as a test for identity with antigens of pregnancy serum.

Fraction 89 contains at least 3 proteins; two precipitation lines show immunological identity with antigens of pregnancy serum. In fraction 90 two precipitation lines are obtained; the inner line of these proves to be identical with proteins of the pregnancy serum. The antigen of fraction 106 is not immunologically identical with pregnancy serum serum antigens. Of two proteins of the fraction 108 one is also contained in pregnancy serum. In the 4 placental fractions, therefore, 3 pregnancy-typical proteins are detectable which also occur in pregnancy serum.

The result of an immunodiffusion of the 4 placental fractions compared to puerperal serum is shown in fig. 5. Fraction 89 yields two precipitation lines which are immunologically identical with antigens of the puerperal serum. Of the 2 proteins of fraction 90 one is not contained in the puerperal serum. The precipitation line of fraction 106 crosses the lines of the puerperal serum, from which it may be deduced that there is no immunological identity. Fraction 108 shows no identity with the antigens of the puerperal serum. In the 4 placental fractions there are thus contained 2 pregnancy-typical proteins which can also be detected in puerperal serum.

The identity of the 4 placental fractions with antigens of the amniotic fluid is evident from fig. 6. In fraction 89,2 precipitation lines are identical with those of the amniotic fluid; one protein of fraction 90 is likewise identical. The precipitates of fractions 106 and 108 show no identity. In the placental fractions 89 and 90, therefore, are contained 2 antigens of the amniotic fluid.

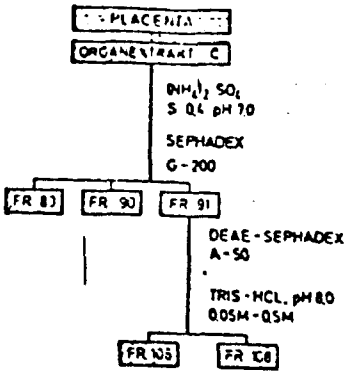
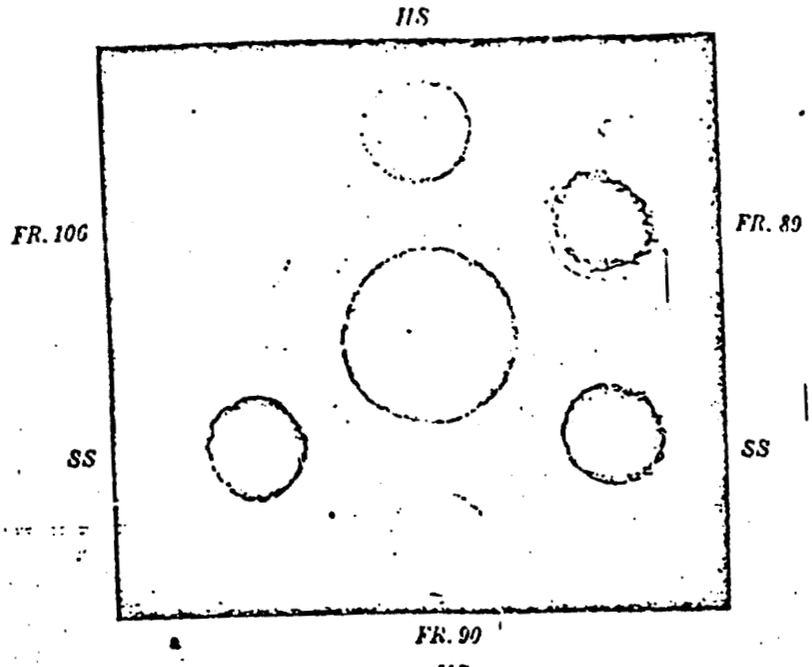
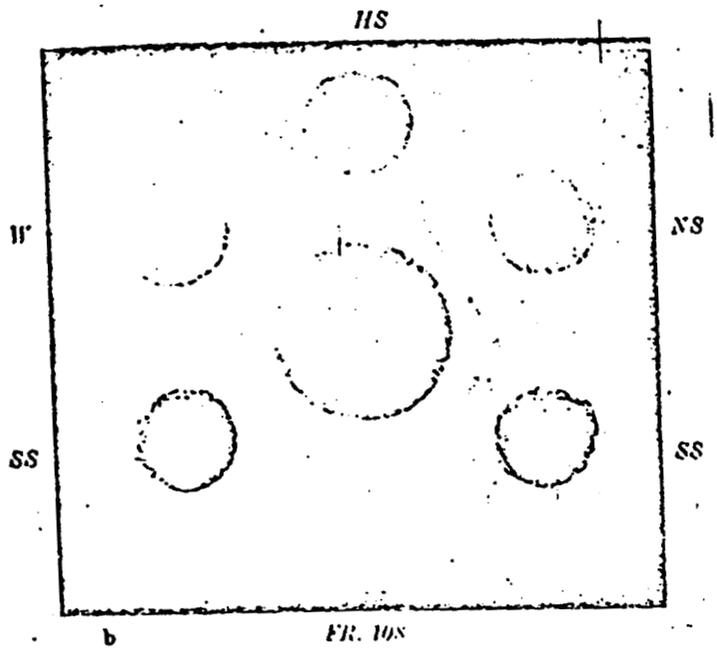


Fig. 3. Diagrammatic summary of fractionation of placental proteins by salt precipitation, gel chromatography and ion exchange.

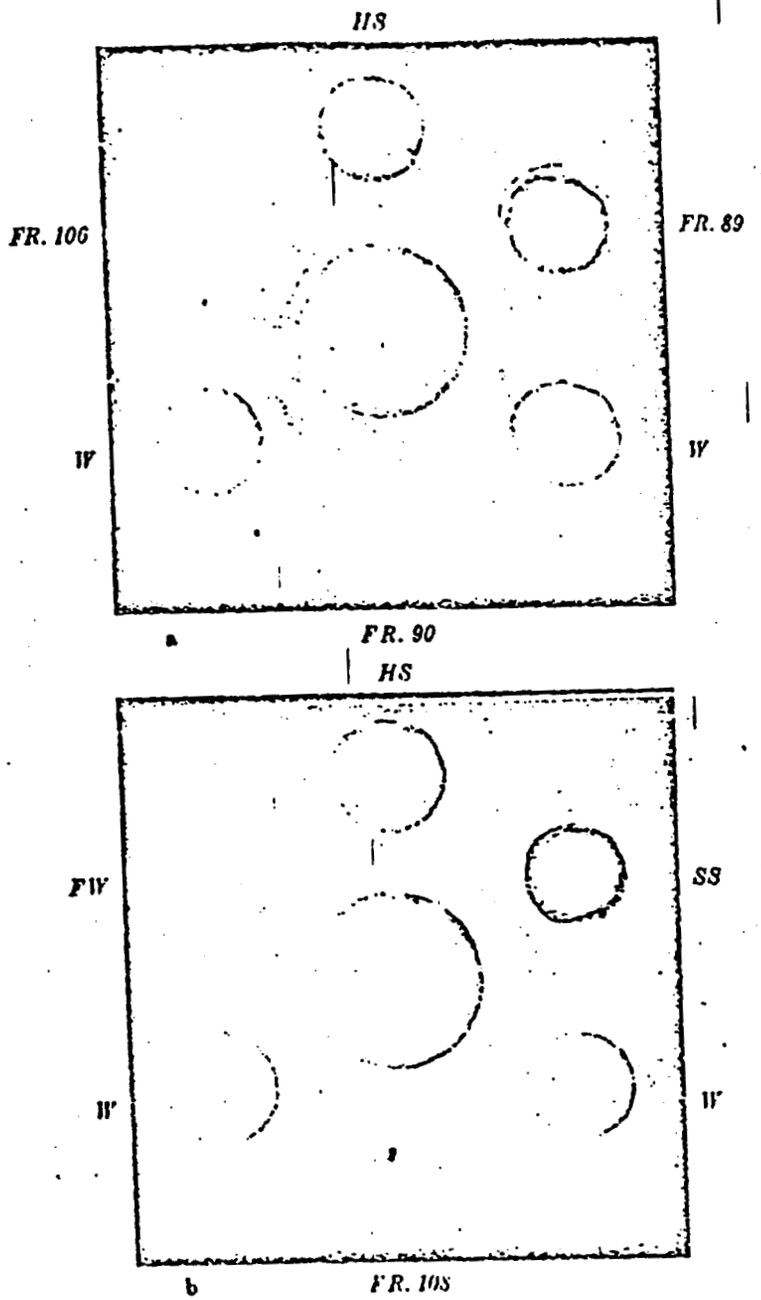
The proof of immunological identity of the umbilical-cord serum antigen with the pregnancy-typical protein of fraction 106 of the placenta is presented in fig. 7. Fractions 89 and 90 are not identical with antigens of the umbilical-cord serum. Fraction 108, in addition to a crossing protein, contains a trace of the umbilical-cord serum antigen. In the four placental fractions, therefore, a protein is contained which is immunologically identical with the pregnancy-typical antigen of the umbilical-cord serum. The bulk of this protein is present in fraction 106, free from other pregnancy-typical proteins.

The occurrence of the pregnancy-typical antigens in the 4 placental fractions is summarized in the table. All 5 of the hitherto described pregnancy-typical antigens (2c) can be isolated from the placenta.





Figs. 4a and 4b. Immunodiffusion. a Antigens: human serum (HS), placenta protein fraction 89 (FR 89), pregnancy serum (SS), placenta protein fraction 90 (FR 90), placenta protein fraction 106 (FR 106). Antiserum: mixture of absorbed antipregnancy and absorbed antiplacenta immune serum (a. ASP). b Antigens: human serum (HS), umbilical-cord serum (NS), pregnancy serum (SS), placenta protein fraction 108 (FR 108), puerperal serum (W). Antiserum: mixture of absorbed antipregnancy immune serum and absorbed antiplacenta immune serum (a. ASP).



Figs. 5a and 5b. Immunodiffusion. a Antigens: human serum (HS), placenta protein fraction 89 (FR 89), puerperal serum (W), placenta protein fraction 90 (FR 90), placenta protein fraction 106 (FR 106). Antiserum: mixture of absorbed antipregnancy and absorbed antiplacenta immune serum (a. ASP). b Antigens: human serum (HS), pregnancy serum (SS), puerperal serum (W), plancenta protein fraction 108 (FR 108), amniotic fluid (FW). Antiserum: mixture of absorbed antipregnancy and absorbed antiplacenta immune serum (a. ASP).

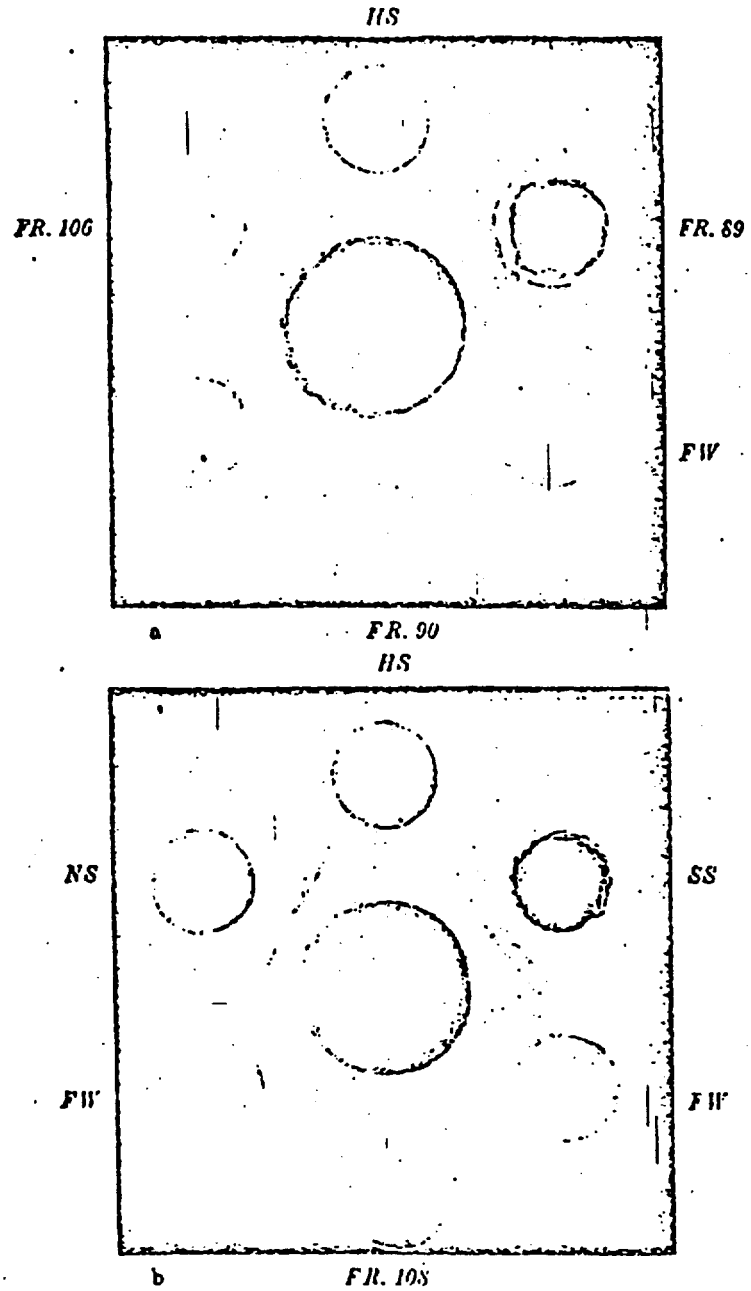


Fig. 6a and 6b. Immunodiffusion. a Antigens: human serum (HS), placenta protein fraction 89 (FR 89), amniotic fluid (FW), placenta protein fraction 90 (FR 90), placenta protein fraction 106 (FR 106). Antiserum: mixture of absorbed antipregnancy and absorbed antiplacenta serum (a. ASP). b Antigens: human serum (HS), pregnancy serum (SS), amniotic fluid (FW), placenta protein fraction 106 (FR 106). Umbilical-cord serum (NS). Antiserum: mixture of absorbed antipregnancy and absorbed antiplacenta immune serum (a. ASP).

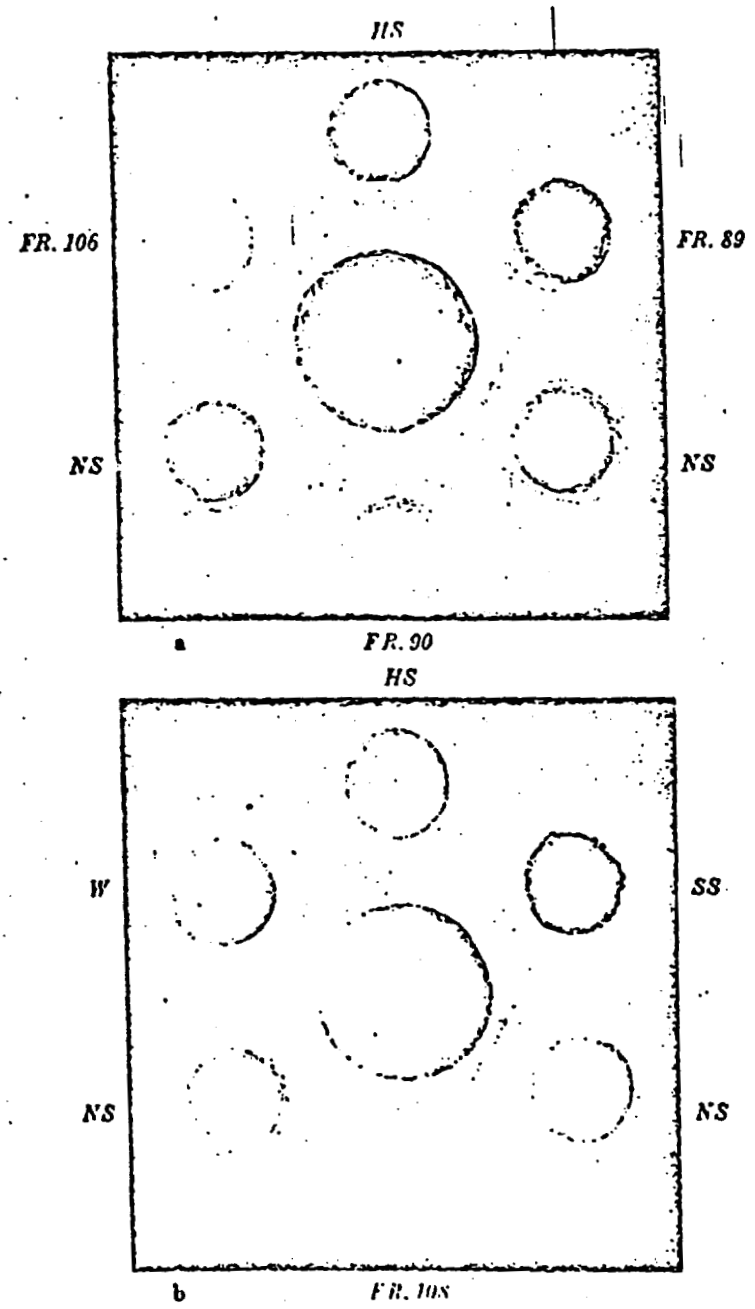


Fig. 7a and 7b. Immunodiffusion. a Antigens: human serum (HS), placenta protein fraction 89 (FR 89), umbilical-cord serum (NS), placenta protein fraction 90 (FR 90). Placenta protein fraction 106 (FR 106). Antiserum: mixture of absorbed antipregnancy and absorbed antiplacenta immune serum (a. ASP). b Antigens: human serum (HS), pregnancy serum (SS), umbilical-cord serum (NS), placenta protein fraction 108 (FR 108), puerperal serum (W). Antiserum: mixture of absorbed antipregnancy and absorbed antiplacenta immune serum (a. ASP).

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Table. Summary concerning occurrence of pregnancy-typical antigens in placenta protein fractions with use of a mixture of absorbed antipregnancy and absorbed antiplacenta immune serum (a. ASP).

Placenta fraction no.	Immune serum	Pregnancy-typical antigen no.				
		1	2	3	4	5
89	a. ASP		+	+		+
90	a. ASP			+		+
106	a. ASP				+	
108	a. ASP	+			+	

By absorption of the immune serum used in nonpregnant serum, all antibodies against normal blood serum proteins were eliminated; so in the present illustrations only pregnancy-typical proteins are precipitated. The blood serum proteins contained in the fractions are not shown. For preparing a monospecific immune serum for the quantitative determination of the pregnancy-typical protein no. 4, placenta fraction 106 is already suitable; the isolation of the other pregnancy-typical antigens from placental fractions 89, 90, and 108 requires further steps of preparation.

The separation of placental proteins by gel chromatography, ion exchange and other preparative steps is suitable for obtaining pregnancy-typical proteins for the preparation of monospecific immune sera.

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OBSTETRICS GYNECOLOGY

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Evaluation of a New Pregnancy Test

Pregnosticon Dri-Dot®

CHARLES A. HORWITZ, MD, MELVIN SINYKIN, MD,
DAVID HILL, MD, ELIZABETH JEROME, MD, and
M. DESMOND BURKE, MD

A new latex agglutination-slide test for pregnancy in which the reagents are incorporated onto the surface of the test slide was evaluated. The test accuracy ranged from 94.68% in pregnant patients (31 to 100 days after the last normal menstrual period) to 98.65% in nonpregnant patients. The advantages and limitations of this latex agglutination-inhibition slide test for pregnancy, which is stable at room temperature, are discussed.

THIS PAPER describes our evaluation of a new pregnancy test (Pregnosticon Dri-Dot®) based on well-known principles of agglutination-inhibition. Unlike most available agglutination–inhibition slide tests for pregnancy, reagents in the Pregnosticon Dri-Dot test are incorporated in dried form onto the surface of the slide—in this case a disposable cardboard slide.

MATERIALS AND METHODS

Group 1. The first study group consisted of 1616 urine specimens collected at random from patients from either Mount Sinai Hospital, attending physician's offices, or the Minneapolis Teenage Medical Service. Of these patients, 724 had uncomplicated pregnancies; 72 had known complications (ectopic pregnancies or spontaneous abortions, threatened, incomplete or complete). Eight hundred ninety-two urine specimens were obtained from nonpregnant female patients (ages 12 to 48) in whom pregnancy had been excluded on clinical grounds, by chart review, or by a review of available endometrial tissue. No specimen was excluded from this study because of proteinuria, hematuria or hyposthenuria.

Group 2. The second study group consisted of 1311 specifically selected urine specimens from males and non-pregnant females in the following categories: a) specimens specifically selected because their protein content could be detected with Labstix® (377), grossly bloody urine (22), or moderate to large amounts of hemoglobin detectable only by Labstix (16); b) there were 64 specimens specifically selected from patients in the early menopause, 33 in late menopause, 54 from patients on oral contraceptives, and 506 from patients on methadone; c) specimens (217) specifically selected from patients with various immunologic disorders in which elevated serum proteins might be expected, including conditions such as rheumatoid arthritis, infectious mononucleosis, primary atypical pneumonia, infectious hepatitis, etc. These specimens were then further subdivided into 42 in which protein was present and 175 in which protein was absent. In addition, 22 specimens were included from patients with multiple myeloma.

Test Procedure

The test procedure was carried out as

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directed by the manufacturer. A positive test for HCG was recorded if no latex particle agglutination was seen at the end of a 2-minute reading period. A negative test result was recorded if agglutination of latex particles was clearly defined within the 2-minute reading period. An *inconclusive* test result was recorded when true agglutination was uncertain. Hemoglobin, protein and urinary pH were determined on each specimen by a simple indicator strip (Labstix®).

Eight hundred and forty-two specimens included in the first study group and 142 specimens from the second study group had been stored for periods of 1 week to 15 months at −10° C were rethawed for test. These frozen urines had been used in a previous pregnancy study of five other immunochemical tests. Prior to inclusion in this study as an incorrect or inconclusive test result, the *validity* of a particular rethawed specimen was reconfirmed by repeating the other tests as indicated. The remainder of the study was performed with fresh urines*. Whenever there were discrepancies between the known clinical diagnosis or other immunochemical test results, the tests were repeated as soon as possible to exclude technical error. When such error was excluded and an incorrect or inconclusive test result seemed likely, follow-up specimens were requested and obtained in most first-study group cases. Follow-up specimens were only used to confirm or exclude pregnancy in particular problem cases.

At least eight different lots of test material were used in this study, including lots D2436, 271134 and 371134. The latter were received in our laboratory in December, 1970; March 1971; and April 1971, respectively. As they were received, portions of each lot were stored at room temperature until they were again used in February 1972. At this time these lots were used on 30 specimens of known HCG content. Fifteen of the 30 specimens tested were from patients known to be pregnant while the other 15 were from nonpregnant patients. Neither hemoglobin nor protein was detectable in the latter specimens with Labstix.

* At least four other agglutination-inhibition pregnancy tests were performed on each of the specimens from this study. The Pregnosticon® tube and Pregnosticon® *liquid* slide tests were performed on most of the problem specimens from this study.

RESULTS

Test accuracy is recorded in Tables 1 to 3 and is based on the number of correct positive or negative results in the particular category under study.

Correct test results were also recorded utilizing the three aforementioned stored lots on the 30 previously described specimens. There were no appreciable differences between endpoints (intensity of latex particle agglutination) when test lot 371134 (received June 1971) was compared with a recently marketed test lot 871134AB (received February 1972).

DISCUSSION

The availability of this latex agglutination-inhibition test for detecting human chorionic gonadotrophin in urine offers certain advantages over other more commonly used test forms: a) it is stable at room temperature and does not require refrigeration for storage; b) the disposable slide, sealed individually in moisture-proof foil envelopes, eliminates the problem caused by residual detergent on glass slides in other latex agglutination-inhibition slide tests; c) the premeasured test reagents incorporated onto the card surface obviate the inherent inaccuracies of dropper pipettes. This change should be a step in the right direction towards eliminating some of the technical errors that can occur in performing slide tests for pregnancy. Such errors can also be attributed to an improper mixing technic.

We confirmed the stability of a small number of Dri-Dot® tests kept in our lab-

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TABLE 1. FALSE-NEGATIVE AND INCONCLUSIVE DRI-DOT TEST RESULTS OBTAINED FROM 724 UNCOMPLICATED AND 72 COMPLICATED PREGNANCIES

Specimen	*No. of specimens*	*False-positive*	*Inconclusive*	*Accuracy** (%)
Total uncomplicated pregnancies	724	33	2	95.17
31–40 days from LNMP	25	16	1	32.00
41–50 days from LNMP	104	8	0	92.31
51–100 days from LNMP	454	5	1	98.68
14½–30 weeks from LNMP	90	0	0	100.00
31–40 weeks from LNMP	51	4	0	92.16
Total complicated pregnancies†	72	14	1	79.17
Threatened or incomplete abortions	58	11	1	79.31
Ectopic pregnancies	14	3	0	78.57

LNMP = last normal menstrual period

* Based on the number of correct positive test results.

† An additional 12 specimens not included in this table were obtained from patients with missed abortions or the dead fetus syndrome; negative test results were obtained in all cases with the Dri-Dot test as well as with all five other immunochemical tests.

oratory at room temperature for periods ranging from 8 to 14 months. Further studies, however, are indicated to determine sensitivity alterations over longer periods.

In the first study group, test accuracy in uncomplicated pregnancies (31 to 100 days after the last normal menstrual period) was 94.68% and in nonpregnancies, 98.65%.

The results from the second study group suggest the need to exercise caution in interpreting Dri-Dot test results on specimens received from patients who are taking methadone. False-positive or inconclusive test results were recorded on 27.9% (141 of 506) of the specimens from patients on methadone. A review of dose records showed that 87.9% (124 of 141) of false-positive or inconclusive test results were obtained with specimens received from patients receiving 100 mg or more of methadone per day. One of the false positive test results in the first study group was recorded on a protein-free specimen from a patient on 130 mg of methadone per day. A warning regarding methadone interference with the Pregnosticon Dri-Dot, however, is incorporated into the test brochure. Methadone has been reported to interfere with other latex agglutination-inhibition slide tests for pregnancy.[1]

Results in the second study group also suggest that Labstix-detectable protein may engender some false-positive tests. Further, of 12 false-positive results among 892 specimens in the first study group, 8 contained Labstix-detectable protein. This problem has also been encountered with other latex agglutination-inhibition slide tests.[2,3]

The reported accuracy of a particular pregnancy test varies considerably with the

TABLE 2. FALSE-POSITIVE AND INCONCLUSIVE DRI-DOT TEST RESULTS FROM 892 SPECIMENS SUBMITTED FROM NONPREGNANT FEMALE PATIENTS (AGES 12–48)

Specimen	*No. of specimens*	*False-positives*	*Inconclusive*	*Accuracy** (%)
Protein and hemoglobin-free	735	4	0	99.46
Proteinuric	111	8	0	92.79
Moderate or large amounts of only hemoglobin detectable by labstix	46	0	0	100.00
TOTAL	892	12	0	98.65

* Based on the number of correct negative test results

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TABLE 3. FALSE-POSITIVE AND INCONCLUSIVE DRI-DOT TEST RESULTS OBTAINED ON 805 SELECTED SECOND STUDY GROUP SPECIMENS

Specimens	*No. of specimens*	*False-positive*	*Inconclusive*	*Accuracy* (%)**
Total proteinuric specimens	377	18	6	93.63
Trace	126	4	0	96.83
1+	132	4	2	95.45
2+	88	5	4	89.77
3+	31	5	0	83.87
Total grossly bloody specimens	22	5	0	77.27
Moderate or large amounts of only hemoglobin detectable by labstix®	16	0	0	100.00
Multiple myeloma	22	1	0	95.45
Early menopause (ages 46–55)	64	0	0	100.00
Late menopause (ages 56–90)	33	0	0	100.00
Patients on oral contraceptives	54	0	0	100.00
Immunologic disorders† (total samples)	217		0	96.77
Protein-free urine	175	3	0	98.29
Urine with protein detectable by labstix®	42	4	0	90.48
Patients on methadone	506	138	3	72.13

* Accuracy based on the number of correct negative test results

† Specimens obtained from patients with diseases such as rheumatoid arthritis, infectious mononucleosis, primary atypical pneumonia, polyclonal gammopathies, etc

source of case material.[4] In the initial phases of this study, many low-titer early pregnancy specimens were received from hospital employees, in contrast to the latter phases of this study when specimens were received primarily from the Minneapolis Teenage Medical Service where patients are encouraged to bring specimens only after they missed two menstrual periods. A review of the test results from this study reflected the source of case material, as the majority of false negatives came from sources at Mount Sinai Hospital who have been encouraged to bring in early specimens for testing.

Although this study was not a comparative pregnancy test evaluation per se, problem specimens were tested by other immunochemical tests. The Pregnosticon Dri-Dot seemed slightly less specific but more sensitive than the liquid form of the Pregnosticon slide test. Neither the Pregnosticon slide tests nor any of the other currently available latex agglutination-inhibition immunochemical slide tests for pregnancy were as accurate as the more sensitive 2-hour Pregnosticon hemagglutination-inhibition tube test in *early* pregnancy.

During the test, occasionally a yellow color persisted on the test slide after the latex Dri-Dot was reconstituted. Despite this rather striking appearance imparted to the test-card, the presence or absence of agglutination could be interpreted without difficulty in all but a few cases. In the latter instances, the tests were repeated using another test card.

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1075735

139