

S. 47,844

712312

888

FORWARDED TO OKO

DATE 8/10/07 BY Law



UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
50125-180	12/15/76	HOLLADAY	

JAMES E. DENNY,
ASSISTANT GEN. COUNSEL FOR PATENT,
FEDERAL ENERGY REG. AGEN. ADMIN.,
WASHINGTON, D. C. 20575

EXAMINER	
PACELSON/A	
ART UNIT	PAPER NUMBER
125	

DATE MAILED: 05-11-02

NOTICE OF ABANDONMENT

This application is abandoned in view of:

1. Applicant's failure to respond to the Office letter, mailed _____.
2. Applicant's letter of express abandonment which is in compliance with 37 C.F.R. 1.138.
3. Applicant's failure to timely file the response received _____ within the period set in the Office letter.
4. Applicant's failure to pay the required issue fee within the statutory period of 3 months from the mailing date of _____ of the Notice of Allowance.
 - The issue fee was received on _____.
 - The issue fee has not been received in Allowed Files Branch as of _____.

In accordance with 35 U.S.C. 151, and under the provisions of 37 C.F.R. 1.316(b), applicant(s) may petition the Commissioner to accept the delayed payment of the issue fee if the delay in payment was unavoidable. The petition must be accompanied by the issue fee, unless it has been previously submitted, the petition fee of \$15, and a verified showing as to the causes of the delay.

If applicant(s) never received the Notice of Allowance, a petition for a new Notice of Allowance and withdrawal of the holding of abandonment may be appropriate in view of *Delgar Inc., V. Schuyler*, 172 U.S.P.Q. 513.

The reason(s) below.

Decision of the PTO Board of Appeals adverse to applicant.

OFFICE OF THE
ASST. GENERAL COUNSEL FOR PATENTS

882 JUN -4 PM 3 31

RECEIVED

Anna P. Fagelson
Primary Examiner
Art Unit 125

PTOL-1432

REPOSITORY Oak Ridge Operations
Records Holding Area
 COLLECTION Documents 81944-1994
 BOX No B-23-8 Bldg. 2714-H
 FOLDER Applications of David W. Holladay
Serial No. 755,100 (70)

1075773

MAILED

JAN 28 1982

Art Unit 125

Paper No. 21

PAT. & T. M. OFFICE
BOARD OF APPEALS

Appeal No. 423-46

rad

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF APPEALS

3 - 47,844
FORWARDED TO ORO
DATE 8/2/83 BY [Signature]

Ex parte David W. Holladay

Application for Patent filed December 28, 1976, Serial No. 755,100. Agent and Method for the Early Detection of Pregnancy.

Allen H. Uzzell et al for appellant.

Before Magil and Merker, 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 12 through 19, all of the claims in the application.

The subject matter on appeal relates to the detection of pregnancy in humans within the first two months after conception through the use of a detection agent containing antibodies to a plurality of pregnancy-specific proteins, said agent's preparation involving the employment of term placental pregnancy-specific proteins. To provide a more detailed description of the detection method, claim 17, the only independent claim, is reproduced as follows:

17. A method of detecting the existence of pregnancy in humans comprising obtaining serum or urine from a patient within about two months of suspected conception, contacting said serum or urine with a pregnancy detection agent containing antibodies to a plurality of pregnancy-specific proteins, and observing whether antibody/antigen reactions occur, the occurrence of at least one antibody/antigen reaction being indicative of pregnancy, said pregnancy detection agent prepared by the method comprising

(a) first providing a mixture of term placental proteins containing antigenic pregnancy-specific proteins;

(b) contacting said mixture of term placental proteins with antibodies raised against either pregnancy-specific proteins or normal human serum proteins to cause said antibodies to react with pregnancy-specific proteins or non-specific antigenic proteins contained in said mixture;

(c) separating the reaction products of step (b) from the remainder of the term placental protein mixture to provide a mixture of antigenic pregnancy-specific proteins isolated from non-specific antigenic proteins;

(d) innoculating a host animal with said mixture of antigenic pregnancy-specific proteins to cause said host animal to raise antibodies to a plurality of pregnancy-specific proteins; and

(e) isolating antibodies to said plurality of pregnancy-specific proteins from antibodies to non-specific human proteins.

As evidence of obviousness, the following references have been cited by the examiner:

Bohn et al (Bohn)	4,065,445	Dec. 27, 1977
Jankowsky (German Offen- legungsschrift)	2,240,327	Mar. 14, 1974
Hofmann et al (Hofmann), <u>Arch. Gynak</u> 208,	187-195	(1969).
Hofmann et al (Hofmann '70), <u>Arch. Gynak.</u>	208,	266-274 (1970).
Lin et al (Lin), <u>Am. J. Obstet. Gynecol.</u> 118,	223-236	(1974).
Brock et al (Brock), <u>Zbl. Gynak.</u> 97,	281-287	(1975).

All of the claims stand rejected for being based upon an insufficient disclosure (35 USC 112, first paragraph). Additionally, all of the claims stand further rejected for being unpatentable (35 USC 103) over Hofmann or Hofmann '70, or over Brock taken with Bohn or Jankowsky.

With respect to the rejection under 35 USC 112, the examiner contends that no specific embodiment is disclosed. A declaration under 37 CFR 1.132 by Dr. John E. Caton has been dismissed as merely setting forth an opinion as to double immunodiffusion, although "the claims are not directed thereto" (Answer, page 4).

Also, the decision in Ex parte Krenzer, 199 USPQ 227 (PTO Bd. App. 1978) has been distinguished on the basis of the relative complexity of the subject matter involved herein.

After considering the examiner's remarks in light of the present disclosure, all of the cited prior art and appellant's arguments, we do not find the rejection based upon 35 USC 112 to be sustainable.

Examples are not needed for everything claimed: In re Anderson, 471 F.2d 1237, 176 USPQ 331 (CCPA 1973). While an application must comply with the disclosure requirements of the statute to be patentable, said disclosure embraces not only what is expressly set forth in words or drawings, but what would be understood by persons skilled in the art. The applicant may begin at the point where his invention begins and describe what he has made that is new and what it replaced of the old. That which is common and well known is as if it were written out in the application: In re Folkers et al, 52 CCPA 1269, 344 F.2d 970, 145 USPQ 390 (1965); In re Howarth, 654 F.2d 103, 210 USPQ 689 (CCPA 1981). Judging the present specification in the manner noted above and giving appropriate weight to Dr. Caton's declaration, we hold that the examiner has failed to support her position. That is, we are aware of no evidence indicating that the present invention is unusually complex and would require an unusually detailed disclosure. On the contrary, with the exception of certain specific variations discussed below, it appears that those skilled in this art were well aware of the preparation and use of the present detection agents. This rejection, therefore, is reversed.

However, we shall sustain the rejection of the claims under Section 103 because we are convinced that the claimed invention would have been obvious from the cited prior art taken as a whole.

Hofmann extracts protein from the placentas of women who have experienced pregnancies of normal term and spontaneous birth (page 10). The author observes that he ultimately obtained a solution "C" which served as innoculating material for the preparation of a

polyvalent antiplacenta sera (page 3). A rabbit was innoculated with the antigenic material and, after checking the antibody titer by the Ouchterlong test and immunophoresis, the author bled the animals and recovered the sera (page 4). After describing other work, Hofmann reports that his "findings prove the existence of several pregnancy-specific antigens" (page 8). While the report fails to state that the polyvalent sera may be used to detect pregnancy within one or two months of conception, such early use is clearly implied by the noted publication item. As acknowledged by appellant, after "2 months, pregnancy is reliably detectable by well-known external symptoms" (specification, page 12, lines 8-9). Accordingly, any useful test would ordinarily be designed for the initial two months of pregnancy to permit prompt prenatal supervision. Compare Bohn, Col. 2, lines 3-11.

At page 9 of the brief appellant asserts that neither "of the primary references describes pregnancy detection." However, one must take into account not only the specific teachings of the references, but also the inferences one skilled in the art would reasonably be expected to draw therefrom: In re Preda, 56 CCPA 706, 401 F.2d 825, ~~4159~~ USPO 342 (1968). See also In re Howarth, supra.

In traversing the rejection, appellant argues that, as shown by his Exhibits 1-3 (accompanying the brief), it would not have been obvious to use antiserum raised against term placental tissue to test for pregnancy within the first two months because the proteins present in a woman's serum and urine are known to vary considerably during the course of her pregnancy (brief, pages 9-10). Nevertheless, we observe that Bohn prepares pregnancy-specific test material from placentas of unspecified periods of pregnancy. Said periods, however, would seem to include both full term (Examples 1 and 3), as well as less than full term (col. 1, lines 16-21 and claims 2 and 8). In this connection, we believe there is good reason to assume that where no period is specified (e.g., Example 1),

full term is involved because less than full term is always identified (e.g., Example 2). Therefore, since Bohn obtains the same pregnancy-specific glycoprotein from all the placentas processed, a person skilled in this art would have reasonably expected that at least more than one of the proteins recovered by Hofmann likewise would be present throughout pregnancy.

A further argument presented by appellant (brief, page 9) is that:

Neither of the primary references isolates the pregnancy-specific protein fraction prior to injection into the host. As pointed out in appellant's specification (page 5, lines 21-32) this step results in an antiserum capable of providing more bands of precipitation in subsequent pregnancy tests because the animal's immune response is directed toward only pregnancy-specific proteins.

Nonetheless, appellant has acknowledged that workers had already determined that some of the proteins recoverable from placentas were non-specific for pregnancy. See page 2 of the instant specification. Moreover, Hofmann '70 eliminates all normal blood serum proteins and recognizes that more than one pregnancy-typical antigen is present in placental fractions. Merely eliminating the non-specific proteins prior to injection into the host, instead of afterwards, by employing an old technique in this art, would have been well within the expertise of a person having only routine skill in the instant field.

Appellant has argued the patentability of claim 18 separately, urging that the use of the pregnancy test within about one month of conception would have been unobvious. While it would have been anticipated that such a test would be less reliable during the first month of gestation, as indicated by appellant's Exhibit I, the two examples at page 15 of the present specification are statistically inadequate to establish unexpectedly better results. Compare In re Boesch, 617 F.2d 272, 205 USPQ 215 (CCPA 1980). Additionally, it will be observed that in said two examples the time since conception

Appeal No. 423-46

was necessarily estimated and was three or four weeks after conception. This further reduces the weight to which such evidence might be entitled.

Accordingly, for the reasons discussed above, the examiner's decision rejecting claims 12 through 19 is affirmed.

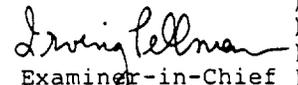
AFFIRMED



Examiner-in-Chief)



Examiner-in-Chief)



Examiner-in-Chief)
(Acting))

BOARD
OF
APPEALS

James E. Denny
U.S. Dept. of Energy
Mail Station CXXI A2-3018 GTN
(GC-42)
Washington, D.C. 20545

S - 47444
FORWARDED TO OKD
DATE 8/26/76 BY z



**U.S. DEPARTMENT OF COMMERCE
Patent Office**

Address Only: COMMISSIONER OF PATENTS
Washington, D.C. 20231

Paper No. 20

Fageelson Art Unit 125
755,100 2/28/76
Daived W. Holladay

MAILED

AUG 27 1980

GROUP 120

James E. Denny
Assistant Gen. Counsel for Pats.
U. S. Energy Res. & Dev. Admin.,
Washington, D. C. 20545

This is in response to the communication re the Power of Attorney filed July 4 1980

assignee.

1. The power of attorney to you in this application has been revoked by the applicant.
2. In view of the notice in this application of the death of _____ his power of attorney is terminated.
3. The power of attorney to you in this application has been accepted by the Commissioner of Patents.

Mary Hawthorne
For Director, Operation

S. 47,844



U.S. DEPARTMENT OF COMMERCE
Patent Office

FORWARDED TO ORO

Address Only: COMMISSIONER OF PATENTS
Washington, D.C. 20231

DATE 8/2/76 BY law

Paper No. 20

Fageelson
755,100
Daived W. Holladay

Art Unit 125
2/28/76

MAILED

AUG 21 1980

GROUP 120

James E. Denny
Assistant Gen. Counsel for Pats.
U. S. Energy Res. & Dev. Admin.,
Washington, D. C. 20545

This is in response to the communication re the Power of Attorney filed _____

[REDACTED]

3. The power of attorney to you in this application has been accepted by the Commissioner of Patents.

[REDACTED]

4. The assignee in this application has intervened and appointed an attorney of his own selection. Further correspondence will be held with said attorney. (Rule 36, Rules of Practice.)

5. The revocation of the power of attorney to _____ has been entered and said attorney has been notified. Further correspondence will be addressed to you.

6. On _____, the applicant appointed _____ assignee as additional attorney in this application. Further correspondence will continue to be addressed to you as specified in the new power of attorney.

7. On _____, the applicant appointed _____ assignee as additional attorney in this application. Further correspondence will be addressed to said attorney. MPEP 4

8. The associate power of attorney to you in this application has been revoked by the attorney of record.

James E. Denny
U. S. Dept. of Energy
Mail Station CXXI A2-3018
GTN (GC-42)
Washington, D. C. 20545

Mary Hawthorn
For Director, Operation

'S - 47,844

James E. Denny
Assistant General Counsel for Patents
United States Department of Energy
Washington, D. C. 20545

FORWARDED TO ORR

DATE 8/20/76 BY law

Applicant :	David W. Holladay)	
Serial No.:	755,100)	
Filed :	December 28, 1976)	GROUP 120
For :	AGENT AND METHOD FOR THE EARLY DETECTION OF PREGNANCY)	



Receipt is acknowledged of the following for the above-identified case:

- | | |
|---|---|
| <input type="checkbox"/> Response to Office Action dated _____ | |
| <input type="checkbox"/> Letter to Draftsman | |
| <input type="checkbox"/> Affidavit/Declaration | <input type="checkbox"/> Appeal Brief |
| <input type="checkbox"/> Fee Authorization | <input type="checkbox"/> Amendment under Rule 312 |
| <input type="checkbox"/> Application with <input type="checkbox"/> Sheets Formal Drawings | <input type="checkbox"/> Petition |
| <input type="checkbox"/> Disclosure u/37 CFR 1.56 | <input type="checkbox"/> Directive u/Sec. 152 |
| <input type="checkbox"/> PTOL-85b Base Issue Fee | <input type="checkbox"/> Withdrawal of Directive |
| <input type="checkbox"/> PTO-1094 with <input type="checkbox"/> Sheets Formal Drawings | <input type="checkbox"/> _____ |
| <input type="checkbox"/> Notice of Appeal | <input type="checkbox"/> _____ |
| <input checked="" type="checkbox"/> Appointment of Attorney | <input type="checkbox"/> _____ |
| <input checked="" type="checkbox"/> Confirmation of Request for Oral Hearing _____ | <input type="checkbox"/> _____ |



1075182

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : David W. Holladay :
Serial No.: 755,100 : APPEAL No. 423-46
Filed : December 28, 1976 : GROUP 120
For : AGENT AND METHOD FOR : Examiner: A. Fagelson
THE EARLY DETECTION OF :
PREGNANCY :
.....

APPOINTMENT OF ATTORNEY

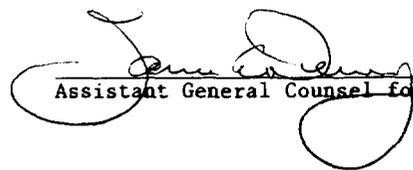
The Commissioner of Patents and Trademarks
Washington, D. C. 20231

Sir:

The undersigned assignee of the entire right, title and interest in and to the above-identified application by virtue of an assignment to the United States Government as represented by the United States Department of Energy, hereby revokes any and all previous powers of attorney in the above-identified application. The assignment was forwarded to the Patent and Trademark Office for recordation February 11, 1977, recorded in Reel 3389, Frame 810 on February 16, 1977.

And the assignee hereby appoints James E. Denny, Registration Number 18863; Richard G. Besha, Registration Number 22770; Jack Q. Lever, Jr., Registration Number 28149; Michael F. Esposito, Registration Number 29506; and Stephen D. Hamel, Registration Number 22220, for whom the address is United States Department of Energy, Mail Station CXXI A2-3018 GTN (GC-42), Washington, D. C. 20545 its attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. All communications are to be addressed to James E. Denny at the above address.

UNITED STATES GOVERNMENT AS REPRESENTED
BY THE UNITED STATES DEPARTMENT OF ENERGY


Assistant General Counsel for Patents

Dated: AUG 1 1977

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : David W. Holladay :
: APPEAL No. 423-46
Serial No.: 755,100 :
: GROUP 120
Filed : December 28, 1976 :
: Examiner: A. Fagelson
For : AGENT AND METHOD FOR :
: THE EARLY DETECTION OF :
: PREGNANCY :
.....

CONFIRMATION OF REQUEST FOR ORAL HEARING

The Commissioner of Patents and Trademarks
Washington, D. C. 20231

Sir:

In accordance with the NOTICE OF HEARING (Paper No. 17) from the Patent and Trademark Office dated July 17, 1980, with respect to the above-identified application, Applicant hereby confirms his request for an oral hearing as scheduled at 9:00 a.m. on November 17, 1980.

Respectfully submitted,



Attorney for Applicant

Dated: AUG 1 1980

Germantown, Maryland

353-5093



**U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office**

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

S. 47,844

FORWARDED TO CRC

DATE 7/22/80 BY JL

Paper No. 17

James E. Denny
Asst. Gen. Counsel For Pat.
U. S. Energy Res. & Dev. Admin.
Washington, DC 20545

Mailed: July 17, 1980
Appeal No: 423-46
Appellant: David W. Holladay
Serial No: 755100

Hearing
Docket: A
Date: Nov. 17, 1980
Time: 9:00 A.M.
Place: Room 11C28 CP2
2011 Jefferson Davis Hwy.
Arlington, Va.

**NOTICE OF HEARING
CONFIRMATION REQUIRED WITHIN TWENTY-ONE DAYS**

Your attention is directed to 37 CFR 1.194(s); July 1, 1977.

The appeal in the above identified case will be heard by the Board of Appeals on the date indicated. Hearings will commence at the time set and as soon as the argument in one case is concluded, the succeeding case will be taken up.

The time allowed for argument is twenty minutes unless additional time is requested and permitted before the argument is commenced.

CONFIRMATION OF THE REQUEST FOR ORAL HEARING, WHICH HAS BEEN SET AS NOTED ABOVE, IS REQUIRED WITHIN TWENTY-ONE DAYS FROM DATE OF THIS NOTICE.

NOTE: Failure to confirm will be construed as a waiver of the request for the hearing. However, the courtesy of actual notification that the request for hearing is being waived will enable the Board to make the most effective use of its hearing facilities.

By Order of the Board of Appeals.

Clerk of Board
(703) 557-3393

Please refer to Appeal Number in all communications concerning this case.

Form PTOL-38 (rev. 10-77)

1075785

S. D. Hanel

James E. Denny
Assistant General Counsel for Patents
United States Department of Energy
Washington, D. C. 20545

Applicant : David W. Holliday)
Serial No. : 759,100)
Filed : December 28, 1976) GROUP 120
For : METHOD AND MEANS FOR THE PROTECTION)
OF PREGNANCY)

Receipt is acknowledged of the following in the above-identified application:

- | | |
|--|---|
| <input type="checkbox"/> Response to Office Action dated _____. | |
| <input type="checkbox"/> Letter to Draftsman | |
| <input type="checkbox"/> Affidavit | <input type="checkbox"/> Petition |
| <input type="checkbox"/> Notice of Appeal | <input type="checkbox"/> Appeal Brief |
| <input type="checkbox"/> Fee Authorization | <input type="checkbox"/> Amendment under Rule 312 |
| <input type="checkbox"/> Appointment of Attorney | <input type="checkbox"/> Directive |
| <input type="checkbox"/> PTOL-85b Base Issue Fee | |
| <input type="checkbox"/> PTO-1094 with <input type="checkbox"/> Sheets Formal Drawings | <input type="checkbox"/> Withdrawal of Directive |
| <input checked="" type="checkbox"/> Reply Brief in triplicate | <input type="checkbox"/> _____ |
| <input type="checkbox"/> _____ | <input type="checkbox"/> _____ |

9-6-79

1075786

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: David W. Holliday :
SERIAL NO.: 755,100(70) : GROUP 120
FILED: December 28, 1976 : EXAMINER
A. Fagelson
FOR: AGENT AND METHOD FOR THE EARLY
DETECTION OF PREGNANCY

REPLY BRIEF TO EXAMINER'S ANSWER

The Commissioner of Patents & Trademarks
Washington, D. C. 20231

Sir:

This is a Reply Brief to the Examiner's Answer of August 21,
1979.

THE NEW ARGUMENTS

In the Examiner's Answer, the following arguments were made for
the first time:

- (a) The Declaration of Dr. John E. Caton is insufficient because
it refers to a double immunodiffusion test not recited in the
claims.
- (b) The claim limitations that the pregnancy detection agent
contains antibodies to a plurality of term placental antigens
are entitled to no weight because the specification did not
disclose the "criticality" of term placentas or the problem
of false results in tests capable of detecting only a single
antigen.

The Examiner's first new argument is without merit. It is clear
that the tests described in the specification at page 14, line 31
through page 16, line 11, were Ouchterlony double immunodiffusion tests
as described in Dr. Caton's Declaration. Note that the immunological
tests using early pregnancy sera were described as double diffusion

1075787

tests (page 15, line 21) and that plates were used as diffusion medium (page 15, line 5). The Examiner has failed to challenge the facts set forth in Dr. Caton's Declaration and has failed to identify any aspect of the claimed method which is not either well known or readily ascertainable by those of ordinary skill in the art with a minimal amount of routine testing.

The Examiner would limit the claims to the specific double diffusion method of the example, or else require a detailed example of each of the myriad methods by which workers in the art routinely carry out immunological tests. Such examples would be redundant and are clearly not required where, as here, applicant has provided a novel immunological pregnancy detection agent which can be used by any of the well-known methods of carrying out antibody/antigen reactions.

The Examiner's distinction of the present case from Ex parte Krenzer is illusory. It is not the step of contacting the serum or urine with the pregnancy detection agent which is complex. This step is simple and is performed routinely by workers in the art as shown by the art of record. Any complexity in the claimed method lies in the preparation of the detection agent, which is described in the minutest detail. The Examiner has failed to specify a single deficiency in the specification which would prevent one of ordinary skill in the art from carrying out the claimed method.

In the second new argument, the Examiner takes the position that specific claim limitations need not be considered because broader language in the specification described certain of the limitations as preferred. This approach is clearly improper and would eliminate

applicant's right to retreat to an otherwise patentable species merely because he thought he was first with the genus. In re Johnson 558 F.2d 1008, 194 USPQ 187 (CCPA 1977); In re Saunders 444 F.2d 599, 170 USPQ 213 (CCPA 1971).

It is immaterial that the specification does not discuss the problems of false negatives prevalent in prior art tests based on a single antigen. This problem is well known in the art and fully documented in the record. Again the Examiner is disregarding the specific claim limitations that a plurality of pregnancy specific antibodies are present and improperly rejecting the claims based on broader language in the specification.

There is no need for a showing of criticality for the use of term placentas as opposed to first month placentas of Jankowsky because the use of term placentas is not prima facie obvious. Even if the Jankowsky method had been fully equivalent to the claimed method, the rejection would be improper because such equivalency was not appreciated in the prior art. If the prior art had considered early pregnancy detection using antibodies to a mixture of term placental proteins to be obvious, then:

- (a) Jankowsky would not have limited his patent to the use of scarce first month placentas;
- (b) the prior art pregnancy tests would not have been based on a single antigen which was known to cause false negatives due to low concentration in early pregnancy; and
- (c) the workers detecting a plurality of term placenta antigens in late pregnancy sera would have suggested their use for early pregnancy detection.

Since the prior art failed to suggest or recognize the clear advantages of the use of a mixture of term placental pregnancy specific antibodies, there is no need for the claims to be limited to the antibodies and antigens used in the examples, especially where any contemplated variation in antibody content is well within the skill of the art.

In view of the above and the arguments previously submitted, the Examiner's rejections under 35 USC 103 and 35 USC 112 are improper and should be overruled.

Respectfully submitted,

LSIAHU
Attorney for applicant

Oak Ridge, Tennessee

Uzzell (FTS-626-1076) (Commercial 615-576-1076)

August 31, 1979

All communications respecting this application should give the serial number, date of filing and name of the applicant.



PAPER NO. _____

**UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office**

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D. C. 20231

A. Fagelson Art Unit 125
12-28-76 755,100
David W. Holladay]

Before the Board of Appeals

James E. Denny
Assistant Gen. Counsel For Pats.
U. S. Energy Res. & Dev. Admin.
Washington, D. C. 20545

MAILED

James E. Denny for Appellant

AUG 21 1979

GROUP 120

Examiner's Answer

This is an appeal from the final rejection of claims 12-19, all of the claims in application.

A correct copy of the claims appears on page 3 of appellant's brief.

The objection to the specification is not^{an} appealable matter for consideration of the PTO Board of Appeals; therefore it will not be presented herein.

THE REFERENCES OF RECORD RELIED ON:

4065445	Bohn et al.	12/1977
2240327	(German) Jankowsky	3/1974
Hofman (1)	<u>Arch. Gynak.</u> , Vol. 208, 1970 pp. 266-274	
Hofman (2)	<u>Arch. Gynak.</u> , Vol. 208, 1969 pp. 187-195	
Lin (1)	<u>Amer. J. Obst. & Gyn.</u> Vol. 118, 1974 pp. 223-236	
Brock	<u>Zentralblatt fuer Gyn.</u> Vol. 97 1975 pp. 281-287	

A BRIEF DESCRIPTION OF THE REFERENCES:

The patent to Bohn et al. shows the isolation of a pregnancy specific β_1 -glycoprotein from placenta, or blood or urine of pregnant women. Antisera may be prepared thereto by the conventional means. Such sera can be employed as an adsorbent in the isolation and purification of the β_1 -glycoprotein from serum or urine, or the antisera may be employed in various tests for pregnancy (column 2, paragraph 1). Phosphate buffers, etc. may be employed in the extracting medium. (column 2, lines 12-25) When the protein is isolated from placenta, it is precipitated from the aqueous extract with inorganic salts such as ammonium sulfate or sodium sulphate (column 3, paragraph 1). The pregnancy specific β_1 -glycoprotein of Bohn et al. is stated to be 90-95% pure (Example 4, column 7).

Jankowsky teaches the preparation of extracts from placenta and mixing same with human chorionic gonadotropin for use as an antigen in the preparation of antisera that is employed in early detection method for pregnancy.

Hofman (1 or 2) shows the isolation of pregnancy specific antigens from placenta by extracting same with aqueous solutions which include phosphate buffer. The proteins are then precipitated from the supernatant with ammonium sulfate. The precipitated proteins are inoculated

Art Unit 125

into animals such as rabbits. The antisera from the rabbit blood is absorbed with normal blood serum which eliminates the antibodies against the normal protein (translation page 11, article pub. 1970 section "IV Demonstration of Pregnancy - Typical Antigens in Proteins of Human Placenta"). The antisera may be employed in detection of antigens in pregnancy serum, or other studies.

Lin shows the preparation of antisera in rabbits against third trimester human pregnancy plasma. The immunoglobulins were salted ^{out} and employed in testing for various pregnancy associated antigen. This reference is deemed cumulative and will not be maintained.

Brock shows the extraction of placenta with phosphate buffer, precipitation of the proteins with ammonium sulfate, immunization of animals with the extracted protein, absorption of the antisera with human serum. Some proteins were found against pregnancy serum as well as placental tissue and fetal serum depending on the on the antigens employed in further absorption studies.

THE REJECTION

The claims stand rejected under 35 USC 112 (paragraph 1) as being based upon an insufficient disclosure for reasons of record. No specific embodiment is noted with respect to any specific method of "detecting the existence of pregnancy in humans", to which the claims

Art Unit 125

are directed. The declaration of Caton under 37 CFR 1.132 is deemed to merely set forth an opinion that he would be able to conduct "double immunodiffusion". However, the claims are not directed thereto and the disclosure only indicates "immunodiffusion" (page 12, paragraph 1). ^{194 USPQ 227} Ex part Krenzer, cited by appellants, relates to the use herbicides and not the development of parameters and conditions for the performance of a test involving a complex system of reagents. Appellants allege their proportion of antigens and sera to somehow differ in kind from the work of others. This is impossible to so determine since it is not known how appellants determined the alleged unexpected results.

The claims are further rejected under 35 USC 103 as obvious over Hoffmann 1 or 2, or Brock considered with Bohn or Jankowsky. No patentable merit is see in securing the proteins to pregnancy by the means known in the art or in removing undesired antigens or antibodies depending on the purification or absorption procedures. The antigen of Bohn, for example, is noted to be relatively pure. It is not known, however, what antigen or antigens appellants are concerned with since the purification means employed may give different end results. It is noted, however, in the specification page 4 various sources may be employed for the pregnancy - specific proteins. Nevertheless the specification page 4, lines 27-29 states that "while it has been found that there

Art Unit 125

are normally several antigenic pregnancy - specific proteins present in pregnancy - associated material, only one need be present for purposes of the invention" (underscoring added). Thus, appellants allegations, for example, on page 10, paragraph 2 of the Brief, that "a single protein produce false negatives" is clearly not supported by the specification. It is also noted on page 3 of the specification lines 17-18 and page 4, lines 22, that the ^{protein} source of "placentas" is merely perferred and not indicated to be critical. Also the only mention of "term placenta" appears on page 12 of the specification line 1 of Example II. This too is not indicated to be a critical matter. Thus, it is not seen that the claimed diagnostic method is rendered patentable particularly in view of the facts 1) that appellants ^{in ject} contend that such method would be within the purview of the routineer in the art; 2) that the proteins employed in the preparation of antisera for such tests are known and 3) that tests for pregnancy employing antisera to placental extracts are known.

For the reasons above set forth the final rejection is deemed to be proper and should be sustained.

AFagelson:fpb

(703) 557-2577

6-16-79

AFagelson
ANNA P. FAGELSON
EXAMINER
ART UNIT 125

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF APPEALS

In the application of :
DAVID W. HOLLADAY : APPEAL NO. _____
Serial No.: 755,100 : GROUP ART UNIT 125
Filed : December 28, 1976 : EXAMINER Anna P. Fagelson
For : AGENT AND METHOD FOR EARLY :
DETECTION OF PREGNANCY :
..... :

BRIEF ON APPEAL

<u>Title</u>	<u>Page Number</u>
Contents.....	1
Brief on Appeal.....	2
Oral Hearing.....	2
Claims on Appeal.....	2
Description of the Claims on Appeal.....	3
The Invention.....	4
The Rejections.....	5
The Teachings of the Prior Art.....	5
The Arguments.....	7
A. The Rejection of Claims 12-19 under 35 USC 112 Is Improper Because the Specification Is Sufficient To Enable One of Ordinary Skill in the Art to Practice the Invention without Undue Experimentation.....	7
B. The Teachings of the Prior Art As a Whole Fail to Suggest Or Render Obvious the Invention As Claimed in Claims 12-17, and 19.....	9
C. Teachings of the Prior Art As a Whole Fail to Suggest Or Render Obvious the Invention As Claimed in Claim 18.....	11
Summary.....	11
Appendix	
Declaration of Dr. John E. Caton	
Exhibit 1	
Exhibit 2	
Exhibit 3	

BRIEF ON APPEAL

This case comes before the Board of Appeals because of the Final Rejection of claims 12-19, all of the claims remaining under consideration in the application.

ORAL HEARING

Applicant requests that an oral hearing be granted in this case.

CLAIMS ON APPEAL

17. A method of detecting the existence of pregnancy in humans comprising obtaining serum or urine from a patient within about two months of suspected conception, contacting said serum or urine with a pregnancy detection agent containing antibodies to a plurality of pregnancy-specific proteins, and observing whether antibody/antigen reactions occur, the occurrence of at least one antibody/antigen reaction being indicative of pregnancy, said pregnancy detection agent prepared by the method comprising

(a) first providing a mixture of term placental proteins containing antigenic pregnancy-specific proteins;

(b) contacting said mixture of term placental proteins with antibodies raised against either pregnancy-specific proteins or normal human serum proteins to cause said antibodies to react with pregnancy-specific proteins or non-specific antigenic proteins contained in said mixture;

(c) separating the reaction products of step (b) from the remainder of the term placental protein mixture to provide a mixture of antigenic pregnancy specific proteins isolated from non-specific antigenic proteins;

(d) innoculating a host animal with said mixture of antigenic pregnancy-specific proteins to cause said host animal to raise antibodies to a plurality of pregnancy-specific proteins; and

(e) isolating antibodies to said plurality of pregnancy-specific proteins from antibodies to non-specific human proteins.

12. The pregnancy detection method of claim 17 in which step (b) comprises contacting said mixture of term placental proteins with antibodies raised against pregnancy-specific proteins.

13. The pregnancy detection method of claim 17 in which step (b) comprises contacting said mixture of term placental proteins with antibodies raised against normal human serum proteins.

14. The pregnancy detection method of claim 17 in which said mixture of term placental proteins is prepared by providing a suspension of term placental tissue in a stabilizing medium, separating suspended solids from said suspension to provide a supernatant solution, adding a salting agent to said supernatant solution to precipitate proteins and dissolving said precipitated proteins to provide said mixture of term placental proteins.

15. The pregnancy detection method of claim 14 in which said stabilizing medium is selected from the group of phosphate buffer and perchloric acid and said salting agent is selected from the group of ammonium sulfate and sodium sulfate.

16. The pregnancy detection method of claim 14 in which said stabilizing medium is phosphate buffer and said salting agent is ammonium sulfate.

18. The method of claim 17 in which said serum or urine is obtained from said patient within about one month of suspected conception.

19. The method of claim 17 in which said method for preparing said pregnancy detection agent further comprises isolating antibodies to said plurality of pregnancy-specific proteins from non-antibody host animal serum proteins.

DESCRIPTION OF THE CLAIMS ON APPEAL

Claim 17 is directed to a method for detecting early pregnancy in which a patient's serum or urine is contacted with an agent containing antibodies to a plurality of proteins specific to pregnancy. The antibody

mixture is produced by inoculating a host animal with a mixture of pregnancy-specific proteins obtained from term placentas and isolated from non-specific antigens.

Dependent claims 12-16 are directed to the pregnancy test of claim 17 employing agents prepared by different methods. Claims 12 and 13 are directed to the use of agents prepared using alternate methods for separating pregnancy-specific placental proteins from non-specific placental proteins prior to inoculation of the host. Claim 14 is directed to the use of an agent prepared by precipitating placental proteins from a tissue suspension prior to isolation of pregnancy-specific placental proteins. Claim 15 is directed to the use of an agent prepared using particular stabilizing media and salting agents. Claim 16 is directed to the use of an agent prepared using the specific stabilizing medium and salting agent exemplified in the specification.

Claim 18 is directed to the use of the pregnancy test of claim 17 for detection of pregnancy within about one month of conception.

Claim 19 is directed to the pregnancy test of claim 17 employing an agent in which the useful antibodies to pregnancy-specific proteins are isolated from non-antibody host animal proteins.

THE INVENTION

The invention is a novel method for early detection of pregnancy by the occurrence of characteristic antibody/antigen reactions. A patient's blood or urine is contacted with a detection agent comprising host-produced antibodies to a plurality of pregnancy-specific tissue proteins occurring in term placentas. Appellant has demonstrated that such an antibody mixture contains antibodies to a plurality of pregnancy-specific proteins which are present at readily detectable concentrations in the very early stages. Consequently, the test is effective for detecting early pregnancy in women whose serum or urine contains any of the several pregnancy-specific placental proteins.

The agent is prepared by inoculating a host animal with a mixture of pregnancy-specific proteins isolated from the other placental antigens.

By isolating the pregnancy-specific mixture prior to inoculation, the animal's immune response is directed toward the proteins of interest. These host-produced antibodies are isolated from antibodies to non-specific human protein, thereby providing a detection agent sensitive to a plurality of serum proteins expressed in the very early stages of pregnancy.

The capability for detecting a plurality of early pregnancy proteins is a considerable improvement over prior art techniques employing antibodies to a single protein, for example human chorionic gonadotropin (HCG). Such tests were prone to false negative results, particularly in early pregnancy, due to low serum levels of the single protein. In the method of this invention positive readings occur when any of several pregnancy-specific proteins are detected. If a plurality of pregnancy-specific proteins are detected, the positive result is confirmed. Reliable positive readings showing a plurality of pregnancy-specific proteins have been obtained as soon as 18 to 22 days after conception.

THE REJECTIONS

Claims 12-19 stand rejected under 35 USC 112 (paragraph 1) as based upon an insufficient disclosure. The specification is objected to as including insufficient exemplary matter to support the claims and for failure to disclose the best mode. Claims 12-19 also stand rejected under 35 USC 103 as obvious in view of the teachings of Hoffman (1), Hoffman (2), Brock, or Lin (1), either singly or in combination with the Bohn patent or Jankowsky. A number of other references have been cited during the prosecution but are not applied against the present claims.

THE TEACHINGS OF THE PRIOR ART

Hoffman (1) describes immunological studies which show that placental tissue contains pregnancy typical proteins also found in puerperal (after childbirth) serum, amniotic fluid, and serum from an unspecified period of pregnancy.

Hoffman (2) describes the preparation of antiplacenta immune serum. The host animal was inoculated with the entire spectrum of placental

proteins without prior separation of pregnancy-specific proteins. Immunological studies using the immune serum found proteins in serum of unspecified periods of pregnancy. The pregnancy proteins were immunologically identical to placental proteins. It was postulated that the pregnancy serum protein had passed over to the mother from the placenta.

Brock describes work similar to that described in Hoffman (1) and (2). Some placental antigens were detected in pregnancy serum, however the period of pregnancy was undisclosed.

Lin (1) discloses that pregnancy associated antigens in third trimester pregnancy serum can be detected with immune serum raised against third trimester pregnancy serum. No placental proteins are described.

The Bohn patent (U. S. Patent 4,065,445) describes the isolation of a single pregnancy-specific protein from placenta, blood, or urine of pregnant women. The protein is said to be useful for the preparation of antiserum for the detection of pregnancy. Any antisera raised against such a protein could detect only that particular protein.

Jankowsky describes a pregnancy detection agent comprising antibodies raised against a mixture of HCG and an extract of first month placental tissue. There is no suggestion that a mixture of pregnancy detection antibodies could be obtained from placental tissue of a later pregnancy period than the period to be detected.

Bell (cited by appellant) describes an evaluation of several prior art immunological pregnancy detection methods. It is seen from Table I that the highest percentages of false negatives or inconclusive results occur when tests are performed 35-49 days after the last menstrual period.

Lamb (cited by appellant) describes an evaluation of prior art immunological pregnancy tests based solely upon HCG. In Fig. 1 (attached hereto as Exhibit 1) it is seen that patients' levels of HCG differ widely and are strongly dependent upon the week of pregnancy.

Dietrich also describes pregnancy tests based upon HCG. Fig. 1 (attached hereto as Exhibit 2) shows that the HCG level in pregnancy

urine increases rapidly from 40-60 days after the last menstrual period and then declines during the remainder of the pregnancy.

Bohn (Blut, Band XXIV, Seite 292-302, 1972) describes a pregnancy-specific beta-glycoprotein. Fig. 7 (attached hereto as Exhibit 3) shows the variation in the concentration of this material during pregnancy.

Seppala describes a study of human placental lactogenic hormone (HPL) in an effort to correlate HPL levels to infant birth weight and to various pregnancy complications. The serum concentration of HPL is said to rise throughout pregnancy and disappear rapidly after birth.

THE ARGUMENTS

- A. The Rejection of Claims 12-19 under 35 USC 112 Is Improper Because the Specification Is Sufficient To Enable One of Ordinary Skill in the Art to Practice the Invention without Undue Experimentation.

In rejecting the claims under 35 USC 112, the Examiner has alleged that the specification is insufficient for failure to contain specific examples of the test methods employed. Such examples are alleged to be necessary in order to disclose the best mode. The Examiner's position is contrary to several decisions of the Patent Office Board of Appeals and the Court of Customs and Patent Appeals which have held that working examples are never required if the invention is otherwise disclosed sufficiently to enable one skilled in the art to practice it without undue experimentation. See, for example, In re Borkowsky 57 CCPA 946, 422 F2d 904, 164 USPQ 642 (CCPA 1970); Ex Parte Krenzer 199 USPQ 227 (P.T.O. Bd. App. 1978).

The present specification is clearly enabling. The claimed method requires simply contacting a sample of blood or urine with a pregnancy detecting agent containing antibodies and observing whether an antibody/antigen reaction occurs. The preparation of the detection agent is described in general terms beginning at page 4, line 17 and continuing through page 9. Example I on pages 10 and 11 describes the preparation of an immunoabsorbent column for preparing the agent. Example II describes a step-by-step procedure for preparing a placenta

extract separating pregnancy-specific proteins and preparing an immune serum. The test method itself is described at page 11, line 20 through page 12, line 6. Gel precipitation tests indicating the duration of the tests and the times of which precipitin bands occurred are described at page 14, line 31 to page 16, line 11. Further exemplification is not necessary. The art of record demonstrates that the entire science of immunology is based upon conducting antibody/antigen reactions.

The Declaration of Dr. John E. Caton further establishes the sufficiency of the specification. The use of declarations under 37 CFR 1.132 to establish the level of skill in the art is in accordance with In re Doyle 482 F2d. 1385, 179 USPQ 227, cert. den. 181 USPQ 417. Dr. Caton pointed out that workers of ordinary skill in the field of immunology and immunoanalytical chemistry are well acquainted with the standard immunological methods for using antibodies to detect the presence of antigens in solutions. If such a worker were given a solution known to contain antibodies to proteins specific to pregnant women, he could easily test serum or urine for the presence of the proteins by the well-known Ouchterlony technique. Such workers often must determine the proper concentration of an antibody solution to use in such tests, and Dr. Caton described a well-known routine method of determining the concentration.

The Examiner has specified no other alleged deficiencies in the specification. Where failure to include a specific operating condition in a specification presents no more than a routine problem to a worker in the art, the specification cannot give rise to a rejection under either the enabling or the best mode requirements of 35 USC 112. In re Karnofsky, 55 CCPA 940, 390 F2d. 994, 156 USPQ 682 (1968) Since a worker of ordinary skill in the art could practice the invention as claimed with only a minimal amount of routine experimentation, the rejection under 35 USC 112 should be overruled.

B. The Teachings of the Prior Art As a Whole Fail to Suggest Or Render Obvious the Invention As Claimed in Claims 12-17, and 19.

The use of immunological methods for pregnancy detection is a relatively crowded art. Perhaps equally crowded is the art of using immunological techniques for the study of placental tissue and other pregnancy associated structures. Notwithstanding the persistent search for correlations between serum protein levels and pregnancy abnormalities, the art has failed to suggest the detection of early pregnancy with antibodies raised against a mixture of pregnancy-specific proteins from term placentas. Appellant has shown that such a detection method has the capability of determining pregnancy by the presence of a plurality of proteins found to be present at detectable levels soon after conception.

Neither of the primary references describes pregnancy detection. The Hoffman references and Brock describe studies which attempt to correlate placental proteins with proteins found in pregnancy serum. Lin (1) teaches only that some antigenic serum proteins are specific to pregnancy. The antibody mixtures used in the primary references are substantially different from the antibody mixture required by the claims. Neither of the primary references isolates the pregnancy-specific protein fraction prior to injection into the host. As pointed out in appellant's specification (page 5, lines 21-32) this step results in an antiserum capable of providing more bands of precipitation in subsequent pregnancy tests because the animal's immune response is directed toward only pregnancy-specific proteins.

Art such as the primary references which describes proteins in "pregnancy serum" without specifying the period of the pregnancy is not pertinent to claims directed to an early pregnancy test. Obviously the pregnancies had already been confirmed. By citing such art the Examiner has taken the position that once placental proteins are shown to exist in serum at any stage of pregnancy, it would be obvious to test for early pregnancy using antiserum raised against term placental tissue. This position ignores the well-recognized fact that the protein content of

serum or urine varies considerably during the course of pregnancy, as shown in Exhibits 1-3 and page 16, lines 4-11 of appellant's specification. In view of the prior art belief that pregnancy associated serum proteins originated from the placenta (Hoffman (2)) and that such proteins disappeared soon after birth, (Seppala and Exhibit 3) it was by no means obvious that antibodies raised against a plurality of term placental pregnancy-specific proteins would be capable of detecting a plurality of proteins present at the very early stages. Claims 12-17 and 19 requiring detection within about 2 months of conception are neither obvious nor inherent in the primary references.

The Bohn patent suggests a pregnancy test based on a single protein obtainable from placentas. As shown in Bell and Dietrich, pregnancy tests sensitive to a single protein produce false negatives in patients deficient in that protein.

Jankowsky, cited as an alternate secondary reference, is closer than any of the primary references yet actually teaches away from the claimed method. Jankowsky teaches the use of an agent prepared from antiserum raised against a mixture of HCG and first month placental tissue. Obviously first month placentas are difficult to acquire, especially those from normal pregnancies with normal proteins. The Jankowsky test highlights the prior art's recognition that placental proteins from a late stage of pregnancy are not necessarily present in the early stages. Had Jankowsky considered it obvious that antibodies raised against readily available term placental tissue could detect a plurality of early ~~placental proteins~~ would hardly have been limited to first month placentas, i.e. placentas from about the same period of pregnancy to be detected.

The Examiner has remarked that the claims are of indeterminate scope in the use of the term "pregnancy-specific proteins". It is not necessary to identify these proteins any further. It is clear from the specification that pregnancy-specific proteins are those not found in normal non-pregnant individuals, see for example, page 6, lines 1-10 where

anti-normal human serum is used to remove non-pregnancy-specific placental proteins. Workers in the art, following appellant's examples, can easily prepare agents sensitive to any desired number of early pregnancy proteins. Since the prior art has never used a pregnancy test employing antibodies raised against a mixture of term placental pregnancy-specific proteins, further identification of the proteins in the claims is unwarranted and would be unduly limiting.

Only the appellant has provided a reliable agent from term placentas which has the capability of detecting a plurality of pregnancy-specific proteins expressed in early pregnancy. Since the prior art used only single protein tests or tests requiring scarce first month placentas, the claimed method cannot be obvious.

C. Teachings of the Prior Art As a Whole Fail to Suggest Or Render Obvious the Invention As Claimed in Claim 18.

Claim 18 is directed to the use of the pregnancy test within about one month of conception. As shown in Bell and Lamb, false negatives in single protein tests are most prevalent within about fifty days of the last menstrual period. By removing non-pregnancy-specific proteins from the term placental mixture prior to inoculation into the host an agent capable of very early detection of a number of proteins is provided. Since the closest prior art (Jankowsky) employed first month placentas for preparing pregnancy detection antiserum, the successful use of term placentas for preparing such antiserum is a clear advantage nowhere suggested in the prior art.

SUMMARY

Appellant has shown that the specification is sufficient to enable one of ordinary skill in the art to successfully practice the claimed method without undue experimentation. Appellant has shown that the prior art as a whole does not teach or render obvious the claimed invention. Accordingly, the Board is respectfully requested to overrule the rejections of claims 12-19 under 35 USC 112 and 35 USC 103.

Respectfully submitted,

Attorney for appellant

APPENDIX

1075808

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS: David W. Holladay :
SERIAL NO.: 755,100(70) : GROUP 120
FILED: December 28, 1976 : EXAMINER
Anna P. Fagelson
FOR: AGENT AND METHOD FOR THE EARLY
DETECTION OF PREGNANCY

DECLARATION UNDER 37 CFR 1.132

I, John E. Caton do hereby declare and affirm:

THAT I hold the degree of Ph.D in Analytical Chemistry from Iowa State University;

THAT I am employed as a Research Staff Member in the Analytical Chemistry Division of the Oak Ridge National Laboratory;

THAT I have published more than fifteen papers in professional journals relating to the fields of analytical and bioanalytical chemistry, including immunochemical techniques;

THAT during the years 1969-1974 I worked as an analytical chemist in the Molecular Anatomy Program of the Oak Ridge National Laboratory, during which time I carried out extensive work in the fields of gel electrophoresis, chromatographic separation of macromolecular solutions such as serum, and the study of techniques utilizing antigen-antibody reactions for analytical purposes;

THAT I am well acquainted with the ordinary level of skill of workers in the field of immunology and immunoanalytical chemistry;

THAT workers of ordinary skill in the field of immunology and immunoanalytical chemistry are well acquainted with the standard immunological methods for detecting the presence of antigens in solutions by contacting the solution with a solution containing antibodies to the antigens sought;

THAT in carrying out immunological tests for detecting the presence of antigens in solutions, workers of ordinary skill in immunology or immunoanalytical chemistry often make determinations of the proper titer of antibody solution to use for a particular concentration of antigen solution;

conception. Neither reference describes detecting antigens in serum or urine from such early stages. The presence of antigens in mid-term or late pregnancy does not indicate that the same antigens would be present sufficiently early to be of interest for pregnancy detection.

Thirdly, no showing of criticality for term placentas is needed since the use of term placentas is not *prima facie* obvious. The only prior art test employing a mixture of antibodies (Jankowsky) required first-month placentas - placentas from the same period of pregnancy as to be detected. It is obvious that such a detection agent would be very expensive since first month human placentas are obtainable only from a very limited number of miscarriages and abortions. Term placentas, on the other hand, are universally available and a detection agent obtained therefrom would be obviously much less expensive than Jankowsky's. The fact that Jankowsky never suggested the use of such an obviously preferable source of antigens clearly demonstrates the unobviousness of the claimed invention.

Fourthly, the Examiner is requested to reconsider the rejection under 35 USC 112 for the absence of an example of specific test protocol. The Board of Appeals rejected the Examiner's position in Ex Parte Krenzer 199 USPQ 227 (1978). A copy of the decision is supplied for the Examiner's convenience. It is clear from Krenzer and cases cited therein that the mere absence of a working example is insufficient to support a rejection under the enablement or best mode clauses of §112. Since the Examiner has failed to provide reasons why one skilled in the art would be unable to carry out the invention as claimed without undue experimentation, it is requested that the rejection be withdrawn.

It is submitted that the claims are in compliance with 35 USC 103 and 35 USC 112 and a Notice of Allowance is requested.

Respectfully submitted,

Attorney for applicant

Oak Ridge, Tennessee
FTS-850-4334 (Commercial AC 615-483,8611, Ext. 34334)
Uzzell:br

fuel in a fuel control. The 1307 and fuel controls seek to regulate fuel as a function of the mass of air flowing through the engine. Both controls are responsive to variations in engine speed to give a signal that is a function of speed. In addition, both are responsive to operational parameters based on temperature and pressure of the air to produce fuel flow as an ultimate function of air flow. Infringement is clear, as the MFC and 1307 fuel controls perform substantially the same function utilizing substantially the same mechanism in essentially the same manner. Therefore, in this case, the claims of the patent are not to be narrowly construed in light of the prior art or the Mock specification and file wrapper, the trial examiner's original opinion with respect to infringement will not be disturbed. Independent of that conclusion, the issue has been reviewed in the light of the record in this case, including the briefs of the parties presented before the court. I hold that infringement has been established.

As to the broader claims 1, 3, 4, infringement does not depend on resort to the doctrine of equivalents. These claims do not contain the "means" language. These claims, being broader than the claims sought to be narrowed, are infringed by the accused de-

Supplemental Conclusion of Law

In the findings of fact and opinion dated June 19, 1975 and the supplemental findings of fact and opinion, supplemental and additional findings of fact filed herewith, all of which constitute a part of the judgment herein, the court concludes as a matter of law that claims 11, 24, 25, and 28 of Kunz Patent No. 2,720,751 are invalid, wherefore the application is dismissed as to that patent, and claims 1, 3, 4, 5, 7, 8, 11, 12, 13, 14, 15 of Mock Patent No. 2,581,275 are not infringed, wherefore defendant is liable for infringement of that patent. Accordingly, plaintiff is entitled to recover judgment is entered to that effect. The amount of recovery is reserved for further findings under Rule 131(c). Findings 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.

Appeal from Group 122

Application for patent of John Krenzer, Serial No. 571,466, filed Apr. 25, 1973. From decision rejecting claims 1-10, applicant appeals (Appeal No. 310-20) Reversed.

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

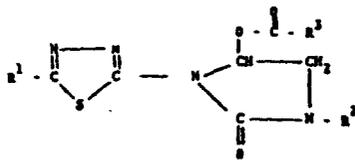
Before Witherspoon and Milestone, Examiners in Chief, and Pellman, Acting Examiner in Chief.

Pellman, Acting Examiner in Chief.

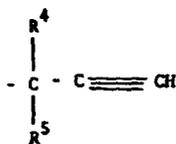
This is an appeal from the examiner's decision finally rejecting claims 1 through 10, all of the claims in the application.

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

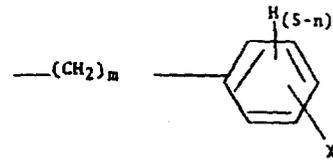


wherein R¹ is selected from the group consisting of alkyl of up to 3 carbon atoms, cycloalkyl of from 3 to 7 carbon atoms, lower alkenyl, lower chloroalkyl, lower bromoalkyl, lower alkoxy, lower alkylthio, lower alkylsulfonyl and lower alkylsulfinyl; R² is selected from the group consisting of lower alkyl, lower alkenyl, lower haloalkyl and



wherein R⁴ and R⁵ are each selected from the group consisting of

hydrogen and alkyl of up to 3 carbon atoms; and R³ is selected from the group consisting of lower alkenyl, lower haloalkyl, lower alkenyl, 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-acyloxy" has no proper antecedent basis in the definition of "R³" 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 Elitroth*, 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')."

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 here. 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



U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20201

MAILED

007-17 1978

GROUP 120

[Anna P. Fagelson Art Unit 125
12/28/76 755,100]
David W. Holladay

James E. Denny
Asst. Gen. Counsel for Patents
U.S. Energy Research &
Development Administration
Washington, D. C. 20545

THIS IS A COMMUNICATION FROM THE EXAMINER
IN CHARGE OF YOUR APPLICATION.

COMMISSIONER OF
PATENTS AND TRADEMARKS

- This application has been examined.
- responsive to communication filed on July 28, 1978
- This action is made final.

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS ACTION IS SET TO EXPIRE Three(3) MONTH(S)
_____ DAYS FROM THE DATE OF THIS LETTER.

FAILURE TO RESPOND WITHIN THE PERIOD FOR RESPONSE WILL CAUSE THE APPLICATION TO BECOME ABANDONED.
35 U.S.C. 133

PART I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

- 1. Notice of References Cited, Form PTO-892.
- 2. Notice of Informal Patent Drawing, PTO-946.
- 3. Notice of Informal Patent Application, Form PTO-152
- 4.

PART II SUMMARY OF ACTION

- 1. Claims 12-19 are pending in the application.
Of the above, claims _____ are withdrawn from consideration.
- 2. Claims _____ have been cancelled.
- 3. Claims _____ are allowed.
- 4. Claims 12-19 are rejected.
- 5. Claims _____ are objected to.
- 6. Claims _____ are subject to restriction or election requirement.
- 7. The formal drawings filed on _____ are acceptable.
- 8. The drawing correction request filed on _____ has been approved.
 disapproved.
- 9. Acknowledgement is made of the claim for priority under 35 U.S.C. 119. The certified copy has
 been received. been filed in parent application;
 not been received. serial no. _____ filed on _____
- 10. Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 OG. 213.
- 11. Other

Art Unit 125

The specification is objected to for the reasons set forth in the last Office action of April 6, 1978.

The claims stand rejected under 35 USC 112 (paragraph 1) for the reasons of record in the absence of teachings as to the specific test method employed.

The claims stand rejected under 35 USC 103 as inherent in the teachings of any one of Hofmann 1 or 2, Brock or Lin (1), either alone or considered with the Bohn patent or Jankowsky for the reasons set forth in the last Office action. There is no evidence of record showing any criticality with respect to the use of "term placental proteins" nor is there any evidence that the instant placental proteins or methods differ in kind from those of the art. The claimed means of securing the proteins and the proteins per se are so indefinite that it is a matter of speculation as to what is contained in any final product. For example, precipitating proteins with salts, depending on concentration, can give various different fractions. Applicant's reference to "plurality" of proteins in the response of July 28, 1978, is not understood. Obviously, the placenta will contain a plurality of proteins. It is not seen that such mixtures of proteins present anything new or unexpected to the routineer in the art.

This action is made FINAL.

APFagelson:mbs

A/C 703

557-2577

10/05/78

ANNA P. FAGELSON
EXAMINER
ART UNIT 125

Stephen P. Hamel

James E. Denny
Assistant General Counsel for Patents
United States Department of Energy
Washington, D. C. 20545

Applicant : David W. Holladay *S-47,844*)
Serial No. : 755,100)
Filed : December 28, 1976) GROUP 120
For : AGENT AND METHOD FOR THE EARLY DETECTION OF)
PREGNANCY)

Receipt is acknowledged of the following in the above-identified application:

- | | |
|--|---|
| <input checked="" type="checkbox"/> Response to Office Action dated <u>April 6, 1978</u> | |
| <input type="checkbox"/> Letter to Draftsman | |
| <input type="checkbox"/> Affidavit | <input type="checkbox"/> Petition |
| <input type="checkbox"/> Notice of Appeal | <input type="checkbox"/> Appeal Brief |
| <input type="checkbox"/> Fee Authorization | <input type="checkbox"/> Amendment under Rule 312 |
| <input type="checkbox"/> Appointment of Attorney | <input type="checkbox"/> Directive |
| <input type="checkbox"/> PTOL-85b Base Issue Fee | |
| <input type="checkbox"/> PTO-1094 with <input type="checkbox"/> Sheets Formal Drawings | <input type="checkbox"/> Withdrawal of Directive |
| <input type="checkbox"/> _____ | <input type="checkbox"/> _____ |
| <input type="checkbox"/> _____ | <input type="checkbox"/> _____ |

7-28-78

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: David W. Holladay :
SERIAL NO.: 755,100(70) : GROUP 120
FILED : December 28, 1976 : EXAMINER
FOR : AGENT AND METHOD FOR THE EARLY DETECTION : Anna P. Fagelson
OF PREGNANCY

AMENDMENT B

The Commissioner of Patents and Trademarks
Washington, D. C. 20231

Sir:

In response to the Office Action of April 6, 1978, please amend
the above-identified application as follows:

IN THE CLAIMS

Cancel claims 3, 7, 9 and 11.

Add new claims 17, 18, and 19.

- 1 17. A method of detecting the existence of pregnancy in humans
2 comprising obtaining serum or urine from a patient within about two
3 months of suspected conception, contacting said serum or urine with a
4 pregnancy detection agent containing antibodies to a plurality of
5 pregnancy-specific proteins, and observing whether antibody/antigen
6 reactions occur, the occurrence of at least one antibody/antigen reac-
7 tion being indicative of pregnancy, said pregnancy detection agent pre-
8 pared by the method comprising
9 (a) first providing a mixture of term placental proteins con-
10 taining antigenic pregnancy-specific proteins;
11 (b) contacting said mixture of term placental proteins with anti-
12 bodies raised against either pregnancy-specific proteins or normal human
13 serum proteins to cause said antibodies to react with pregnancy-specific
14 proteins or non-specific antigenic proteins contained in said mixture;
15 (c) separating the reaction products of step (b) from the remainder
16 of the term placental protein mixture to provide a mixture of antigenic
17 pregnancy-specific proteins isolated from non-specific antigenic
18 proteins;

19 (d) innoculating a host animal with said mixture of antigenic
20 pregnancy-specific proteins to cause said host animal to raise anti-
21 bodies to a plurality of pregnancy-specific proteins; and

22 (e) isolating antibodies to said plurality of pregnancy-specific
23 proteins from antibodies to non-specific human proteins.

1 18. The method of claim 17 in which said serum or urine is
2 obtained from said patient within about one month of suspected concep-
3 tion.

1 19. The method of claim 17 in which said method for preparing
2 said pregnancy detection agent further comprises isolating antibodies
3 to said plurality of pregnancy-specific proteins from non-antibody
4 host animal serum proteins.

Amend claims 12-16 as follows:

1 12 (Amended). The [agent] pregnancy detection method of claim
2 [11] 17 in which step (b) comprises contacting said mixture of term
3 placental proteins with antibodies raised against pregnancy-specific
4 proteins.

1 13 (Amended). The [agent] pregnancy detection method of claim
2 [11] 17 in which step (b) comprises contacting said mixture of term
3 placental proteins with antibodies raised against normal human serum
4 proteins.

1 14 (Amended). The [agent] pregnancy detection method of claim
2 [11] 17 in which said mixture of term placental proteins is prepared by
3 providing a suspension of term placental tissue in a stabilizing
4 medium, separating suspended solids from said suspension to provide a
5 supernatant solution, adding a salting agent to said supernatant
6 [solutions] solution to precipitate proteins and dissolving said pre-
7 cipitated proteins to provide said mixture of term placental proteins.

Claim 15, line 1, after "The" delete "agent" and replace it with
---pregnancy detection method---

Claim 16, line 1, after "The" delete "agent" and replace it with
---pregnancy detection method---

REMARKS

Claims 3, 7, 9 and 11 through 16 stand rejected under 35 USC 112 and 35 USC 102 or 103. The specification is objected to as containing insufficient exemplary matter. The present amendment cancels claims 3, 7, 9 and 11 and adds new claims 17, 18 and 19. New claims 17, 18 and 19 are previously submitted claims 7, 9, and 3, respectively, with the additional limitation that the agent contains antibodies to a plurality of pregnancy-specific proteins and the placental proteins used in preparing the pregnancy detection agent are from term placentas. All of the present claims are directed to a method of detecting pregnancy using an agent defined by its method of preparation, i.e. to a method of use of a product-by-process.

The Examiner requested a more complete citation of references identified as "Intersociety." The complete citation of the references is as follows:

"Proceedings of the American Society of Experimental Biology", Vol. 33:3, No. 443 (1974), and

"Proceedings of the American Society of Experimental Biology", Vol. 33:3, No. 490 (1974).

In the Office Action, the Examiner cited U. S. Patent 4,065,445 to Bohm, et al. as a secondary reference. As pointed out in the January 10, 1978 letter, applicant's attorney has no copy of this reference. This reference is not believed to be closer than the other art of record and will not be discussed further in this response. The Examiner is requested to provide a copy of this reference if she intends to rely upon it further.

THE INVENTIVE CONCEPT

Applicant's inventive concept as set forth in the presently submitted claims is based upon the discovery that term placentas contain a plurality of antigenic pregnancy-specific proteins, several of which are present in detectable amounts in the serum or urine of women in the early stages of pregnancy. While workers in the prior art were apparently aware of the existence of a number of pregnancy-specific

proteins in placental tissue and pregnancy serum, it is only the applicant who has discovered that a host animal inoculated with a mixture of term placental pregnancy-specific proteins can raise antibodies to a plurality of proteins which appear in pregnancy serum sufficiently early to be useful in a pregnancy test. The operability of the claimed pregnancy tests for detecting a plurality of antigens in the very early stages of pregnancy is demonstrated by a number of double-diffusion tests described on pages 14-16. The use of the claimed pregnancy-detection agent represents a significant advance in the art. Those women prone to false negatives in prior art tests based on only one serum antigen are now provided with a test capable of detecting any of several pregnancy-specific antigens. The improvement in reliability over the prior art "single antibody" tests is self-evident.

THE 35 USC 102 OR 103 REJECTIONS

The present claims are directed to a pregnancy detection method for use in detecting pregnancy within two months (claims 12 through 17 and 19) or within one month (claim 18) of suspected conception. Nowhere in the art of record is any pregnancy detection method described for the early stages of pregnancy which utilizes antibodies to a plurality of pregnancy-specific proteins obtained from term placenta.

The Lin (1) reference deals with third trimester serum and is not concerned with pregnancy detection. Hofmann I, Hofmann II and Brock do not describe the use of antibodies to placental proteins for the detection of early pregnancy. Though pregnancy serum was used in some of the immunological tests of the references, the condition of pregnancy had already been confirmed. There is no suggestion that serum from women one or two months after conception would contain a plurality of antigens detectable by applicant's claimed method. Consequently, pregnancy detection within two months of conception is not inherent in the Hofmann references. It is unjustified speculation on the Examiner's part to assert that pregnancy serum used in the Hofmann work was

obtained within two months of conception, especially since the serum had already been identified as pregnancy serum. In the absence of any teaching that pregnancy can be initially detected by the use of antibodies to a plurality of term placental proteins, the application of the Hofmann, Brock or Lin references to the presently submitted claims is unwarranted.

The Jankowsky reference describes a pregnancy detection method employing antibodies to first month placental proteins. The fact that an antigen might be present in first month placentas does not indicate that it would be present in term placentas, especially in view of numerous teachings on the art of record that some placental proteins do not occur in the serum at all. See Brock et al. As pointed out in applicant's specification, p. 16, lines 6-10, the precipitin bands which are strongest for early pregnancy show identity with the weakest bands from third trimester pregnancy serum. It should be apparent term placentas are more readily available and consequently less expensive than first month placentas. If Jankowsky had recognized that a useful detection agent could be prepared from term placentas, he certainly would have used term placentas or at least suggested their use. It is only by the use of applicant's own teachings that one is motivated to use ~~term~~ placentas to prepare a detection agent.

The art of record, when taken as a whole, evidences the unobviousness of the claimed method. Even after some fifteen years of intensive immunochemical investigation of pregnancy related antigens, none of the references cited suggests the use of antibodies to a mixture of term placental proteins in a pregnancy test. Furthermore, none of the references or combinations of references suggest that term placentas even contain a plurality of antigens also expressed in serum or urine in the first one or two months after conception. Absent such teachings, it is a clear distortion of the prior art as a whole to modify the reference processes to perform the claimed invention. If the Examiner intends to rely further on the art of record, she is

requested to identify the specific location of the teachings essential to establish the prior arts' knowledge or suggestion of the method as claimed.

THE 35 USC 112 REJECTIONS

The Examiner's continuance of the rejection under 35 USC 112 is specifically traversed. This rejection appears to be based upon the general allegation that the specification includes insufficient exemplary matter to support the claims and, more specifically, the lack of specific examples of test methods with protocol and proportions employed.

It is not clear whether the Examiner is continuing her original rejection that there is insufficient disclosure of the characteristics of pregnancy-specific proteins to determine the bounds of the claims. Nevertheless, the present claims are directed to the use of a product prepared by a specified process and in that context the description of pregnancy-specific proteins is made clear, especially in view of the definition of pregnancy-specific proteins on page 6 of the specification. The method of isolating pregnancy-specific proteins from non-specific human proteins indicates that the pregnancy-specific proteins specified in the claims are those proteins which are not bound on a column prepared with anti-normal human serum as opposed to those proteins, i.e. normal serum proteins, which react with such a column. The fact that the proteins might prove useful for the detection of cancer does not detract from their character as pregnancy-specific as defined in the specification. Furthermore, the term "pregnancy-specific" is employed frequently in the prior art of record to define a similar class of proteins.

It is not understood how the specification can be defective under the enablement or best mode clauses of 35 USC 112. The pregnancy test as presently claimed is described in the specification first at page 3, lines 21-25 which states that the method comprises

"Contacting serum or urine from a patient with an antibody to a pregnancy-specific protein isolated from non-specific antibodies, and observing whether an antibody/antigen reaction occurs, the occurrence of said reaction being indicative of pregnancy."

Again on page 11, line 31-page 12, line 6 it is stated

"All that is necessary for this pregnancy detection method is that serum from a patient be contacted with an antibody raised against a pregnancy-specific protein and the results observed to detect signs of antibody/antigen reaction. The occurrence of the reaction, of course, indicates pregnancy. It can be readily appreciated that any of the several methods for detecting antigen/antibody reaction is suitable for detecting pregnancy according to my method. Examples of such methods are gel precipitation, gel electropherograms, immunoelectrophoresis, crossimmuno-electrophoresis, and immunodiffusion."

One need only review the art of record to see that such methods are common and well within the skill of workers in the art.

Applicant does not regard the mechanical steps of these well-known immunological techniques as his invention and need not describe them in the application. What applicant has invented is a pregnancy test employing the use in prior art test methods of an antibody agent prepared using a mixture of term placental antigens. Applicant has described the method of preparing this agent in great detail (pp. 3-14) and has demonstrated its operability for pregnancy detection by prior art techniques (p. 14, line 31-p. 16, line 11). On page 12, lines 7-11 it is stated that the pregnancy detection method is useful for detecting pregnancy within about two months of conception, especially within about one month of conception. It is described therein that gel precipitation was used to test the antiserum to placental antigens for the presence of antigens in the sera of pregnant women. In women of the third trimester of pregnancy, two to four bands were visible after 40 hours compared to a control which showed no reaction. On page 15, line 21, serum samples were tested by double diffusion against antiserum to placental antigens using placental extract and serum from a woman in the third trimester of pregnancy as a reference. After 16 hours one sharp band and one faint band of precipitation were observed in both samples and the two bands merged with bands from both placental extract and third trimester pregnancy serum sample. For both samples a precipitin band was observed as early as one hour after the start of immunodiffusion with three precipitation bands observed in the second sample within 24 hours of loading. Beginning on page 16, it is stated

that another pregnancy serum sample was tested against the antiserum and gave two precipitin bands within 16 hours.

In view of the above statements in the specification, it is not understood where the deficiency lies. It is stated in the specification that the test can be performed by double diffusion. The declaration of John E. Caton establishes that if a person of ordinary skill in the field of immunology and immunoanalytical chemistry were given a solution known to contain antibodies to proteins specific to the serum or urine of pregnant women (such as described by the applicant), that he would be able to test the samples of serum or urine for the presence of these proteins specific to pregnancy by the well-known Ouchterlony technique of double diffusion. Dr. Caton further declared that workers of ordinary skill in the art often determine the proper concentration of antibody solution used for detecting the presence of antigens in solutions such as urine or serum and that such a person would be able to determine the proper concentration by a simple well-known method which requires only that an agar plate be left overnight. Since no undue experimentation is required in carrying out the test as claimed, no additional information is needed.

The rejection was based in part on the lack of a specific example. The lack of a specific example is of no moment in the present case where it is well within the skill of the art to carry out the claimed method. The Examiner's position that when one claims a method one must have a specific example carrying out the method is not understood. The method applicant claims involves the simple contacting of serum or urine with the specified pregnancy detection agent. Applicant described this contacting via a double diffusion test and suggested that other methods are also suitable. Applicant has not carried out such other methods and should not be required to formulate examples based upon these other procedures in order to claim patent protection commensurate in scope with his discovery.

The Examiner's requirement for a specific example of a claimed method is contrary to current case law. In In Re Borkowski,

164 USPQ 642, CCPA, 1970, the CCPA held quite differently. The claimed invention was a process for producing oxygenated hydrocarbons by reacting hydrocarbons with ferric chloride in vapor phase and hydrolyzing the resulting material. The claims were rejected under 35 USC 112 as based upon insufficient disclosure particularly with reference to a chlorination step. The Examiner believed that relative amounts of the hydrocarbon and the magnitude of reaction times were two parameters which the appellant should have disclosed more fully. While acknowledging that the specification need not read as instructions to a technician and that one might after a few hours of experimentation determine how to carry out and control the chlorination of methane, the Board affirmed the Examiner stating:

"...the asserted novelty in the mode of operation which involves a careful balance of a number of distinct reactions makes illustration particularly necessary. Desirably and necessarily, such illustrations should provide an exemplary correlation of the times of reaction, rates of reactant, feed and material removal (chlorinated product, ferric oxide, HCl, etc.). This would inform a man skilled in the art of the actual feasibility of appellant's process and provide some sort of jumping off place in a plunge into the unknown when planning a series of experiments from which the necessary operating parameters of the process may be determined."

The CCPA criticized the Board's position saying that the exemplary corrolation the Board thought necessary was apparently no more than a specific working example. The Court stated:

"However, as we have stated in a number of opinions 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. Here, while it may be that an 'exemplary corrolation' of parameters such as times of reaction and rates of reactant feed and product removal will give the worker in the art some useful information and provide a 'jumping off place,' we see no basis for concluding that without such information the worker in the art would not be enabled by the specification to practice the invention; i.e. to 'balance' the several reactions involved in the appellants' process. The 'few hours' experimentation mentioned by the Examiner certainly would not seem to be an undue amount of time considering the nature of the claimed invention."

In the present case, the only work which a worker in the art might need to perform is set forth in Dr. Caton's declaration. Since workers in the art are well-acquainted with the needed tests and accustomed to performing them, the rejection is improper. Since the mere lack of an

example is not proper grounds for rejection, the Examiner is requested to either withdraw the rejection or point out specific deficiencies which would require undue experimentation. As the Court of Customs and Patent Appeals pointed out in In re Budnick, 190 USPQ 422, CCPA (1976),

"Where an applicant asserts that a specification contains enablement commensory in scope with the protection sought by the claims but the Examiner is of the opinion that the disclosure is not enabling, he has the burden of substantiating his doubts concerning the enablement with reasons or evidence."

The best mode rejection is equally unwarranted. In In re Karnofsky, 156 USPQ 682, CCPA (1968), it was held that a specific example is not needed to fulfill the best mode requirement. The claims in issue were directed to a process for manufacturing vinyl acetate monomer including a number of steps and a number of separations involving rectification, distillation and recycling of various components. In support of the Board's affirmance of a 35 USC 112 rejection it was argued that one wishing to practice the claimed invention would have to determine a number of variables, such as the richness of the feed to the splitter column and expected variations in that richness; temperatures at top and bottom of the splitter column; feed rates; etc. and would have to corrolate these values with pressure recycle rate and design of the column itself before he could determine the method of operating his process to achieve appellant's result. In answer to this charge the Court pointed out:

"Assuming all this to be true, it does not concern the question before us. Our concern is whether one of ordinary skill in the art would know how to adjust these operating variables to achieve the desired result.

Applicant has asserted throughout the prosecution of his application that such adjustments would present no more than a routine operating problem to a chemical engineer. He has noted the fact that the materials involved have widely separated boiling points. He has explained the physical phenomenon of the law of partial pressure in multicomponent distillations and argued that a distillation engineer of ordinary skill being conversant with these physical facts would know how to operate a still to achieve the results of the specification."

After finding that the Board had no controverting facts the Court held in favor of the applicant pointing out:

"Where one of ordinary skill in the art would know how to select operating conditions so as to achieve a particular result, the failure to include a recitation of some specific operating conditions in the specification cannot give rise to a rejection either under the 'enabling' or under the 'best mode' requirement of 35 USC 112."

In view of the above decisions it is submitted that the Examiner has failed to meet her burden to specifically point out information that is needed to carry out the invention and is both lacking in the specification and incapable of determination with only a small amount of routine testing well within the skill of the art.

It is believed that the presently submitted claims are in compliance with 35 USC 102, 103 and 112 and a Notice of Allowance is requested. If the Examiner has any suggestions or questions as to claim language, a telephone call to applicant's attorney is suggested.

Respectfully submitted,

Allen H. Uzzell

Attorney for applicant

Oak Ridge, Tennessee

FTS-850-4334 (Commercial AC 615-483-8611, Ext. 34334)

Uzzell:br



U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

Fagelson Art Unit 125
755,100 12/28/76
David W. Holladay,

MAILED
MAILED

APR 6 1978

James E. Denny,
Assistant Gen. Counsel For Pats.,
U.S. Energy Res. & Dev. Admin.,
Washington, D.C. 20545

GROUP 120

THIS IS A COMMUNICATION FROM THE EXAMINER
IN CHARGE OF YOUR APPLICATION.

COMMISSIONER OF
PATENTS AND TRADEMARKS

- This application has been examined.
- Responsive to communication filed on Jan 17, 1978.
- This action is made final.

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS ACTION IS SET TO EXPIRE Three (3) MONTH(S)
 DAYS FROM THE DATE OF THIS LETTER.

FAILURE TO RESPOND WITHIN THE PERIOD FOR RESPONSE WILL CAUSE THE APPLICATION TO BECOME ABANDONED.
35 U.S.C. 133

PART I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

- 1. Notice of References Cited, Form PTO-892.
- 2. Notice of Informal Patent Drawing, PTO-948.
- 3. Notice of Informal Patent Application, Form PTO-152
- 4.

PART II SUMMARY OF ACTION

- 1. Claims 3, 7, 9, 11-16 are pending in the application.
Of the above, claims are withdrawn from consideration.
- 2. Claims have been cancelled.
- 3. Claims are allowed.
- 4. Claims 3, 7, 9, 11-16 are rejected.
- 5. Claims are objected to.
- 6. Claims are subject to restriction or election requirement.
- 7. The formal drawings filed on are acceptable.
- 8. The drawing correction request filed on has been approved, disapproved.
- 9. Acknowledgement is made of the claim for priority under 35 U.S.C. 119. The certified copy has been received, not been received. been filed in parent application; serial no. filed on
- 10. Since this application appears to be in condition for allowance except for formal matters, prosecution at the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11, 453 O.G. 213.
- 11. Other

Art Unit 125

The specification is again objected to as including insufficient exemplary matter to support the claims for the reasons set forth in the last Office action. The statutes require a full disclosure of the best mode contemplated. The fact that those skilled in the art may develop a test employing the placental antigens is of no moment where here applicant is claiming such method and seeks patent rights for such method he now states may be developed by others.

The claims therefore stand rejected under 35 USC 112 (par 1) for the reasons above set forth.

The claims are rejected under 35 USC 102/103 as inherent in the teachings of any one of Hofmann 1 or 2, Brock, or Lin (1) either alone or considered with the Bohn patent, newly cited, or Jankowsky. Hofmann, Brock and Lin are deemed to teach the use and means of preparing antigenic pregnancy specific proteins. Moreover, it is not seen that such proteins are rendered newly patentable by the recitation of the means of preparing same. The use of such placental proteins in detecting pregnancy while inherent in the teachings of Hofmann, Brock or Lin is further taught by Bohn and Jankowsky, the latter in particular noting the early detection.

Fagelson:cvn

A/C 793

557-2577

4/4/78

ANNA P. FAGELSON
EXAMINER
ART UNIT 125

1. J 2, 1975

FORM PTO. 46-42 (REV. 12-76) (FORMERLY PTO. 892)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	SERIAL NO. 753700	GROUP ART UNIT 125	ATTACHMENT TO PAPER NUMBER 7
NOTICE OF REFERENCES CITED		APPLICANT (S) D W Holladay		

U.S. PATENT DOCUMENTS																	
•	A	B	C	D	E	F	G	H	I	J	K	DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	X											A 4065445	12/1/77	Bahn	421	12X	

FOREIGN PATENT DOCUMENTS														
•	L	M	N	O	P	Q	DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS DWC	PP SVC
							L 2240327	3/1974	Germany	Jankowsky			-	1

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)	
•	Brock, Zentralblatt fuer Gyn. Vol. 97, 1975 pp 281-287
X	Hofmann, Arch Gynec Vol. 208, 1970 pp 266-274
X	Hofmann ⁽²⁾ , Arch Gynec Vol. 208, 1969 pp 187-195
X	Linn, Amer J Obstet Gynec, Vol. 118, 1974 pp 223-236
X	Bell, J Clin Pathol Vol. 22, 1969 pp 79-83
X	Korenitz, Obstet Gynec, Vol. 40, Oct. 1972 pp 563-566
X	Lamb, Obstet Gynec, Vol. 39, May 1972 pp 665-672
X	Dietrich, CMA Journal Aug 3, 1974/Vol. III pp 235-237
EXAMINER	DATE
A F [signature]	12/1/75

* A copy of this reference is not being furnished with this office action.
(See Manual of Patent Examining Procedure, section 707.05 (a).)

pg 2 of 2 pages

FORM PTO 44-47 (REV. 12/76) (FORMERLY PTO. 892)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	SERIAL NO 755100	GROUP ART UNIT 125	ATTACHMENT TO PAPER NUMBER 7
NOTICE OF REFERENCES CITED		APPLICANT (S) DW Holladay		

U.S. PATENT DOCUMENTS						
	DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
A						
B						
C						
D						
E						
F						
G						
H						
I						
J						
K						

FOREIGN PATENT DOCUMENTS								
	DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS. PP.	
							DWG	SPEC
L								
M								
N								
O								
P								
Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)	
* R	Hodgen, J. Clin Endocrinol. Metab. Vol. 38, 1974 pp 927
* S	Berne, Intersociety 490 (incomplete information supplied by applicant)*
* S	Lin, Intersociety 443
* S	Bohn, Blut, Band XXIV, 1972 pp 292-302
T	
U	

EXAMINER A Fوجلson	DATE 3/24/78	* Question if there is a publication named "Intersociety"
-----------------------	-----------------	---

* A copy of this reference is not being furnished with this office action.
(See Manual of Patent Examining Procedure, section 707.05 (a).)

W. E. Denny

James E. Denny
Assistant General Counsel For Patents
United States Department of Energy
Washington, D. C. 20545

Applicant : David W. Hollaway)
Serial No. : 755,100)
Filed : December 28, 1976) GROUP 120
Inventor : AGENT AND METHOD FOR THE EARLY DETECTION)
OF PREGNANCY)

Receipt is acknowledged of the following in the above-identified application:

- Response to Office Action dated September 19, 1977 .
- Letter to Draftsman
- Affidavit () Petition
- Notice of Appeal () Appeal Brief
- Fee Authorization () Amendment under Rule 312
- Appointment of Attorney () Directive
- PTO-85b Base Issue Fee
- PTO-1094 with () Sheets Formal Drawings () Withdrawal of Directive
- Declaration under 37 CFR 1.132 ()
- Prior Art Letter w/cys of 11 cited references ()

1-17-78

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT : David W. Holladay :
SERIAL NO.: 755,100(70) : GROUP 120
FILED : December 28, 1976 : EXAMINER
FOR : AGENT AND METHOD FOR THE EARLY : Anna P. Fagelson
DETECTION OF PREGNANCY

AMENDMENT A

The Commissioner of Patents & Trademarks
Washington, D. C. 20231

Sir:

In response to the Office Action dated September 19, 1977,
please amend the above-identified application as follows:

IN THE SPECIFICATION

Page 15, line 26, delete "placnetal" and replace it with
---placental---

IN THE CLAIMS

Cancel claims 1, 2, 4, 5, 6, 8, and 10.

Claim 3, line 1, after "claim" delete "1" and replace it with
---11---

cancel per R
1 7 (Amended). A method of detecting the existence of pregnancy in
2 humans comprising [contacting] obtaining serum or urine from a patient
3 within about two months of suspected conception, contacting said
4 serum or urine with the agent of claim [1] 11 and observing whether [an]
5 antibody/antigen [reaction occurs] reactions occur, [and] the occurrence
6 of said [reaction] reactions being indicative of pregnancy.

cancel per B
1 9 (Amended). The method of claim 7 wherein said serum [is with-
2 drawn] or urine is obtained from said patient within about two months of
3 suspected conception.

Add new claims 11-16.

cancel per R
1 11. A pregnancy detection agent prepared by the method comprising
2 a. first providing a mixture of placental proteins containing
3 antigenic pregnancy-specific proteins;

4 b. contacting said mixture of placental proteins with antibodies
5 raised against either pregnancy-specific proteins or normal human serum
6 proteins to cause said antibodies to react with pregnancy-specific
7 proteins or non-specific antigenic proteins contained in said mixture;
8 c. separating the reaction products of step (b) from the remainder
9 of the placental protein mixture to provide a mixture of antigenic
10 pregnancy-specific proteins isolated from non-specific antigenic
11 proteins;
12 d. inoculating a host animal with said mixture of antigenic
13 pregnancy-specific proteins to cause said host animal to raise anti-
14 bodies to said pregnancy-specific proteins;
15 e. isolating antibodies to said pregnancy-specific proteins from
16 antibodies to non-specific human proteins.

*amended
per
B*

1 12. The agent of claim 11 in which step (b) comprises contacting
2 said mixture of placental proteins with antibodies raised against
3 pregnancy-specific proteins.

*amended
per
B*

1 13. The agent of claim 11 in which step (b) comprises contacting
2 said mixture of placental proteins with antibodies raised against normal
3 human serum proteins.

*amended
per
B*

1 14. The agent of claim 11 in which said mixture of placental
2 proteins is prepared by providing a suspension of placental tissue in a
3 stabilizing medium, separating suspended solids from said suspension to
4 provide a supernatant solution, adding a salting agent to said super-
5 natant solutions to precipitate proteins and dissolving said precipi-
6 tated proteins to provide said mixture of placental proteins.

per B

1 15. The ^{*pregnancy detection method*} agent of claim 14 in which said stabilizing medium is
2 selected from the group of phosphate buffer and perchloric acid and said
3 salting agent is selected from the group of ammonium sulfate and sodium
4 sulfate.

per B

1 16. The ^{*pregnancy detection method*} agent of claim 14 in which said stabilizing medium is
2 phosphate buffer and said salting agent is ammonium sulfate.

REMARKS

Claims 1-10 stand rejected under 35 USC 102 and 35 USC 103 on various combinations of Fisk, the Japanese patent, the German patent, the Schuyler article and the Seppala article. Claims 1-10 also stand rejected under 35 USC 112 based upon insufficient exemplary matter and on insufficient disclosure as to specific test methods. The present amendment cancels claims 1, 2, 4, 5, 6, 8, and 10, and adds new claims 11-16. Claims 3 and 7 are made dependent on new claim 11.

In a telephone interview on December 5, 1977, applicant's attorney argued that the presently submitted product-by-process and use claims are sufficiently specific in scope to be in compliance with 35 USC 112, first paragraph and that the specification contained sufficient disclosure to support the claims under 35 USC 112, second paragraph. Applicant's attorney pointed out that Title 35 USC has no requirement for specific examples such as the detailed pregnancy test procedure suggested by the Examiner. The Examiner is referred to the case of In re Borkowsky, 164 USPQ 642 CCPA (1970) which clearly sets forth the requirements of the specification. The Court held that 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 this connection, applicant's attorney pointed out that workers in the field of immunology are well acquainted with tests such as immuno-diffusion and could easily determine proper test conditions such as the appropriate concentrations of antibody solution. The Examiner indicated that such an argument would be persuasive and suggested that the evidence be submitted.

The accompanying declaration of John E. Caton, one of several years experience in the fields of immunology and immunoanalytical chemistry, establishes that workers of ordinary skill in the field of immunology and immunoanalytical chemistry are well acquainted with double diffusion techniques for detecting the presence of antigens in a sample by the use

of antibody solutions. The use of declarations under 37 CFR 1.132 to establish the level of skill in the appropriate art is in conformance with the Court of Custom and Patent Appeals decision of In re Doyle, 179 USPQ 227 (1973). The declaration points out that it would be a simple matter for one to determine how to carry out the pregnancy test of the present invention if one were given a solution known to contain antibodies to proteins specific to the serum or urine of pregnant women, such as the solutions demonstrated in the specification. The declaration also sets forth the simple procedures customarily used in the art so that the Examiner can herself judge the level of skill of the art. In view of the fact that applicant's pregnancy detection method can be carried out with well-known test procedures with only a minimal amount of experimentation and that workers in the relevant art are accustomed to determining the proper conditions for conducting such tests, it is urged that the rejection under 35 USC 112 be withdrawn.

THE INVENTIVE CONCEPT

Applicant's inventive concept is based upon the discovery that antibodies raised against a mixture of pregnancy-specific placental proteins constitutes a highly effective pregnancy detection agent capable of reliably detecting the very early stages of pregnancy. Applicant's pregnancy detection agent is prepared by first providing a mixture of placental proteins; separating antigenic pregnancy-specific proteins from said placental protein mixture by immuno-chemical techniques and raising antibodies to the isolated pregnancy-specific proteins. The antibodies to pregnancy-specific proteins are isolated from antibodies to non-specific human proteins and can also be isolated from animal proteins to provide a highly effective pregnancy detecting agent. Applicant's invention constitutes the pregnancy detecting agent prepared by the described process and a method of use for the detection of pregnancy. The advantages of applicant's test are especially realized when it is used for the detection of pregnancy within about two months and within about one month of conception.

PRIOR ART

Nowhere in the prior art cited by the Examiner is any pregnancy detection agent or method described which makes use of antibodies to a mixture of pregnancy-specific proteins known to be present in early pregnancy. The use of a mixture causes the pregnancy detection agent of this invention to be more effective than prior art agents which contain antibodies to only one pregnancy-specific protein. Since the levels of pregnancy-specific proteins can be expected to vary among individual women, it can be readily seen that applicant's agent which detects more than one pregnancy-specific protein will be significantly more accurate than the prior art methods. The accuracy of the claimed pregnancy detecting agent in the claimed method is demonstrated on pages 14-16 of the specification. The agent of this invention proved positive against third trimester serum from three women in their third pregnancy, three women in their second pregnancy, and three women in their first pregnancy, as compared to a previously pregnant woman taking an estrogen-based birth control pill. The presence of 2 to 4 precipitation bands indicated the presence of at least two to four pregnancy-specific proteins. The detection agent gave negative tests against a succession of batches of pooled normal human serum and was positive against all of a series of forty samples of third trimester pregnancy serum. The agent tested negative against the sera of ten non-pregnant women including those previously pregnant. Of four cases tested, no reaction was shown with sera from women taking estrogen related contraceptives. The utility of claimed tests for the early detection of pregnancy was demonstrated successfully in a woman at 18-22 days after conception, 22-26 days after conception and at 28-32 days after conception. At least 10 distinguishable precipitation bands developed for the positive samples. The fact that the serum from different women demonstrated different numbers of early precipitation bands demonstrates the superiority of the pregnancy detection agent and method over the prior art. In the prior art methods, if a pregnant patient had an unusually low level of the single

detectable antigen, a false negative would result. If an irregular condition caused a high level of the detectable antigen in the non-pregnant patient, a false positive would result. The detection of multiple pregnancy-specific antigens with the claimed agent clearly reduces the possibility of either false result.

The Fisk method utilizes antibodies to only human chorionic gonadotropin (HCG). The pregnancy detecting agent of Fisk is prepared from purified HCG and there is no suggestion that pregnancy associated tissues would contain other pregnancy-specific antigens. There is no motivation from Fisk for one to prepare a pregnancy detecting agent utilizing antibodies to more than one protein. The Japanese reference L teaches only that HCG is present in human placenta and is useful as an antigen for preparing an antibody. There is no suggestion that placental tissue contains a mixture of pregnancy-specific antigens which can be used to raise antibodies useful in a pregnancy detecting method. The German patent teaches that pregnancy-specific proteins can be isolated from placental tissue. It should be noted that the beta-glycoprotein of the German patent is isolated from other placental proteins before it is used to raise antibodies. There is no suggestion that several pregnancy-specific proteins from placental tissue can be injected into a host and raise a detection agent capable of detecting the presence of a plurality of pregnancy-specific proteins in serum or urine.

The Schuyler reference actually teaches away from applicant's concept. While Schuyler suggests that a pregnancy detecting agent can be prepared from antibodies to HCG Schuyler specifically suggests that highly purified hormones be used to prepare the antiserum, rather than a mixture of several distinct antigens as shown by applicant. The Seppala reference describes the human placental lactogenic hormone, HPL, is present in pregnancy, and that HPL is antigenic. All of the data concerning the levels of HPL are for periods after the 30th week of gestation and do not indicate that HPL was present sufficiently early in gestation to be useful in a pregnancy test.

The T reference does not appear to deal with pregnancy detection at all but to the presence of alpha-feto proteins and their possible relationship to cancer. Chemical abstract 25559 apparently does not concern the detection of pregnancy but merely the presence of antigens in pregnancy serum and fetal serum. It should be noted that the antisera against placental tissue in pregnancy serum apparently were raised against the entire spectrum of serum or placental proteins and that no attempt was made to separate pregnancy-specific proteins from non-specific antigenic proteins prior to injection in the host. As indicated in applicant's disclosure such methodology would produce very low titer antiserum and may mask the host's response to the pregnancy-specific antigens. Again there is no suggestion that such an antiserum would be useful for detecting early stages of pregnancy and there is no discussion as to when the described proteins might have appeared during the gestation period. Chemical abstract 25702 describes the detection of beta-glyco proteins in maternal serum. There is no description of the method of preparing whatever antibodies were used to detect a beta-glyco protein; certainly no suggestion to use a mixture of antigenic pregnancy-specific placental proteins to prepare antibodies useful for detection of pregnancy.

THE CLAIMS

Claim 11 is directed to a pregnancy detection agent prepared by applicant's specific process of initially providing a mixture of placental proteins and separating antigenic pregnancy-specific proteins from the mixture prior to inoculation of a host animal and isolating the host produced antibodies to the specific proteins from antibodies to non-specific human proteins. The detection agent of claim 11 then is capable of detecting the presence of a variety of pregnancy-specific antigens in pregnancy serum or urine. Since there is no teaching in the cited references that a host could produce a sufficient amount of antibodies to a variety of pregnancy-specific proteins to be useful in a

pregnancy test then applicant's claimed detection agent which contains antibodies to a mixture of pregnancy-specific proteins could not be obvious. The presence of a mixture of antibodies is demonstrated in applicant's examples by the formation of several precipitation bands in tests against pregnancy serum. Claim 3 is directed to the agent of claim 1 prepared by the method which further comprises isolating antibodies to pregnancy-specific proteins from non-antibody host animal serum proteins. This procedure produces an antiserum which would be relatively more concentrated in the antibodies to pregnancy-specific proteins by the elimination of other animal proteins.

Claim 7 is directed to the use of the agent of claim 11 to detect pregnancy within about two months of supposed conception. It is in the detection of early pregnancy that the claimed pregnancy test excels. The use of the claimed agent to detect pregnancy within about 2 months of gestation requires a knowledge that several of the specific antigens present in early pregnancy are present in placental tissue and can evoke a combined antibody response sufficient to make the antiserum capable of detecting a mixture of early expressed antigens. Since this knowledge was apparently unavailable in the prior art, claim 7 could not be obvious. Likewise, claim 9, directed to the detection of pregnancy within about one month of conception is even more unobvious in view of the prior art methods.

Claims 12 and 13 are directed to the agent prepared using particular methods of immunologically separating pregnancy-specific proteins from a mixture of placental proteins. As described earlier, neither of the prior art methods make a separation but separates a single pregnancy-specific protein prior to injecting into the host. It is only the present applicant who recognizes that highly accurate antiserum can be raised against a mixture of pregnancy-specific proteins recovered from placental tissues and that such an antiserum is substantially superior for the early detection of pregnancy to prior art antiserum raised against a single antigen.

Claim 14 is directed to the agent of claim 11 prepared from the mixture of specific proteins provided by the precipitation techniques as shown in applicant's disclosure, pages 4-5. As indicated in the specification at page 13, lines 3-5, some of the unprecipitated proteins have shown to be pregnancy-specific. Claims 15 and 16 are directed to the agents prepared by the method of pages 4-5 and Example II.

The superiority of applicant's test methods using applicant's claimed detection agent is demonstrated by comparing applicant's results in the detection of very early pregnancy with the results of prior art immunological pregnancy tests. Some of the references accompanying the prior art letter, (Lamb, Bell, Dietrich et al., and Horwitz et al) clearly show the improved accuracy of applicant's invention. According to the Bell reference prior art pregnancy tests based on HCG range from 2-24% error or inconclusiveness. The greatest percentage of false positives appears during the period 35-49 days after the last menstrual period, indicating the reduced accuracy for the detection of early pregnancy. The Horwitz et al. article more dramatically illustrates the reduced accuracy during early stages of pregnancy. The particular test studied is only 32% accurate at 31-40 days from the last normal menstrual period and only 92% accurate in 41-50 days from the last normal menstrual period. The Lamb reference indicates that the tests investigated were based on HCG. The tests described had inaccuracies ranging from 12-26% for all periods of gestation testing.

The Dietrich article also demonstrates the accuracy of pregnancy tests based on HCG. It is stated that in early pregnancy there may not be enough HCG present in a urine sample to neutralize anti-HCG added, thereby giving false readings. Figure 1 of the Dietrich reference indicates the level of HCG normally present in pregnant women. It is apparent that a pregnancy test must be capable of detecting levels less than about 5,000 IU/L in order to reliably detect pregnancy sooner than about 50 days after the last menstrual period (which would be about 35 days after conception.) This sensitivity requirement creates complications when one considers the problem described in the Lamb reference;

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS: David W. Holladay :
SERIAL NO.: 755,100(70) : GROUP 120
FILED: December 28, 1976 : EXAMINER
FOR: AGENT AND METHOD FOR THE EARLY : Anna P. Fagelson
DETECTION OF PREGNANCY

DECLARATION UNDER 37 CFR 1.132

I, John E. Caton do hereby declare and affirm:

THAT I hold the degree of Ph.D in Analytical Chemistry from Iowa State University;

THAT I am employed as a Research Staff Member in the Analytical Chemistry Division of the Oak Ridge National Laboratory;

THAT I have published more than fifteen papers in professional journals relating to the fields of analytical and bioanalytical chemistry, including immunochemical techniques;

THAT during the years 1969-1974 I worked as an analytical chemist in the Molecular Anatomy Program of the Oak Ridge National Laboratory, during which time I carried out extensive work in the fields of gel electrophoresis, chromatographic separation of macromolecular solutions such as serum, and the study of techniques utilizing antigen-antibody reactions for analytical purposes;

THAT I am well acquainted with the ordinary level of skill of workers in the field of immunology and immunoanalytical chemistry;

THAT workers of ordinary skill in the field of immunology and immunoanalytical chemistry are well acquainted with the standard immunological methods for detecting the presence of antigens in solutions by contacting the solution with a solution containing antibodies to the antigens sought;

THAT in carrying out immunological tests for detecting the presence of antigens in solutions, workers of ordinary skill in immunology or immunoanalytical chemistry often make determinations of the proper titer of antibody solution to use for a particular concentration of antigen solution;

THAT if a person of ordinary skill in the art of immunology or immunoanalytical chemistry were given a solution known to contain antibodies to proteins specific to the serum or urine of pregnant women, said person would be able to test a sample of the serum or urine for the presence of these proteins specific to pregnancy serum or urine by the well-known Ouchterlony technique of double diffusion;

THAT one of ordinary skill in the art of immunology or immunoanalytical chemistry would normally carry out the Ouchterlony technique as follows:

The test would be carried out in a plate containing agar gel, with prepunched sample wells. Such plates are available commercially from several sources; for example, Hyland Laboratories of Costa Mesa, California. A sample of urine or serum would be placed in one well and the solution containing antibodies to proteins specific to pregnancy serum or urine would be placed in an adjacent well. The plate would then be set aside overnight and subsequently examined under indirect light. The presence of antigens specific to pregnancy serum or urine would then be indicated by a precipitant band which would appear as a white line between the two wells. No white line would indicate that the serum or urine had contained no antigens specific to pregnancy.

THAT, in general, the length of time necessary to carry out Ouchterlony double diffusion tests is not critical, and the plates with filled wells are usually set aside and examined the next day; however, if one wished to determine some minimum time, one could simply watch for the appearance of the white precipitant band.

THAT workers of ordinary skill in the art of conducting double immunodiffusion tests must often determine the proper concentration of antibody solution to use for detecting the presence of antigens in solutions such as urine or serum:

THAT if a person of ordinary skill in the art of conducting double immunodiffusion analysis were given a solution known to contain antibodies to proteins specific to the serum or urine of pregnant women, he would be able to determine the proper concentration or titer of antibody solution to use in a double diffusion test for the presence of these proteins specific to pregnancy by the following method with which he would be familiar:

Using a plate filled with agar containing a central well and five surrounding wells equidistant from the central well, a known sample of pregnancy serum or urine would be placed in the central well. A different concentration of antibody solution would be placed in each of the five surrounding wells. Upon being left overnight, precipitant bands form an irregular pentagon in the gel. A side of the pentagon which is located about equidistant between the central well and an antibody solution well indicates that the antibody solution in that well was the optimum concentration of antibody solution to use for a double diffusion test for pregnancy. For high concentrations of antibody solution, the precipitant band will be closer to the antibody solution well, and for low concentrations of antibody solution, the precipitant band will be closer to the central well.

THAT all statements herein made of my own knowledge are true and all statements made on information and belief are believed to be true;

THAT I am aware that willful false statements and the like are punishable by law or imprisonment or both (18 USC 1001) and may jeopardize the validity of the application or any patent issuing thereon.

/s/ John E. Caton
John E. Caton

December 30, 1977
Date

Date December 5, 1971

Application No. 10 157,100

Attny. Allen H. Hall

Examiner Page 2/5

RECORD OF INTERVIEW WITH EXAMINER

1. Brief description of any exhibit or demonstrations:

Reference to be tested which demonstrated low reliability in the early stages were discussed. He agreed to submit a 132 affidavit that one of ordinary skill in the art would know how to perform a simple immunodiffusion test.

2. Identification of claims discussed:

Revised proposed claims directed to the use of the product by process for detecting pregnancy within 30 days.

3. Identification of specific prior art discussed:

Only the general teachings of the cited art was discussed.

4. Identification of principal proposed amendments:

It was proposed to amend the claim to cover a specific product prepared by a specific process, and to require testing within 30 days.

5. Brief presentation of arguments made (general nature and thrust):

The general thrust of the argument was that the
want to see a policy of detentions, a minimum of
prosecution, specific protection, which is not
the present case, unless it is necessary, only one.

6. Other pertinent matters discussed:

The Examiner stated that he intended that the
were experienced, such as police officers, in which
which claimed that the present tests were effected
within 30 days.

7. General results or outcome of interview:

The Examiner indicated that he would withdraw
the §112 rejection as a result of the applicant's
the use of which in the art, and that he would not
withdraw the §102+103 rejection.

10/77
SDH

1075846

Allen Rizzell, CR

NO S.C.

copy of Request

James E. Denny
Assistant General Counsel for Patents
United States Department of Energy
Washington, D. C. 20545

Applicant :	David W. Holladay)	
Serial No. :	755,100)	
Filed :	December 28, 1976)	GROUP 170
For :	AGENT AND METHOD FOR THE EARLY DETECTION OF PREGNANCY)	

Receipt is acknowledged of the following in the above-identified application:

- | | |
|--|---|
| <input type="checkbox"/> Response to Office Action dated _____ | <input type="checkbox"/> Petition |
| <input type="checkbox"/> Letter to Draftsman | <input type="checkbox"/> Appeal Brief |
| <input type="checkbox"/> Affidavit | <input type="checkbox"/> Amendment under Rule 312 |
| <input type="checkbox"/> Notice of Appeal | <input type="checkbox"/> Directive |
| <input type="checkbox"/> Fee Authorization | <input type="checkbox"/> Withdrawal of Directive |
| <input type="checkbox"/> Appointment of Attorney | <input type="checkbox"/> _____ |
| <input type="checkbox"/> PTOL-85b Base Issue Fee | <input type="checkbox"/> _____ |
| <input type="checkbox"/> PTO-1094 with <input type="checkbox"/> Sheets Formal Drawings | <input type="checkbox"/> _____ |
| <input checked="" type="checkbox"/> Request for Extension of Time | <input type="checkbox"/> _____ |
| <input type="checkbox"/> _____ | <input type="checkbox"/> _____ |

12-14-77

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: David W. Holladay :
SERIAL NO.: 755,100(70) : GROUP 120
FILED: December 28, 1976 : EXAMINER
FOR: AGENT AND METHOD FOR THE EARLY : Anna P. Fagelson
DETECTION OF PREGNANCY :

REQUEST FOR EXTENSION OF TIME

The Commissioner of Patents & Trademarks
Washington, D. C. 20231

Sir:

It is respectfully requested that the period for response to the Office Action of September 19, 1977, be extended for an additional month to January 19, 1978. In a telephone interview with the Examiner on December 5, 1977, applicant's attorney argued that the specification was not defective under 35 USC 112 for failure to contain a detailed example of the pregnancy test procedure. It was pointed out that workers in the field of immunology and biochemical analysis are well acquainted with such tests and could easily determine the proper titer or concentrations of an antibody solution to use to detect the presence of antigens in a serum or urine sample. The Examiner indicated that such an argument would be persuasive if supported by evidence. Accordingly, applicant intends to submit expert testimony in the form of a declaration under 37 CFR 1.132 as to the level of skill in the relevant art. The additional time is needed for the preparation and execution of the declaration.

Respectfully submitted,

Allen H. Uzzell

Attorney for applicant

Oak Ridge, Tennessee

FTS-850-4334 (Commercial AC 615-483-8611, Ext. 34334)

Uzzell:d1m

APPROVED
DEC 14 1977

S/E L. Lott - RR
SUPERVISORY PATENT ASSISTANT
GROUP 8

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: David W. Holladay :
SERIAL NO.: 755,100(70) : GROUP 120
FILED: December 28, 1976 : EXAMINER
FOR: AGENT AND METHOD FOR THE EARLY : Anna P. Fagelson
DETECTION OF PREGNANCY :

REQUEST FOR EXTENSION OF TIME

The Commissioner of Patents & Trademarks
Washington, D. C. 20231

Sir:

It is respectfully requested that the period for response to the Office Action of September 19, 1977, be extended for an additional month to January 19, 1978. In a telephone interview with the Examiner on December 5, 1977, applicant's attorney argued that the specification was not defective under 35 USC 112 for failure to contain a detailed example of the pregnancy test procedure. It was pointed out that workers in the field of immunology and biochemical analysis are well acquainted with such tests and could easily determine the proper titer or concentrations of an antibody solution to use to detect the presence of antigens in a serum or urine sample. The Examiner indicated that such an argument would be persuasive if supported by evidence. Accordingly, applicant intends to submit expert testimony in the form of a declaration under 37 CFR 1.132 as to the level of skill in the relevant art. The additional time is needed for the preparation and execution of the declaration.

Respectfully submitted,

Allen H. Uzzell

Attorney for applicant

Oak Ridge, Tennessee

FTS-850-4334 (Commercial AC 615-483-8611, Ext. 34334)

Uzzell:d1m



**U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office**

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

Magelson Art Unit 125
12/28/76 755,100
David W. Holladay

MAILED

MAILED: **SEP 19 1977**

GRUP 120

James E. Denny
Assis. Gen. Coun. For Pats.
U.S. Energy Res. & Dev. Admin.
Washington, D.C. 20545

THIS IS A COMMUNICATION FROM THE EXAMINER
IN CHARGE OF YOUR APPLICATION.

COMMISSIONER OF
PATENTS AND TRADEMARKS

- This application has been examined.
- Responsive to communication filed on _____.
- This action is made final.

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS ACTION IS SET TO EXPIRE Three (3) MONTH(S)
_____ DAYS FROM THE DATE OF THIS LETTER.

FAILURE TO RESPOND WITHIN THE PERIOD FOR RESPONSE WILL CAUSE THE APPLICATION TO BECOME ABANDONED-
35 U.S.C. 133

PART I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

- 1. Notice of References Cited, Form PTO-892.
- 2. Notice of Informal Patent Drawing, PTO-948.
- 3. Notice of Informal Patent Application, Form PTO-152
- 4.

PART II SUMMARY OF ACTION

- 1. Claims 1-10 are pending in the application.
Of the above, claims _____ are withdrawn from consideration.
- 2. Claims _____ have been cancelled.
- 3. Claims _____ are allowed.
- 4. Claims 1-10 are rejected.
- 5. Claims _____ are objected to.
- 6. Claims _____ are subject to restriction or election requirement.
- 7. The formal drawings filed on _____ are acceptable.
- 8. The drawing correction request filed on _____ has been approved.
 disapproved.
- 9. Acknowledgement is made of the claim for priority under 35 U.S.C. 119. The certified copy has
 been received. been filed in parent application;
 not been received. serial no. _____ filed on _____
- 10. Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
- 11. Other

08-30-77

PART III

SERIAL NUMBER 755100

GROUP ART. UNIT 125

NOTIFICATION OF REJECTION(S) AND/OR OBJECTION(S) (35 USC 132)

	CLAIMS (1)	REASONS FOR REJECTION (2)	REFERENCES * (3)	INFORMATION IDENTIFICATION AND COMMENTS (4)
1	1-10	35USC102 35USC103	A	Terminology deemed to include any and all materials that may be employed as an antigen such as HCG, HPL etc.
2	1-4, 7,10	35USC102 35USC103	R/ RvA	R method deemed to meet claimed method for the reasons above set forth. Detection in one or two months would be inherent in R since HCG is then detectable as noted by A.
3	1-10	35USC103	L/M+A	No patentable merit seen in preparing the antisera to the placental extract of L/M by the means of injecting animals and adsorbing unwanted antibodies as taught by A, which is conventional in the art.
4	1-10	35USC103	A+S	No patentable merit is seen in preparing the antisera of S by the method of A.
5	1-10	35USC112 (par. 1&2)	---	Disclosure insufficient to support claims. See 5, below

6 Specification is objected to as including insufficient exemplary matter to support the claims. There are no specific examples of test methods with protocol and proportions employed. There appears to be no disclosure of characteristics of the "pregnancy specific proteins". Source of proteins includes numerous materials. Thus, impossible to determine bounds of the claims. See cited reference as to examples of materials found in pregnancy, which may be termed "pregnancy specific proteins". The disclosure, pg. 16 indicates that the antisera may also be employed for detecting cancer; thus, it appears that the proteins are no more "specific" than those shown in the art.

* Capital letters representing references are identified on accompanying Form PTO-892.
The symbol "v" between letters represents - in view of -.
The symbol "+" or "&" between letters represents - and -.
A slash "/" between letters represents the alternative - or -.

NOTE- Sections 100, 101, 102, 103, and 112 of the Patent Statute (Title 35 of the United States Code) are reproduced on the back of this sheet.

EXAMINER
A. Fagelson

TEL. NO.
(703) - 557 - 2577

ANNA P. FAGELSON
EXAMINER
ART UNIT 125

TO SEPARATE, HOLD TOP AND BOTTOM EDGES, SNAP—APART AND DISCARD CARBON

FORM PTO-46-42 (REV. 12-76) (FORMERLY PTO-892)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	SERIAL NO. 755100	GROUP ART UNIT	ATTACHMENT TO PAPER NUMBER 2
NOTICE OF REFERENCES CITED		APPLICANT (S) Holladay DW		

U.S. PATENT DOCUMENTS						
	DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
A	3171783	3/1965	FISK	424	12	
B						
C						
D						
E						
F						
G						
H						
I						
J						
K						

FOREIGN PATENT DOCUMENTS							
	DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT PAGES OR SPEC
L	42-10369	6/1967	Japan	TRIKOKU	424	100	- 3
M	2157610	5/1973	Germany	Bohn	424	12	- 7
N							
O							
P							
Q							

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)	
R	Schuyler, PSEBM, Vol. 75, 1950 pp 517-555
S	Seppala, Acta Obstet. Gynec. Scand, Vol. 49, 1970, pp 143-147
T	Uriel, CR Acad. Sc. Paris, Vol. 265 3 July, 1967, Ser. D, pp 758
U	Brock, Chem Abs. Vol. 83, 1975 Ab. No. 25559a
V	Tatra, Chem Abs. Vol. 83, 1975 Ab. No. 25702g
U	

EXAMINER AFagelson	DATE 8/22/77
------------------------------	------------------------

S.N. 755,100(7)

AGENT AND METHOD FOR THE EARLY DETECTION OF PREGNANCY

Inventor: David W. Holladay ..

1075853

ABSTRACT OF THE DISCLOSURE

An agent capable of detecting the early stages of pregnancy in humans is provided by preparing an antiserum to proteins isolated from pregnancy-associated material such as placentas. By conventional techniques of detecting antigen-antibody reactions, the antiserum is used to detect the presence of pregnancy-specific proteins in women at the early stages of pregnancy.

Background of the Invention

This invention was made in the course of, or under, a contract 10 with the Energy Research and Development Administration. It relates in general to pregnancy detection and, more specifically, to an agent and method for detecting pregnancy in women during the very early stages. Pregnancy has been detected as early as 18 days after conception according to this invention.

There has long been a need for a simple, reliable test which is capable of detecting pregnancy within a short time after conception. Such a test would be of great value for those women with an ancestral history of genetic disorders so that preventive therapy could be initiated as soon as possible. Those women working in potentially toxic or radio- 20 active environments or taking potentially harmful medication also have a need for early pregnancy information. More recently, it has become important for women fitted with intrauterine devices to have them removed soon after conception.

In the prior art pregnancy has been detected by a variety of well-known methods. Most of the prior art methods are unreliable when administered sooner than sixty days after conception. One method which has been shown effective for early pregnancy detection is described by B.B. Saxena et al. in the article "Radioreceptor Assay of Human Chorionic Gonadotropin: Detection of Early Pregnancy" Science, Vol. 8, pp. 793FF. (1974). This 30 method is rather complex and requires the use of radioactive substances, and, therefore, might be unsuitable for routine clinical use. The present invention involves the use of host-produced antibodies to detect the presence

of pregnancy-specific proteins in the sera of women in the early stages of pregnancy. The occurrence of pregnancy-specific components in sera from pregnant women was first demonstrated by Thornes and reported in an article by MacLaren et al. in Am. J. Obstet & Gynecol. 78:939 (1959). Smithies in Adv. Protein Chem. 14:65(1959) observed the presence of a pregnancy-associated alpha-globulin. Hirschfeld and Soderberg in Nature 187:332(1960) found two precipitates on immunoelectrophoresis of pregnancy sera. Gall and Halbert, Int. Arch. Allergy Appl. Immunol. 42:503(1972); and Lin et al., Am. J. Obstet, Gynecol. 118:223(1974) 10 observed and characterized four sequential pregnancy-associated plasma proteins found in the third trimester of pregnancy. Antisera has been raised against pregnancy plasma and then absorbed exhaustively with non-pregnancy plasma. Bohn, in Arch. Gynaekol. 210:440(1971), used rabbit antihuman placenta antiserum absorbed with male serum to detect four pregnancy-associated plasma proteins. Only one was considered pregnancy-specific and two could sometimes be detected in sera from non-pregnant female subjects, especially those taking oral contraceptives. Berne, in Clin. Chem (Winston Salem, N.C.) 19:657, Abstr. 093, 1973 found pregnancy zone protein in women six weeks pregnant. The protein was also found in 20 men and non-pregnant women. While the prior art was aware of the presence of proteins associated with pregnancy, there was no awareness of proteins which were specific to pregnancy and which could also be detectable sufficiently early in the gestation period to be useful as a pregnancy test.

Summary of the Invention

It is an object of this invention to provide an agent capable of detecting the early stages of pregnancy in humans.

It is a further object to provide an agent capable of detecting early pregnancy by simple and well-known clinical operations.

It is a further object to provide a method for the detection of pregnancy which requires no radioactive material or complex procedures. 30

It is a further object to provide an agent and method for detecting pregnancy which is effective less than thirty days after conception.

These and other objects as will be apparent are provided according to this invention by a pregnancy detection agent comprising an antibody to a pregnancy-specific protein isolated from non-specific antibodies and prepared by the method comprising:

a) first providing a mixture of proteins occurring in pregnancy-associated material and containing an antigenic pregnancy-specific protein;

b) isolating said antigenic pregnancy-specific protein from non-specific antigenic proteins;

c) inoculating a host animal with said isolated pregnancy-specific antigenic protein to cause said host animal to raise antibodies to said pregnancy-specific antigenic protein;

d) harvesting serum proteins from said inoculated host animal, said serum proteins containing antibodies to said pregnancy-specific antigenic protein, and;

e) isolating said antibodies to said pregnancy-specific antigenic protein from antibodies to non-specific human proteins.

The preferred pregnancy-associated material for obtaining the proteins is placentas. More concentrated pregnancy-specific antibodies are obtained by separating pregnancy-specific antibodies from the remaining animal serum proteins and/or antibodies.

A method of detecting the existence of pregnancy in humans according to this invention comprises contacting serum or urine from a patient with an antibody to a pregnancy-specific protein isolated from non-specific antibodies, and observing whether an antibody/antigen reaction occurs, the occurrence of said reaction being indicative of pregnancy. It is possible that other body fluids such as lymphatic fluid might contain pregnancy-specific proteins according to this invention, however, urine and serum are most suitable for routine clinical testing.

Detailed Description

An important aspect of this invention is the discovery that structures and substances produced during the course of pregnancy in women contain proteins which are also present in detectable quantities in the very early

stages of pregnancy. Another important aspect is the discovery that at least some of these pregnancy-associated proteins are both highly specific to pregnancy and sufficiently antigenic to raise antibodies in host animals which can then be isolated and used to detect the presence of the proteins in pregnant women relatively soon after conception, by standard immunological techniques.

For purposes of this invention, the terms specific and non-specific relate to pregnancy-specific and non-pregnancy-specific. A non-specific protein is one which is not specific to pregnancy. A pregnancy-specific
10 antibody is an antibody to a pregnancy-specific protein. A non-specific antibody is an antibody to a non-specific protein. The term "antigenic" protein refers to a protein capable of producing detectable antibodies in a host animal. For purposes of this invention, a non-antigenic protein is one which raises no more than miniscule amounts of antibody which would not significantly interfere with the detection of pregnancy-specific proteins by pregnancy-specific antibodies.

The first step in preparing the pregnancy detection agent of this invention is to prepare a mixture of proteins occurring in pregnancy-associated materials. For purposes of this invention the term "pregnancy-associated material"
20 is defined as tissues or fluids such as placentas, umbilical, amniotic fluid, fetal tissue, etc. which are produced during pregnancy. Placentas are preferred because of their ready availability. The separation of proteins from pregnancy-associated material need not be quantitative. The mixture of proteins can be prepared from the pregnancy-associated material by a number of well-known biochemical techniques for concentrating proteins from biological tissues or fluids. This protein mixture will contain antigenic pregnancy-specific protein. While it has been found that there are normally several antigenic pregnancy-specific proteins present in pregnancy-associated material, only one need be present for purposes of this invention.
30 For example, pregnancy-associated tissues can be diced and homogenized in a stabilizing medium to minimize precipitation and agglomeration of proteins. Suitable stabilizing media include pH7 phosphate buffer, or a mild acid such

as 0.1 M perchloric acid. The homogenized suspension is then separated such as by centrifugation, filtration, etc. to provide a supernatant liquid. Proteins can be separated from the supernatant by adding a precipitant. Suitable precipitants include ammonium sulfate, sodium sulfate, or other salting agents as are known in the art. The precipitated proteins are separated from the solution, e.g., by centrifugation and redissolved in a buffer solution containing a bacteriostat or bactericide; for example, 0.1% sodium azide to prevent growth of unwanted bacteria. Other suitable reagents include merthiolate, toluene, butanol, chloroform, etc. The protein solution can then be dialyzed against the same buffer to remove the traces of the precipitant. Dialysis can be carried out with any of the well-known dialysis membranes such as viscose dialysis tubing sold by Union Carbide Corporation under the trademark VISKING. The protein solution is separated from precipitated material to provide a relatively pure solution of proteins.

The next step in preparing the agent of this invention is to isolate antigenic pregnancy-specific protein from non-specific antigenic proteins. Preferably, this isolation should be complete, however, small amounts of antigenic non-specific proteins can be tolerated with the specific proteins since subsequent processing is capable of removing non-specific antibodies. It has been found that the removal of most or all of the antigenic non-specific proteins from the mixture of proteins prior to injection into a host animal will yield a substantially higher titer antisera in the host. When non-specific antigenic proteins are removed prior to inoculation, the host animal's immune response is directed toward the pregnancy-specific protein. When non-specific antigenic proteins are not removed prior to inoculation, the normal human proteins are likely to shield out the response to the pregnancy-specific proteins. Preliminary tests have shown that when non-specific antibodies are not at least partially removed prior to injection into the host, fewer bands of precipitation are observed in the pregnancy test and only at later times than in the tests with antisera raised against partially or highly purified pregnancy-specific proteins.

There are at least two methods by which antigenic pregnancy-specific proteins can be isolated from non-specific antigenic human proteins. In one instance, the mixture can be contacted with antibodies raised against human serum containing no pregnancy-specific proteins, hereinafter termed anti-normal human serum. Suitable serum can be collected and pooled from a large sample of males and non-pregnant females and used to raise host-produced antibodies. The antibodies to this pooled serum will react with non-specific antigenic proteins in the mixture, and the reaction products can be separated from the mixture, thereby providing a mixture of pregnancy-
10 specific proteins and non-antigenic, non-specific proteins. For purposes of this invention, such non-antigenic proteins can be regarded as non-interfering material. Alternatively, the protein mixture obtained from the pregnancy-associated material can be contacted with antibodies raised against pregnancy-specific proteins, whereby only antigenic pregnancy-specific proteins react with the antibodies and are recoverable from the mixture. This method will provide an inoculant more concentrated in pregnancy-specific protein.

The preferred method of carrying out antibody/antigen reactions for isolating the pregnancy-specific proteins from antigenic non-specific
20 proteins is by passing the protein mixture through an immunoabsorbent column prepared with appropriate antibodies, e.g., anti-normal human serum or anti-pregnancy-specific proteins. When the column is prepared with anti-normal human serum (which reacts with normal serum proteins), contaminating normal serum proteins are bound to the column and thus separated from pregnancy-specific proteins. Accordingly, the unbound fraction contains pregnancy-specific proteins isolated from antigenic non-specific protein. If the column is prepared with antiserum raised against pregnancy-specific protein, the normal proteins pass through unbound and the pregnancy-specific proteins are bound to the column and may be eluted with a suitable eluent
30 capable of separating antibody/antigen complexes, such as a 2.5 M sodium thiocyanate solution.

The next step in preparing the agent of this invention is to inoculate a host animal with the isolated pregnancy-specific antigenic proteins to cause the host animal to raise antibodies to the pregnancy-specific proteins. All that is required prior to inoculation is that the proteins be in a solution capable of injection. The solution from the immunoadsorbent step can be concentrated and directly injected. If the pregnancy-specific proteins had been isolated by being bound to the column and eluted with thiocyanate, the thiocyanate should be removed to return the proteins to their native structure. This can be accomplished by dialysis against
10 phosphate buffer. Of course, antibodies could be raised less efficiently against dilute solutions. Suitable hosts are virtually any warm-blooded animals such as goats, cattle, horses, rabbits, chickens, etc. Goats are preferred because they have an extensive and multi-varied antibody response, normally superior to other animals. Of course, it can be readily appreciated that the terms "antigenic" and "non-antigenic" refer to the particular host animals being used since an antigenic protein to one animal may be non-antigenic to others. No warm-blooded animal has proven incapable of producing antigens to at least one pregnancy-specific protein. As is customary in the raising of antibodies, conventional adjuvants can be
20 employed to accelerate antibody-production. The injection schedule can be expected to vary for different types of hosts and it is well within the skill of those acquainted with the manufacture of vaccines, etc. to work out a suitable injection schedule. The same injection schedule will be appropriate for producing anti-normal human serum.

The next step in preparing the agent of this invention is harvesting serum proteins from the inoculated host animal. After an inoculation schedule (e.g., weekly) of several months, the host animals are bled and their sera harvested. The serum is separable from whole blood by conventional means such as centrifugation after clotting. The serum contains
30 host serum proteins comprising antibodies to at least one of the pregnancy-specific proteins.

The next step in preparing the agent of this invention is isolating antibodies to the pregnancy-specific proteins from antibodies to non-specific human proteins. Even if the proteins injected into the host were completely free of antigenic non-specific human proteins, it is necessary that this isolation be performed to remove traces of antibodies to non-specific human proteins which were native to the host or which were raised against marginally non-antigenic human proteins. The preferred method of carrying out this isolation step is by passing the host serum through an immunoabsorbent column prepared with normal human serum proteins. The material which is not adsorbed on the column is thus a mixture containing an antibody to a pregnancy-specific protein isolated from non-specific antibodies, and as such is now an agent capable of detecting the presence of pregnancy-associated proteins in women, in accordance with this invention. It is preferred that this column be loaded with serum proteins obtained from a large sample of males and non-pregnant females, having all of the blood types. Females taking estrogen-based oral contraceptives should be included in the sample, if possible. The serum, of course, is typically collected as plasma separated from whole blood and lyophilized or freeze-dried for storage. This pooled serum is also preferred for injection into a host to produce anti-normal human serum as used for carrying out this adsorption of non-specific antigenic human proteins from the original protein mixture. The anti-normal human serum can be prepared by any of a number of well-known serological methods. The preferred method is to make balanced antisera which contains sufficient antibodies to remove all of the non-specific antigenic proteins in a protein mixture. The procedure for carrying out immunoabsorption chromatography and for preparing a balanced antisera is described in N.G. Anderson et al., "Cyclic Affinity Chromatography: Principles and Applications," Analytical Biochemistry 68, 371-393 (1975), which is herein incorporated by reference.

It is generally desirable that pregnancy-specific antibodies be further isolated from some or all of the animal serum proteins. This can be accomplished by passing the animal protein mixture, either before or after

pregnancy-specific antibodies are isolated from antibodies to non-specific proteins, through a conventional column containing diethylaminoethyl (DEAE) cellulose beads. Such beads are capable of adsorbing immunoglobulin from the remainder of the animal's proteins. Diethylaminoethyl cellulose suitable for this process is commercially available from Whatman Corporation as cellulose microfiber bundles, 40-300 microns, and as microgranules with an average equivalent spheres diameter of about 40 microns. Differential chromatography using DEAE cellulose for separating placental proteins and antibodies to placental proteins is described in Molecular Anatomy Program, 10 First Semi-Annual Progress Report (1971) ORNL-4733, pp. 384-394, available from National Technical Information Service, Department of Commerce, Springfield, Virginia and which is herein incorporated by reference. Antibodies to pregnancy-specific proteins can be further separated, if desired, from other animal antibodies by passing the antiserum through an immunoadsorbent column loaded with pregnancy-specific proteins. The pregnancy-specific antibodies are adsorbed thereon and can be eluted with a solution capable of dissociating antigen/antibody complexes. An example of such a solution is a chaotropic ion solution such as thiocyanate or trichloroacetate solution. Ammonium and sodium salts are preferred. The preferred antibody eluent is 20 a 2.5 M sodium thiocyanate, 0.1 M sodium phosphate pH 7.0, 0.1% sodium azide (bacteriostat) solution. Below 1.0 M thiocyanate does not give quantitative elution. It can be readily seen that this adsorption/elution step is capable of effecting the isolation of pregnancy-specific antibodies from non-specific human proteins as well as from animal protein.

The immunoadsorbent columns useful for preparing the agent of this invention can be prepared by conventional techniques. What is necessary is that the column contain the desired antibodies or antigens immobilized so that they are reactive with their corresponding antigen or antibody. The immunoadsorbent columns therefore provide both reaction contact between antigen 30 and antibody as well as separation of unreacted components which exit the column as the unbound fraction. Such immunoadsorbent columns are well-known in the art of serology; see, for example, J. Porath et al. Nature Vol. 215

p. 1491 (1967). For purposes of illustration, the following example is provided for the preparation of an immunoabsorbent. The method is suitable for binding any desirable protein to the packing material. Those skilled in the art can prepare similarly effective immunoabsorbent columns by a variety of different methods.

Example I

Protein to be bound (either antigen, antibody, or non-antigenic) is concentrated in aqueous solution to 70 ml and dialyzed against the coupling buffer to provide a protein solution in coupling buffer. Coupling
10 buffer is the buffer in which the binding reaction ultimately occurs, and in this case is 0.1 M phosphate, pH 6.5. Other coupling buffers such as carbonates can be used. Seventy ml (a like volume as the protein) of agarose gel beads is washed with at least 20 volumes of 0.1 M phosphate pH 6.5. Suitable agarose beads are available under the trademark Sepharose and are available from Pharmacia Fine Chemicals, Piscataway, New Jersey. The agarose gel beads (Sepharose 4B) used in this example contained 4% agarose and had particle sizes of 40-190 microns in the wet state. The preparation of Sepharose gels is described by Hjerten in "The Preparation of Agarose Spheres for Chromatography of Molecules and Particles," Biochim.
20 biophys. Acta 79, pp. 393-398 (1964). The washed agarose is mixed with an equal volume of coupling buffer in a 400 ml beaker with magnetic stirrer, and in an ice bath to form a slurry. The slurry was titrated to a pH of 11.5 with 6 M NaOH. To the cooled slurry is added 21 g. of cyanogen bromide (0.3 g/ml Sepharose) and the pH was maintained at 11.5 with NaOH and the temperature was maintained at about 20°C. Under these conditions the cyanogen bromide reacts with the Sepharose to cause activation. The reaction was allowed to proceed until no more decrease in pH was noted, to ensure the end of the activation. The activated slurry was washed with 4°C coupling buffer on a coarse Buchner Funnel. This washing must proceed
30 within 2 minutes from the completion of activation to prevent excessive crosslinking of active sites. The washed CnBr-activated Sepharose 4B was mixed with the solution of protein to be bound and the mixture was slowly

tumbled overnight at 4°C. Additional mixing (i.e. over 24 hours) would normally provide more extensive attachment of proteins. As is customary, spacers or chemically active ligands may be used if desired. After mixing, the unbound protein is washed off in coupling buffer (0.1 M 6.5 pH phosphate) and washing is continued until no background absorbance is detected in the wash water. After this washing, the loaded Sepharose is tumbled in 1M ethanolamine pH 10 for 2 hours to load any remaining sites on the resin. The ethanolamine is washed off with water or pH 7 phosphate buffer and the loaded Sepharose is washed alternately with 0.10 M sodium acetate pH 4, 10 0.1% sodium azide to prevent bacterial growth, 1.0 M sodium chloride, 0.1 M sodium borate pH 8.5, 0.1% sodium azide and 1.0 M sodium chloride. An additional wash with 0.1 M glycine NaOH (pH 11.0) can also be included. These washes serve to remove materials which are not covalently bound to the support material. The protein-loaded Sepharose is finally washed with 2.0 M sodium thiocyanate, 0.06 M sodium phosphate pH 7.0 and 0.1% sodium azide, followed by 0.1 M sodium phosphate pH 7.0 and 0.1% sodium azide until background absorbance is obtained. The protein is now firmly bound to the Sepharose to provide an immunoabsorbent particle ready for use as column packing.

20 The pregnancy detection method of this invention comprises contacting serum from the patient with antibodies to pregnancy-specific proteins isolated from non-specific antibodies. Since the existence of the antigenic pregnancy-specific proteins has herein been demonstrated, the source of the protein is not an essential feature of the pregnancy test. The desired antibodies can be prepared by the methods disclosed herein, or it is conceivable that at least one of the pregnancy-specific proteins can be identified and synthesized, thereby avoiding the necessity of separating the pregnancy-specific protein from antigenic non-specific human proteins. The synthetic protein could then be used directly to raise antibodies useful for detecting 30 pregnancy.

All that is necessary for this pregnancy detection method is that serum from a patient be contacted with an antibody raised against a pregnancy-specific protein and the results observed to detect signs of antibody/antigen

reaction. The occurrence of the reaction, of course, indicates pregnancy. It can be readily appreciated that any of the several methods for detecting antigen/antibody reaction is suitable for detecting pregnancy according to my method. Examples of such methods are gel precipitation, gel electropherograms, immunoelectrophoresis, crossimmunoelectrophoresis, and immunodiffusion.

The primary utility of the pregnancy detection method of this invention is for detecting pregnancy within about 2 months of conception. After 2 months, pregnancy is reliably detectable by well-known external symptoms. The method of this invention is particularly useful for detecting pregnancy within one month of conception, when other tests are inconclusive. It is likely that it will prove effective within one week of conception.

In order to demonstrate a preferred method for preparing the pregnancy detection agent of this invention and the operability of the agent for detecting pregnancy, a detailed example is presented. It will be apparent that substantial variation from the procedures described therein can be made without departing from the spirit and scope of the invention. For example, it will be apparent to those skilled in the art that plasma can be substituted for serum in many instances and can be considered equivalent for purposes of this invention.

Example II

Preparation of crude placental extract. Term placentas were obtained at delivery and chilled in 0.85% sodium chloride solution for preservation. The placentas were washed for 1 hr. in tap water to remove a maximum of blood elements and serum proteins. While the mother's or infant's serum would likely contain traces of pregnancy-specific proteins, the placenta contains substantially more of those proteins. Fat was cut away since it is superfluous and non-antigenic, and the placentas were cut into pieces about 1 cm on a side and frozen. Subsequently, the frozen material was homogenized in three volumes of 0.1 M pH 7 phosphate buffer. The homogenate was centrifuged at 17,000 x g (acceleration of gravity) for 10 min. in an angle-head centrifuge, the supernatant was collected, and the pellet was rehomogenized in two volumes of the same buffer, and a second

centrifugation at 17,000 x g for 10 min. was done. The proteins of interest were precipitated from the pooled supernatants by adding solid ammonium sulfate to 55% saturation and separated by centrifugation. Some of the unprecipitated proteins have since been shown to be pregnancy-specific and can be recovered, if desired. Following centrifugation, the material was dissolved in a minimal amount of 0.1 M pH 7 phosphate buffer containing 0.1% sodium azide, and the solution was dialyzed against this same buffer, using viscose dialysis tubing such as is sold by Union Carbide Corporation. Any material that precipitated was removed by centrifugation. The precipitate was mostly large macromolecules representing the connective tissue of the placenta. Any serum proteins left in the solution were removed by cycling it over an immunoabsorbent column loaded with anti-normal human serum. The cycling was carried out automatically by an apparatus as disclosed in Anderson et al., Analytical Biochemistry 66, 159-174 (1975). During cycling, the normal serum antigenic proteins were eluted after each loading with ammonium or potassium thiocyanate and the column was re-equilibrated with phosphate buffer prior to the loading of the next sample.

The unbound fraction emerging from this treatment was still a complex mixture, containing at least 20 different proteins, as judged by gel electrophoresis. The solution could not be concentrated beyond 20 A_{280} units per ml without undesired precipitation occurring on storage, especially on freezing and thawing. An A_{280} unit is the quantity of protein per ml of water or buffer giving an absorbance of 1 at 280 nm in a cell with a light path of 1 cm. Apparently, the bulk of the material that precipitated was placental tissue proteins, mostly acid in nature. If desired, this material could be separated into several subfractions by chromatography over diethylaminoethyl cellulose. Though not essential to the operability of the process, some separations of placental tissue proteins that appear in the serum from those that do not could be effected to provide a more concentrated agent.

Only 10 to 15 of the proteins in the mixture have proved to be sufficiently antigenic to result in detectable antibody production to them. It is likely possible to modify the extraction procedure (for example, mild acid extraction

of diced placenta, e.g., with 0.1 M perchloric acid, without homogenization) in order to minimize the extraction of proteins that do not appear in the plasma; however, this is unnecessary for the operability of the detection method.

The placental extracts, minus non-specific serum proteins but still containing the relatively non-antigenic tissue proteins mentioned, were concentrated to about 15 A_{280} units per ml for injection purposes. An estimate of the antigen content was 0.1 to 1 mg/ml, based on intensity of reaction in immunodiffusion. The extract was mixed with equal volume of Freund's complete adjuvant, and the preparation was injected subcutaneously into goats and rabbits, either in the hips or behind the neck (4 ml for goats, 1 ml for rabbits). Freund's complete adjuvant is commercially available from Difco Laboratories and contains sterile light mineral oil plus emulsifier and heat killed *Mycobacterium butyricum* (0.5 mg dry weight per ml).

Intravenous injection should normally be avoided due to the increased likelihood of anaphylactic shock and sudden death of the host. Thereafter, the same amounts of extract without adjuvant were injected subcutaneously at weekly intervals. Every fourth week, injection was done with antigen plus adjuvant. First bleeding was at 5 weeks (40 ml for rabbits and 150 ml for goats) and was biweekly thereafter to test for antibody production. High-titer antiserum was obtained, usually in about 7 to 8 months. The antiserum was absorbed on a conventional immunoabsorbent column loaded with lyophilized, pooled serum obtained from normal human males (100 mg/ml of antiserum) to remove non-specific antibodies. The unbound fraction contained pregnancy-specific antibodies and normal animal protein, isolated from antibodies to non-specific human protein. If desired, i.e., for commercial preparation, the bulk of the animal protein can be removed by DEAE cellulose chromatography, in which the unbound fraction can be passed through a DEAE cellulose column whereby the antibodies load the column and the bulk animal protein passes through.

Gel precipitation was used to test the antiserum-to-placental-antigens for the presence of antigens that appeared in the sera of pregnant women.

The antiserum was first tested against sera from women in the third trimester of pregnancy. Tests against the sera from three different women in their first pregnancy, three women in their second pregnancy, and three women in their third pregnancy showed positive reactions within 5 hours with 2 to 4 bands clearly visible after 40 hours. The control for all three plates was from a 28 year-old woman who had one successful pregnancy 5 years previously, and who since that time had been taking an estrogen-based birth control pill. No reaction was noted at any time with the control.

The antiserum gave negative tests against a succession of batches of 10 pooled normal human male sera. It was positive against all of a series of 40 samples of third-trimester pregnancy sera. It was negative against the sera of ten non-pregnant women, including those previously pregnant. Of four cases tested, no reaction has been shown with sera from women taking estrogen-related contraceptives. In order to demonstrate the detection of pregnancy early enough to be valuable for a pregnancy test, several runs were made on sera from women who suspected pregnancy. A serum sample was obtained from a 29 year-old woman 6 days after she had failed to begin her menstrual period. A good estimate of the time duration since conception was 18 to 22 days. A serum sample was obtained from a 25 year-old woman 10 days after she had 20 failed to begin her menstrual period, giving a time duration of 22 to 26 days since conception. The samples were tested by double diffusion against anti-serum to placental antigens. Placental extract and serum from a woman in the third trimester of her third pregnancy were used as reference.

After 16 hours one sharp band and one faint band of precipitation were observed in both samples, and the two bands merged or showed identity with bands from both ^{placental} ~~placental~~ extract and the third-trimester pregnancy serum sample. For both samples, a precipitin band could be observed as early as 1 hour after the start of immunodiffusion. Three precipitin bands were observed for the second sample within 24 hours after loading. Improved 30 immunodiffusion techniques would likely permit a decision on pregnancy on the same day the blood sample is withdrawn.

Another early pregnancy serum sample, taken at 28 to 32 days after conception, was tested against the antiserum and gave two precipitin bands within 16 hours. Pregnancy was confirmed at 8 weeks of gestation by routine examination for all three of the women. The human placental antigen preparation gave at least ten distinguishable precipitin bands with the antiserum. The precipitin bands that are the strongest for very early pregnancy samples show identity with the weakest bands from samples from third-trimester pregnancies. This indicates that the antigens involved reach a concentration peak early in pregnancy, and then slowly decrease during gestation. It is possible that some early appearing antigens may not be detectable in the second and third trimester.

While the particular pregnancy-specific antigenic proteins utilized for this invention have not yet been characterized, their identification is not essential to manufacturing the agent of this invention or to performing the pregnancy test of this invention.

Recent advances in cancer research have intimated that certain proteins present in pregnancy-associated material such as placentas, are re-expressed in the early stages of cancer. This is consistent to the idea that the formation of the fetus is physiologically similar to the formation of tumor cells in cancer patients. For a description of the observed relationship between fetal and placental proteins see, for example, Ruoslahti et al., *Int. J. Cancer*: 7 218-225 (1971), Thompson et al., *Medical Sciences* 64 164-6 (1969), Gold, *Preg. Exp. Tumor Res.* 14: 43-58, and *Am. Assn. Cancer Res. Abstracts* 490, 568, 584, 842 (1974). Accordingly, a possible utility for the pregnancy detection agent of this invention is the early detection of at least some forms of cancer.

What is claimed is:

*Cancelled
per
A*

- 1 1. A pregnancy detection agent comprising antibodies to a pregnancy-specific protein isolated from non-specific antibodies and prepared by the
- 3 method comprising:
- 4 a) first providing a mixture of proteins occurring in pregnancy-associated material and containing an antigenic pregnancy-specific protein;
- 6 b) isolating said antigenic-pregnancy-specific protein from non-specific
- 7 antigenic proteins;
- 8 c) innoculating a host animal with said isolated pregnancy-specific
- 9 antigenic protein to cause said host animal to raise antibodies to said
- 10 pregnancy-specific antigenic protein;
- 11 d) isolating antibodies to said pregnancy-specific proteins from anti-
- 12 bodies to non-specific human proteins;

*Cancelled
per
A*

- 1 2. The agent of claim 1 in which said proteins occurring in pregnancy-associated material are recovered from placentas.

*per
A
Cancelled
per
B3*

- 1 3. The agent of claim 1 wherein said method further comprises isolating
- 2 antibodies to said pregnancy-specific proteins from non-antibody host animal
- 3 serum proteins.

*Cancelled
per
A*

- 1 4. A method for detecting the existence of pregnancy in humans comprising
- 2 contacting serum or urine from a patient with an antibody to a
- 3 pregnancy-specific protein isolated from non-specific antibodies, and
- 4 observing whether an antibody/antigen reaction occurs, the occurrence of
- 5 said reaction being indicative of pregnancy.

*Cancelled
per
A*

- 1 5. The method of claim 4 wherein said serum or urine is withdrawn
- 2 from said patient within about two months of conception.

*Cancelled
per
A*

- 1 6. The method of claim 4 wherein said serum or urine is withdrawn
- 2 from said patient within about one month of conception.

*Cancelled
per
A
Cancelled
per
B*

- 1 7. A method of detecting the existence of pregnancy in humans comprising
- 2 contacting serum or urine from a patient with the agent of claim
- 3 1 and observing whether an antibody/antigen reaction occurs, and occurrence
- 4 of said reaction being indicative of pregnancy.

*Cancelled
per
A 2*

~~8. The method of claim 7 wherein said serum is withdrawn from said patient within about two months of conception.~~

*amended
per cancelled
A 2*

~~9. The method of claim 7 wherein said serum is withdrawn from said patient within about one month of conception.~~

*Cancelled
per
A 2*

~~10. The method of claim 7 wherein said proteins occurring in pregnancy-associated material are recovered from placentas.~~

*claims (11), (12, 13, 14), 15 + 16 added per A
Cancelled per B amended per B*

claims 17, 18 + 19 added per B

As the below named inventor, I declare that the information in item 201 below is true.
that I believe that I am the original, first and sole inventor of the invention described and claimed in

- the attached application (declaration accompanying the specification), or
 my patent application Serial Number _____, filed _____ (supplemental
declaration - not accompanying the specification),

as titled in item 301, below,

that as to the subject matter of this application which is common to my earlier copending application(s) filed in the United States of America (i.e. common subject matter), if any, described in item 105 below, I do not know and do not believe, that said common subject matter was ever known or used in the United States of America before my invention thereof, or that said common subject matter was ever patented or described in any printed publication in any country either before my invention thereof or more than one year prior to the filing date(s) of such earlier copending application(s), or that said common subject matter was ever in public use or on sale in the United States of America more than one year prior to the filing date(s) of such earlier copending application(s), that said common subject matter has not been patented or made the subject of an inventor's certificate issued before the filing date(s) of such earlier copending application(s) in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months prior to the filing date(s) of such earlier application(s), and that no application for a patent or an inventor's certificate on such common subject matter has been filed by me or my legal representatives or assigns in any country foreign to the United States of America, except as those identified in item 601-603 below, if any;

that as to any subject matter of this application which is not common to my said earlier copending application(s) (i.e. non-common subject matter) described in item 105 below, I do not know and do not believe, that the non-common subject matter was ever known or used in the United States of America before my invention thereof, or that the non-common subject matter was ever patented or described in any printed publication in any country either before my invention thereof or more than one year prior to the filing date of this application, or that the non-common subject matter was ever in public use or on sale in the United States of America more than one year prior to the filing date of this application, that said non-common subject matter has not been patented or made the subject of an inventor's certificate issued before the filing date of this application in any country foreign to the United States of America on an application filed by me or my legal representative or assigns more than twelve months prior to the filing date of this application, and that no application for a patent or an inventor's certificate on said non-common subject matter has been filed by me or my legal representatives or assigns in any country foreign to the United States of America. It is further declared that no foreign filing has been made which is contrary to 35 U.S.C. 184.

FULL NAME OF APPLICANT (Including at least one unabbreviated First or Middle Name):

2	LAST	Holladay	FIRST	David	MIDDLE	W.
	RESIDENCE:					
0	CITY	Knoxville	STATE (OR FOREIGN COUNTRY)	Tennessee	COUNTRY OF CITIZENSHIP	
	U.S.A.					
1	POST OFFICE ADDRESS:					
	STREET ADDRESS		CITY	STATE (OR FOREIGN COUNTRY)	ZIP CODE	
	Rt. 15, Roland Lane		Knoxville	Tennessee	37921	

105 This application is a CONTINUATION CONTINUATION-IN-PART DIVISION APPLICATION of my earlier filed U.S. application Serial Number _____ Filed _____ Specific reference to my related earlier filed U.S. application(s) is made on page _____ of the specification for the purpose of receiving benefit of earlier filing date(s) 35 USC 120.

301 TITLE OF INVENTION:

Foreign applications directed to COMMON SUBJECT MATTER if any, filed prior to the filing date of this application.

	COUNTRY	APPLICATION NO.	PATENT NO. (if any)	DATE OF FILING (day, month, yr)
601				
602				
603				

Foreign applications continued on page attached hereto YES NO

500 SEND CORRESPONDENCE TO: NAME Mr. James E. Denny Assistant General Counsel for Patents U.S. Energy Research and Development Administration Washington, D.C. 20545

DIRECT TELEPHONE CALLS TO: (name and telephone No.)

4 POWER OF ATTORNEY: As the named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

	NAME	REGISTRATION NUMBER	TELEPHONE NUMBERS
0	Allen H. Uzzell	27,603	80- 615-483-8611, ext. 34334 853-5093
0	D. S. Zachry	16,234	
	James E. Denny	18,863	
	John A. Horan	16,077	
	Dean E. Carlson	18,537	

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

201 SIGNATURE OF APPLICANT /s/ David W. Holladay DATE OF SIGNATURE Dec. 16, 1976

PATENT ASSIGNMENT FORM

WHEREAS, I, David W. Holladay, a citizen of the United States residing in Knoxville, County of Knox, State of Tennessee, have invented certain new and useful improvements in AGENT AND METHOD FOR THE EARLY DETECTION OF PREGNANCY, for which I am about to file an application for United States Letters Patent identified as ERDA CASE S-47,844

~~XXXX~~ and executed by me on Dec. 16, 1976, 1976; and

WHEREAS, the Government of the United States desires to acquire the entire right, title, and interest in and to the said invention and in and to any Letters Patent wherever they may be issued thereon:

NOW, THEREFORE, to all whom it may concern, be it known that for and in consideration of the sum of One Dollar to me in hand paid by the Government of the United States, and for other good and valuable consideration, the receipt of which is hereby acknowledged, I by these presents do sell, assign, and transfer unto the said Government of the United States, as represented by the United States Energy Research and Development Administration, and/or its assigns, the entire right, title, and interest in and to the said invention and in and to any and all Letters Patent wherever they may be granted thereon as well as reissues and extensions of said Letters Patent, the same to be held and enjoyed by the said Government of the United States to the full end of the term or terms for which Letters Patent are or may be granted, reissued, or extended, as fully and entirely as the same would have been held or enjoyed by me had this assignment not been made.

I agree to make, execute, and deliver unto the Government of the United States, or to the United States Energy Research and Development Administration, any and all papers, documents, affidavits, renewal, divisional and reissue applications, statements, or other instruments in such usual or other forms, terms, and contents as may be required by the United States Energy Research and Development Administration, or its duly authorized representative, in or incident to the prosecution or conduct of any and all applications, before as well as after the issuance of any Letters Patent thereon, or in the adjustment or settlement of any interferences or other actions or proceedings that said applications may encounter or in which they may become involved, and I agree that I will aid the Government of the United States in every way in protecting the invention as may be requested by the United States Energy Research and Development Administration or its assigns, except that any expenses arising through extending such assistance will be paid for by proper arrangement with the Government of the United States.

WITNESS:

/s/ W.D. Cline

INVENTOR:

/s/ David W. Holladay (SEAL)

Date December 16, 1976

Approved and consented to this 27th day of December, 1976

ATTEST:

/s/ M. W. Kirstowsky

UNION CARBIDE CORPORATION
NUCLEAR DIVISION

(SEAL)

By /s/ P. R. Vanstrum dcm

Its Vice-President

Accepted for the Benefit of the Government of the United States.

UNITED STATES ENERGY RESEARCH AND
DEVELOPMENT ADMINISTRATION,

WITNESS:

By _____

Draft
Uzzell/cad
11/12/76
AGENT AND METHOD FOR THE EARLY DETECTION OF PREGNANCY
Inventor: David W. Holladay, Rt. 15, Roland Lane, Knoxville, Tennessee 37921

INSTRUCTIONS FOR DRAFT PATENT APPLICATION

Please provide the requested information, if possible. If a statement is incorrect, please replace it with a correct statement. I have tried to describe the invention as broadly as possible in order to obtain the broadest patent coverage. I apologize for the extent of the questions, but it is necessary that everything be explained as completely as possible to prevent the invention from being limited to the particular reagents or process steps used. Feel free to make any changes, deletions, etc. which you deem advisable. Also, please point out any additional surprising or unpredictable results which were observed.

ABSTRACT OF THE DISCLOSURE

An agent capable of detecting the early stages of pregnancy in humans is provided by preparing an antiserum to proteins isolated from pregnancy associated material such as placentas. By conventional techniques of detecting antigen-antibody reactions, the antiserum is used to detect the presence of pregnancy specific proteins in women at the early stages of pregnancy.

Background of the Invention

This invention was made in the course of, or under, a contract with the Energy Research and Development Administration. It relates in general to pregnancy detection and, more specifically, to an agent and method for detecting pregnancy in women during the very early stages. Pregnancy has been detected as early as 18 days after conception according to this invention

There has long been a need for a simple, reliable test which is capable of detecting pregnancy within a short time after conception. For example, such a test would be of great value for those women with an ancestral history of genetic disorders so that preventive therapy could be initiated as soon as possible. Those women working in potentially toxic or radioactive environments or taking potentially harmful medication also have a need for early pregnancy information.

In the prior art pregnancy has been detected by a variety of well-known methods. Most of the prior art methods are unreliable when administered sooner than sixty days after conception. One method which has been shown effective for early pregnancy detection is described by B.B. Saxena et al. in the article "Radioreceptor Assay of Human Chorionic Gonadotropin: Detection of Early Pregnancy" Science, Vol. 8 pp. 793FF. (1974). This method is rather complex and requires the use of radioactive substances, and, therefore, might be unsuitable for routine clinical use. The present invention involves the use of host-produced antibodies to detect the presence of pregnancy-specific proteins in the sera of women in the early stages of pregnancy. The occurrence of pregnancy-specific components in sera from pregnant women was first demonstrated by Thornes and reported in an article by MacLaren et al. in Am. J. Obstet & Gynecol. 78:939(1959). Smithies in Adv. Protein Chem. 14 65,(1959) observed the presence of a pregnancy-associated alpha-globulin. Hirschfeld and Soderberg in Nature 187:332(1960) found two precipitates on immunoelectrophoresis of pregnancy sera. Gall and Halbert, Int. Arch. Allergy Appl. Immunol. 42:503(1972); and Lin et al., Am. J. Obstet. Gynecol. 118:223(1974) observed and characterized four sequential pregnancy-associated

plasma proteins found in the third trimester of pregnancy. Antisera had been raised against pregnancy plasma and then absorbed exhaustively with non-pregnancy plasma. Bohn in Arch. Gynaekol. 210:440(1971) used rabbit antihuman placenta antiserum absorbed with male serum to detect four pregnancy-associated plasma proteins. Only one was considered pregnancy specific and two could sometimes be detected in sera from non-pregnant female subjects, especially those taking oral contraceptives. Berne, in Clin. Chem (Winston Salem, N.C.) 19:657, Abstr. 093, 1973 found pregnancy zone protein in women six weeks pregnant. The protein was also found in men and non-pregnant women. While the prior art was aware of the presence of proteins associated with pregnancy, there was no awareness of proteins which were specific to pregnancy and which could also be detectable sufficiently early in the gestation period to be useful as a pregnancy test.

Summary of the Invention

It is an object of this invention to provide an agent capable of detecting the early stages of pregnancy in humans.

It is a further object to provide an agent capable of detecting early pregnancy by simple and well-known clinical operations.

It is a further object to provide a method for the detection of pregnancy which requires no radioactive material or complex procedures.

It is a further object to provide an agent and method for detecting pregnancy which is effective less than thirty days after conception.

These and other objects as will be apparent are provided according to this invention by a pregnancy detection agent comprising an antibody to a pregnancy-specific protein isolated from non-specific antibodies and prepared by the method comprising:

1075876

a) contacting a first mixture of proteins occurring in pregnancy-associated material with antibodies raised against human serum containing no antigenic pregnancy-specific proteins;

b) separating non-specific antigenic human proteins from said first mixture to provide a second mixture containing pregnancy specific proteins isolated from non-specific antigenic proteins;

c) inoculating a host animal with said pregnancy-specific proteins to cause said host animal to raise antibodies to at least one of said pregnancy specific proteins;

d) harvesting serum proteins from said host animal, said serum proteins containing antibodies to at least one of said pregnancy specific proteins;

e) contacting serum proteins from said host animal with non-specific human serum proteins to effect the separation of non-specific antibodies to human proteins from antibodies to pregnancy specific proteins to provide a mixture containing an antibody to a pregnancy specific protein isolated from non-specific antibodies.

A method of detecting the existence of pregnancy in humans according to this invention comprises contacting serum of (very possibly) urine but this has not been demonstrated. from a Patient with an antibody to a pregnancy specific protein isolated from non-specific antibodies, and observing whether an antibody/antigen reaction occurs, the occurrence of said reaction being indicative of pregnancy.

1075877

OTHER FLUIDS AT LATER STAGES OF PREGNANCY (AMNIOTIC for example) would certainly react, BUT PREGNANCY WOULD BE OBVIOUS. IN SUMMATION, BEST TEST FLUIDS ARE SERUM, CONFIRMED: URINE, UNCONFIRMED

Detailed Description

An important aspect of this invention is the discovery that structures and substances produced during the course of pregnancy in women contain proteins which are also present in detectable quantities in the very early stages of pregnancy. Another important aspect is the discovery that at least some of these pregnancy-associated proteins are both highly specific to pregnancy and sufficiently antigenic to raise antibodies in host animals which can then be isolated and used to detect the presence of the proteins in pregnant women relatively soon after conception. [Question: Could it be predicted with certainty that antibodies raised against proteins would be specific

to those proteins? Please explain.] *ONE OF THE SALIENT CHARACTERISTICS OF THE IMMUNE RESPONSE IS THE UNIQUE SPECIFICITY OF THE MATCHUP BETWEEN AN ANTIGEN MOLECULE AND THE ANTIBODY WHICH THE BODY MANUFACTURES TO BIND SPECIFICALLY WITH THAT ANTIGEN, AND NO OTHER. HOWEVER, SOMETIMES PROTEINS CAN BE ISOLATED WHICH SIMPLY DO NOT ~~ELICIT~~ AFFECT THE IMMUNOLOGICAL SYSTEM IN SUCH A MANNER AS TO ELICIT THE PRODUCTION OF ANTIBODIES. THERE ARE SEVERAL REASONS FOR THIS FAILURE TO ELICIT ANTIBODY PRODUCTION: 1) THE PROTEIN MAY BE SEEN AS BEING "LIKE" THE HOST ANIMAL'S PROTEIN, TOO MUCH ALIKE TO MAKE ANTIBODY. 2) THE PROTEIN MAY BE DAMAGED ALONG THE SEGMENTS WHICH FORM THE ANTIGENIC DETERMINANT, 3) THE PROTEIN COULD BE AT SUCH LOW LEVELS THAT THE ANTIBODY TITER WOULD BE SIMPLY TOO LOW TO DETECT BY STANDARD IMMUNOLOGICAL TECHNIQUES*

For purposes of this invention, the terms specific and non-specific relate to pregnancy specific and non-pregnancy specific. A non-specific protein is one which is not specific to pregnancy. A non-specific antibody is an antibody to a non-specific protein. The term "antigenic" protein refers to a protein capable of producing detectable antibodies in a host animal. For purposes of this invention, a non-antigenic protein is one which raises no more than miniscule amounts of antibody which would not significantly interfere with the detection of pregnancy specific proteins by pregnancy specific antibodies.

The first step in preparing the pregnancy detection agent of this invention is to prepare a mixture of proteins occurring in pregnancy associated materials such as placentas, umbilici, amniotic fluid, fetal tissue, (others?) none practical, etc.

Placentas are preferred because of their ready availability. The mixture of proteins can be prepared from the pregnancy-associated material by a number of well-known biochemical techniques for concentrating proteins from biological tissues or fluids. For purposes of this invention, the separation of proteins from pregnancy associated material need not be quantitative. For example, pregnancy associated tissues can be diced and homogenized in phosphate

buffer. (Why phosphate buffer? What else would serve? Phosphate buffer is a known stabilizing media for body fluid proteins, offering a very benign pH to minimize precipitation, agglutination, etc. As noted in the journal article, it was believed that mild acid such as 0.1 M perchloric acid should also be tried.)

The homogenized suspension is then separated such as by centrifugation, filtration, etc. to provide a supernatant liquid. Proteins can be separated from the supernatant by adding a precipitant such as ammonium sulfate. (What else would work?) This was such a standard and well tried method that no other salting out techniques were tried. I can say other methods, different salting agents would be successful, without detailing them, unless you find it absolutely necessary.

medium culture is acceptable, final precipitation is likely acceptable, consider Gel fraction, but this show and not very efficient

The precipitated proteins are separated from the solution e.g., by centrifugation and redissolved in a buffer solution containing, for example, 0.1% sodium azide (Why? What else would work?) This is an agent to prevent unwanted bacteria from multiplying in this excellent growth media. Among other possible bacteriocides or bacteriostats are MERTHIOGATE, toluene, ~~starch~~.

Dialysis is a routine laboratory technique. MAY be accomplished with dialysis tubing or a similar substitute, or may be done in Bio Rad's Dialysis Cartridges.

The protein solution can then be dialyzed against the same buffer (Why?) How?

Dialysis is desirable to remove any traces of the ammonium sulfate used to precipitate the antigenic proteins. Presence of the sulfate can complicate handling and affinity chromatography in subsequent steps.

and separated from precipitated material to provide a relatively pure solution of proteins.

The next step in preparing the agent of this invention is to contact the proteins mixture isolated from pregnancy associated structures with antibodies raised against human serum containing no antigenic pregnancy-specific proteins (normal human serum). [Is this step necessary in view of the adsorption of

^{Antiserum} goat ~~serum~~ on normal human serum after raising antibodies?] Please explain.

STUDIES were made both with and without this step and it appeared that this first absorption to remove contaminating normal human proteins, ^{obviously} not specific for pregnancy, yielded a higher titer antiserum in the host animal. You see, if you can remove those proteins, such as normal proteins, to which the goat will make a quick and extensive antibody response, you greatly enhance the likelihood that the goat's response can then be directed towards the obscure and dilute proteins which are unique for pregnancy. It is like a competition, in that the contaminating normal proteins shield out the desired response to the specific pregnancy proteins.

This can be easily accomplished, for example, by cycling the isolated proteins

over an immunoabsorbent column loaded with antibodies raised against normal

human serum (anti-normal human serum). [How would such an immunoabsorbent

See ATTACHMENT 1!

column be prepared? Please provide a procedure for loading a column material

with anti-normal human serum. In addition is there any other way of separating

pregnancy specific proteins from normal human proteins? Please explain.]

Also See ATTACHMENT 2, for story of first isolating anti-normal human serum antibodies with a normal human serum column, and the subsequent attachment of the anti-normal serum antibodies to the Sepharose (p-4, attachment 2).

Attachment 3, pp. 316-317, talk about balancing the antiserum.

1075880

After contacting the isolated proteins with anti-normal human serum, the raffinate solution from the column (the unbound fraction) contains pregnancy specific proteins and non-pregnancy specific proteins which are non-antigenic, i.e., insufficiently antigenic to cause the production of appreciable amounts of antibodies in warm-blooded animals. For purposes of this invention, such non-pregnancy-specific proteins can be regarded as non-interfering material.

The unbound fraction can then be concentrated to provide a serum suitable for inoculation into the host animal. Concentration is usually necessary prior to injection, but, of course, antibodies could be raised less efficiently against dilute solutions. Suitable hosts are virtually any warm-blooded animal, such as goats, cattle, horses, rabbits, chickens, etc.

Goats are preferred because of the convenience of size, ease of handling, and they have an expensive and multivariant antibody response normally superior to other mammals

All that is necessary is that at least one pregnancy specific protein causes the host animal to raise antibodies. ^{actually} ~~Actually~~ It is believed that about

^{at least} 3 different pregnancy specific proteins cause the production of antibodies. As is customary in the raising of antibodies, conventional

adjuvants can be employed to accelerate antibody production. The injection schedule can be expected to vary for different types of hosts, and it is well within the skill of those acquainted with manufacturing vaccines, etc. to work out a suitable injection schedule for a particular type of host. [Aside from your specific example, is there any additional guidance you can give the practitioner concerning the injection schedule?]

page 5 of attachment 5 is a succinct summation of the ~~schedule~~ ^{schedule}. If the ~~serum~~ ^{serum} does not develop too much local infection from the insult of the adjuvant, the schedule could possibly be ~~for~~ shortened by more frequent injection with adjuvant. Bleeding was from the jugular ^{vein} through a large cannula.

see also attachment 8!

After a suitable period of time, usually several months, the host animals are bled and their plasma is harvested. The plasma contains ^{host} serum proteins comprising antibodies to at least one of the pregnancy-specific proteins. These ^{host} serum proteins (either concentrated or as plasma) are then contacted with normal human serum, for example, by passing the antiserum through an immunosorbent column containing (What support?) ^{puted} normal serum proteins immobilized on (attachments 1 & 2) SEDHARSE 4B loaded with lyophilized (What is this process?) ^{essentially} freeze-drying gas in instant coffee; The ^{blood slurry} ~~liquid~~ is frozen, and the frozen ~~liquid~~ ^{water} sublimates away under a vacuum, leaving only normal human serum. [Why ^{is this step necessary since the serum was already passed through the column containing normal human serum prior to injection} NO! the placental extract preparation was first passed over a column of anti-normal serum proteins to remove as many contaminating normal proteins as possible prior to injection to the host.

The Procedure of first removing isolated serum proteins prior to ~~any~~ placental material injection into host, and the removal of any subsequently formed anti-normal serum protein antibodies after harvesting of the antiserum from the host animal is completely consistent. It has one goal, production of an antiserum which reacts with only the abnormal and unusual pregnancy specific proteins, but not with normal proteins. OTHERWISE THE TEST WOULD OBVIOUSLY ^{detect normalcy, NOT pregnancy.}

The material which is not adsorbed on the column is a mixture containing an antibody to a pregnancy specific protein isolated from non-specific antibodies, and as such is now an agent capable of detecting the presence of pregnancy-specific proteins. [How is this material different from the agents used by

Bohn, Berne, Lin, and other workers in the prior art to isolate pregnancy associated proteins?] ^{and neither would anyone else,} FRANKLY, we don't know as noted, we

suspect ^{that} there are at least 3 of these early proteins.

We do know that none of these workers ever reported any of their "specific" proteins prior to 6 weeks. ^{that reported in 2nd and 3rd trimesters.}

All we can say for sure is that ~~our~~ our proteins occur much earlier. EXTENSIVE WORK WOULD BE REQUIRED, and probably an interchange of proteins among the various workers would be required, to even sort out if our early proteins are the same as the ones they could detect ^{only} much later than we.

It may be that ^{we at the pregnancy specific proteins apparently and because the pregnancy detection agent of this invention can be prepared by conventional serologically undetectable} The normal human serum and anti-normal human serum used in preparing ^{in 3rd trimester} serological techniques well-known to the science of immunology. To provide a

pregnancy detection agent suitable for the general public, the normal human

serum should be collected and pooled from a large sample of non-pregnant

human donors having all of the blood types. These would most likely be

limited to males to ensure that serum from unknowingly pregnant women did

not contaminate the serum. It would be desirable that at least some non-

pregnant women be included in the sample to provide non-pregnancy related

1075883

attachment to page 15
After centrifugation of the anti-normal human serum (anti-NHS) antiserum, there were two methods for isolating the specific antibody molecules directed against normal serum proteins.

FIRST, the antiserum could be run over a DEAE Column, a simple ion exchange support, so that the whole IgG set, containing as a subset our specific anti-NHS molecules, could be separated away from the balance of the host animal's proteins. Recall that only about 20% of the goat's protein is total antibody, ^(while IgG set) with the remaining 80% being protein for his own functions. An immunosorbent column could then be prepared using the entire IgG set of antibodies, of which it was likely ^{that only} some 20-30% would be our specific antibodies to normal human serum. The other ~~70~~ ⁷⁰ odd % of the goat's antibodies would be directed against ^(for weeks later see) other antigenic challenges that the goat had experienced in his life. Using this DEAE method, we were assured of setting all of the antibodies that the goat had produced ^{in response} ~~to~~ our normal serum injection. However, the presence of other nonreactive antibodies simply reduced the capacity of our anti-normal human serum immunosorbent column.

So, Serum 1, the best high capacity anti-NHS 2
immunoabsorbent was produced by first preparing
a column (immunoabsorbent) using pooled normal human
serum. The whole antiserum could be taken from
the goat, centrifuged, and run through the normal
human serum column. Obviously then, those goat
proteins ~~and~~ (80%) and goat antibodies not reactive
with the normal serum proteins (another 70% of the
remaining 20% of the goat proteins) would pass through
the column without reacting. However, utilizing the
remarkable immunological specificity that is the basis
of all of this work, the subset of ~~ant~~ goat
antibodies (~30% of the 20% of the goat proteins
that is total antibody), directed against our normal
serum proteins, experience a strong binding reaction with
the normal proteins of the immunoabsorbent. The anti-normal
serum protein antibodies can be broken loose from their
bond to the column using a solution known as a
chaotropic" ion. See attachment 6.

The difference in the methods is apparent. Method
2 gives an immunoabsorbent with about 3-5 times the
active capacity as method 1, since for method 1, only 20 %
of antibodies are specific to normal proteins.

the agent of this invention. It should be noted that immunoabsorbent columns used according to this invention provide both reaction contact between antigen and antibodies as well as separation of unreactive components which exit the column as the unbound fraction.

The pregnancy detection method of this invention comprises contacting serum from the patient with antibodies to pregnancy specific proteins isolated from non-specific antibodies. Since the existence of the antigenic pregnancy specific protein has herein been demonstrated, the source of the protein is not an essential feature of the pregnancy test. The desired antibodies can be prepared by the method disclosed herein, or it is conceivable that at least one of the pregnancy specific proteins can be identified and synthesized, thereby avoiding the necessity of separating the pregnancy specific protein from antigenic non-specific human proteins. The synthetic protein could then be used directly to raise antibodies useful for detecting pregnancy.

All that is necessary is that serum from a patient be contacted with an antibody raised against a pregnancy specific protein and the results observed to detect signs of antibody/antigen reaction. The occurrence of the reaction of course, indicates pregnancy. It can be readily appreciated that any of the several methods for detecting antigen/antibody reaction is suitable for detecting pregnancy according to my method. Examples of such methods are gel precipitation, gel electropherograms, immunoelectrophoresis, _____

cross immunoelectrophoresis, immunodiffusion

[Do each of the tests require blood serum? Do each require contact between the serum and antiserum? Would other body fluids such as lymphatic fluid,

urine, etc. be suitable?]

Each of these tests require that an antigenic mixture and an antibody mixture, with certain antibodies specifically reactive with some of the antigens, be available. They are located in ports or holes some distance from each other, and then either by natural diffusion or by a speeded-up diffusion in the presence of an electrical current, the antigens migrate to meet their migrating specific antibodies. Upon contact, the antigens and antibodies form their strong bond, ~~and~~ increasing numbers of them stack up, agglomerate, and form a concentration band readily detectable by eye, or optical techniques, density scanners, etc. These bands can be enhanced by dye staining.

As noted above, urine is the most likely candidate other than ^{blood serum} that

The primary utility of the pregnancy detection method of this invention is for detecting pregnancy within about 2-4 weeks of conception. After

2 months, pregnancy is reliably detectable by well known external

symptoms. It is likely this ^{could} be reduced to within a week of conception.

In order to demonstrate a preferred method for preparing the pregnancy detection agent of this invention and the operability of the agent for detecting pregnancy, a detailed example is presented. It will be apparent that substantial variation from the procedures described therein can be made without departing from the spirit and scope of the invention.

Example

Preparation of crude placental extract. Term placentas were obtained at delivery and chilled in 0.85% sodium chloride solution for preservation.

The placentas were washed for 1 hr. in tap water to remove a maximum of blood

elements and serum proteins. Why? We did not desire to obtain either the mother's blood or the fetal blood, although they might contain traces of pregnancy specific proteins, we believed that the placenta was the best source of early pregnancy associated serum proteins. Fat was then cut away (Why?) It is superfluous, and non antigenic, having other functions in pregnancy.

and the placenta ^{was} cut into pieces about 1 cm on a side and frozen. Subsequently, the frozen material was homogenized in three volumes of 0.1 M, pH 7, phosphate buffer. The homogenate was centrifuged at 17,000 x g for 10 min. in an angle-head centrifuge, the supernatant was collected, and ^{the} pellet was rehomogenized in two volumes of the same buffer, and a second centrifugation at 17,000 x g for 10 min. was done. [Question: What is x g?]

denotes an acceleration factor for centrifugation (accel. w/ gravity)

The proteins of interest were precipitated from the pooled supernatants by adding solid ammonium sulfate to 55% saturation. [Were some proteins unprecipitated? Explain.]

Certainly. Some of the proteins later shown to be pregnancy specific were always lost during these fractionations, part being discarded with the dilute throwaway fractions. It must suffice to say that we may have left others behind during our procedure of extraction, but this is ~~an~~ entire study of

Following centrifugation, the material was dissolved in a minimal amount of

0.1 M, pH7, phosphate buffer containing 0.1% sodium azide, the solution was dialyzed against this same buffer, (what sort of membrane? Anything critical about the dialysis?)

*Visking tubing from Union Carbide.
Nothing critical.*

and any material that precipitated was removed by centrifugation. (What was the

precipitate probably?) *Mostly very large macromolecules representing the connective tissue of the placenta. Since they were not very soluble, they were not likely to handle well during immunoadsorption, further dialysis, or ultimately as injectable material into the host.*

Any serum proteins left in the solution were removed by cycling it over

an immunoadsorbent column of antibodies to normal human serum. (How was it

cycled? What sort of column support?) *For Description of*

the automatic cycling apparatus see

Anderson et al, ANALYTICAL BIOCHEMISTRY, 66, 159-~~169~~¹⁷⁴, 1975

THE CYCLUM WAS NOT MAGIC, BUT SIMPLY SERVED AS A SUBSTITUTE FOR MANPOWER, AS IT ALLOWED FOR 24 hr. operation

At the immunosorbent column cycling had the
ADDITIONAL CONVICTION THAT ONCE ~~BE~~ ALL THE MATERIAL HAD
BEEN PASSED THROUGH THE COLUMN (WHICH ITSELF WAS CYCLED
THROUGH A REPETITIVE SEQUENCE BY THE MACHINE) / THE CONCENTRATE
product will be passed through the column again for enhanced
The unbound fraction emerging from this treatment was still a processing

complex mixture, containing at least 20 different proteins, as judged
by gel electrophoresis. The solution could not be concentrated beyond
20 A_{280} units per ml without undesired precipitation occurring on storage,
especially on freezing and thawing. An A_{280} unit is the quantity of
protein per ml of water or buffer giving an absorbance of 1 at 280 nm in
a cell with a light path of 1 cm. Apparently, the bulk of the material that
precipitated was placental tissue proteins, mostly acid in nature. This
material could be separated into several subfractions by chromatography over
DEAE cellulose. [How? What sort of eluents?] See ATTACHMENT 7

Though not essential to the operability of the process, some separations
of placental tissue proteins that appear in the serum from those that do
not could be effected. Only 10 to 15 of the proteins in the mixture have
proved to be sufficiently antigenic to result in detectable antibody production
to them. It is likely possible to modify the extraction procedure (for example,
0.1M perchloric acid, as noted earlier.
mild acid extraction of diced placenta, without homogenization) in order to
minimize the extraction of proteins that do not appear in the plasma; however,
this is unnecessary for the operability of the process.

Raising of antisera. The placental extracts, minus non-specific serum
proteins but still containing the relatively nonantigenic tissue proteins
mentioned, were concentrated to about 15 A_{280} units per ml for injection
purposes. An estimate of the antigen content was 0.1 to 1 mg/ml, based on
intensity of reaction in immunodiffusion. The extract was mixed with equal

1075890

... complete adjuvant - commercial
available from Difco Laboratories [Do you know the
address?]
and contains sterile light mineral oil plus emulsifier
and heat killed Mycobacterium bovis (0.5 mg.
dry weight per ml)

volume of Freund's complete adjuvant, and the preparation was injected
subcutaneously into goats and rabbits, either in the hips or behind the
neck (4 ml for goats, 1 ml for rabbits). [Would intravenous injection

See attachment 8

also work?] Quite often, intravenous injection results in

→ anaphylactic shock and sudden death of the
host animal. The other methods allow slow and ^{release of the} safe
Thereafter, the same amounts of extract without adjuvant were injected
subcutaneously at weekly intervals. Every fourth week, injection was
done with antigen plus adjuvant. First bleeding was at 5 weeks (40 ml for
rabbits and 150 ml for goats) and was biweekly thereafter to test for
antibody production. High-titer antiserum was obtained, usually in about
7 to 8 months. The antiserum was absorbed on a conventional immunoabsorbent
column with lyophilized, pooled serum obtained from normal human males (100
mg/ml of antiserum) to remove non-specific antibodies. The unbound fraction
contained pregnancy specific antibodies and normal animal protein. [Is
any further treatment necessary or desirable for commercial manufacture?
Should the goat fraction be removed?]

*Now you are cooking! This is an obvious method
of providing a purer more highly concentrated antiserum
reagent. Oddly enough, we never proceeded that far. So,
certainly the F₂G fraction should be removed from the bulk of
the goat (animal) protein. DEAE CHROMATOGRAPHY WOULD BE A
Simple effective 1st METHOD of choice.*

Results and Discussion

Gel precipitation was used to test the antiserum to
placental antigens for the presence of antigens that appeared in the sera of
pregnant women. The antiserum was first tested against sera from women in the
third trimester of pregnancy. Tests against the sera from three different
women in their first pregnancy, three women in their second pregnancy, and
three women in their third pregnancy showed positive reactions within 5 hours

with 2 to 4 bands clearly visible after 40 hours. The control for all three plates was from a 28 year old woman who had had one successful pregnancy 5 years previously, and who since that time had been taking an estrogen-based birth control pill. No reaction was noted at any time with the control.

The antiserum gave negative tests against a succession of batches of pooled normal human male sera. It was positive against all of a series of 40 samples of third-trimester pregnancy sera. It was negative against the sera of ten nonpregnant women, including those previously pregnant. Of four cases tested, no reaction has been shown with sera from women taking estrogen-related contraceptives. In order to demonstrate the detection of pregnancy early enough to be valuable for a pregnancy test, several runs were made on ^{sera} serum from women who suspected pregnancy. A serum sample was obtained from a 29-year-old woman 6 days after she had failed to begin her menstrual period. A good estimate of the time duration since conception was 18 to 22 days. A serum sample was obtained from a 25-year-old woman 10 days after she had failed to begin her menstrual period, giving a time duration of 22 to 26 days since conception. The samples were tested by double diffusion against antiserum to placental antigens. Placental extract and serum from a woman in the third trimester of her third pregnancy were used as reference.

After 16 hr. one sharp band and one faint band of precipitation were observed in both samples, and the two bands merged or showed identity with bands from both placental extract and the third-trimester pregnancy serum sample. For both samples, a precipitation band could be observed as early as 1 hr after

1075892

the start of immunodiffusion. Three precipitin bands were observed for the second sample within 24 hr after loading.

Improved immunodiffusion techniques would likely allow for a decision on pregnancy on the same day as blood sample withdrawn

Another early-pregnancy serum sample, taken at 28 to 32 days after conception, was tested against the antiserum and gave two precipitin bands within 16 hr. Pregnancy was confirmed at 8 weeks of gestation by routine examination for all three of the women. The human placental antigen preparation gave ten *at least*

distinguishable precipitin bands with the antiserum. The precipitin bands that are the strongest for very early pregnancy samples show identity with the weakest bands from samples from third-trimester pregnancies. This indicates

that the antigens involved reach a concentration peak early in pregnancy, and then slowly decrease during gestation. *Some early appearing antigens may not be detectable in 2nd & 3rd trimester.*

While the particular pregnancy specific antigenic proteins utilized for this invention have not yet been characterized, their identification is not essential to manufacturing the agent of this invention or to performing the pregnancy test of this invention.

Recent advances in cancer research have intimated that certain proteins present in pregnancy are ⁽²⁾reexpressed in the early stages of cancer. This is consistent to the idea that the formation of the fetus is physiologically similar to the formation of tumor cells in cancer patients. Accordingly, an expected utility for the pregnancy detection agent of this invention is the early detection of cancer. [If possible, could you elaborate on this idea, giving additional references or evidence of the possible utility?]

*See the ATTACHMENTS
CONCERNING THE CANCER CONNECTION*

What is claimed is:

1. A pregnancy detection agent comprising an antibody to a pregnancy specific protein isolated from non-specific antibodies and prepared by the method comprising:

a) contacting a first mixture of proteins occurring in pregnancy-associated material with antibodies raised against human serum containing no antigenic pregnancy-specific proteins;

b) separating non-specific antigenic human proteins from said first mixture to provide a second mixture containing pregnancy specific proteins isolated from non-specific antigenic proteins;

c) inoculating a host animal with said pregnancy-specific proteins to cause said host animal to raise antibodies to at least one of said pregnancy specific proteins;

d) harvesting serum proteins from said host animal, said serum proteins containing antibodies to at least one of said pregnancy-specific proteins;

e) contacting serum proteins ^(as host antiserum) from said host animal with non-specific human serum proteins to effect the separation of non-specific antibodies to human proteins from antibodies to pregnancy specific proteins to provide a mixture containing an antibody to a pregnancy specific protein isolated from non-specific antibodies. _{agent, claim 1}

2. The agent of claim 1 in which said proteins occurring in pregnancy associated material are recovered from placentas.

3. A method of detecting the existence of pregnancy in humans comprising contacting serum or urine from a

patient, with an antibody to a pregnancy specific protein isolated from non-specific antibodies, and observing whether an antibody/antigen reaction occurs, the occurrence of said reaction being indicative of pregnancy.

1075894
hb851

4. The method of claim 3 wherein said serum or urine

_____ is withdrawn from said patient within about
1/2 1-4 weeks
_____ months of conception.

5. A method of detecting the existence of pregnancy in humans comprising contacting serum or urine _____ from a patient with the agent of claim 1 and observing whether an antibody/antigen reaction occurs, the occurrence of said reaction being indicative of pregnancy.

6. The method of claim 5 wherein said serum is withdrawn from said patient within about 1/2 1-4 weeks _____ months of conception.

7. The method of claim 5 wherein said proteins occurring in pregnancy associated material are recovered from placentas.

Preparation of Immunoabsorbents

Campbell et al probably prepared the first antigen-antibody immunoabsorbent material in 1951 (see reference in attachment 1). The support material to which the antigen (human serum albumin) was attached was our old friend DEAE. This work languished in the literature for 15 years or so. Then in 1964 Campbell and Weetall resurrected the concept, using instead of DEAE (diethylaminoethyl cellulose) a similar support CMC (carboxy methyl cellulose). Feasibility of using immobilized antigens to remove specific antibodies from a complex antiserum had been shown.

However, the complexity of the chemistry in preparation of the ~~is~~ DEAE immunoabsorbents, and the poor handling and flow properties of DEAE immunoabsorbents have combined to relegation of this initial work to the status of "first efforts," so that more desirable column supports and attachment techniques were the goal of workers in the field. The exploitation of antigen-antibody immunoabsorbents has never taken place. By far the largest efforts to ~~develop~~^{develop} this powerful tool into large scale biomedical ^{use}, drug manufacture, and clinical applications were conducted at the MAN Program from 1971-1974.

What happened to "affinity chromatography" was the birth and boom of affinity columns for use in separating and ~~the~~ utilizing, not antigens or antibodies, but drugs, drug metabolites, and mostly enzymes. Weetall who had been involved in the early work with Campbell (attachment 1), was a prime mover in the enzymatic affinity preparation developments. More on him later.

As it became obvious in the research field of enzymology that enzyme purification by "affinity

chromatography was a very powerful and specific tool, a great interest ^{grew} in developing the best type of affinity support. Among these ^{supports} were (instead of using DEAE & CMC) ethyl chloroformate matrices, ethylene maleic anhydride matrices, immunodial, poly styrene, and so on. However, in the late 60's two techniques were introduced which ~~was to remain~~ have served to the present as the most applicable and widely accepted affinity chromatography systems. These procedures are the use of cyanogen bromide activated Sepharose ^{Amersham Pharm} and the use of silane activated glass beads (Weetall). There are several variations on utilizing these supports for various chemical moieties, as will be outlined now.

In attachment 2³, PERATH outlined the initial work in preparing protein-Sepharose affinity supports. This was the basis of the first patent on these affinity supports. It is noted in this article that Sepharose was selected as the support media after some considerable screening of candidate materials. To digress, SEPHAROSE is ~~the~~ PHARMACIA'S (FINE CHEMICALS, INC., 800 CANTANIEL AVE, PISCATAWAY N.J., but it is a Swedish-based company) patented form of agarose. AGAROSE is the more general family of chemicals which are basically linear polysaccharides consisting of D-galactose and 3,6 anhydro-L-galactose units. The gelling of agarose is attributed to hydrogen bonding. Because of their hydrophilic nature and the nearly complete absence of charged groups agarose gels, like dextran gels, cause very little denaturation and adsorption of sensitive biochemical substances.

the production of a spherical agarose bead is the heart of PHARMACIA'S process. Could agarose be used by anyone to produce a workable affinity support? I really believe you would be as well off to buy SEPHAROSE. Remember once the immuno adsorbents are made, they can be used

for years.

Well, Sepharose affinity columns were easy to produce and had excellent flow properties, ^{even} after all the chemical procedures were completed, which chemistry itself was fairly simple. So quickly the research community adopted this technique to many different biological specific reactions. As to the matter of alterations in the basic cyanogen bromide activation of Sepharose, many different variations have evolved.

PORATH et al had used sodium bicarbonate as their coupling solution without clear explanation of the chemical reaction/mechanistic reasons for ^{that} choice. Then CAUTRECAUS, WILCHER and ANFINSEN FOLLOWED WITH THEIR VERY DEFINITIVE WORK ON AFFINITY CHROMATOGRAPHY ~~AS~~ AS UTILIZED FOR ENZYME PURIFICATION AND ENZYMIC PROCESSING VIA IMMOBILIZED ENZYMES.

CAUTRECAUS in attachment 4 described several different chemical forms of activated Sepharose that were useful for biologically specific separations. Additionally, he specified various coupling buffers which could be applied in the attachment of protein to Sepharose (see Table I, attachment 4). However, in the conclusions to his paper CAUTRECAUS points out that for various reasons, the Table I results are misleading and the optimum pH for coupling may be in the 6-7 range. Now possibly useful buffers in this range are sodium phosphate, sodium citrate, TRIS-HCl (^{TRIS IS} ~~the~~ hydroxymethylaminomethane)

Brock (Geman)

This reference is concerned with the ^{behavior} presence of placental proteins in

There is no evidence that a plurality of pregnancy specific antigens are expressed in the early stages of pregnancy.

Lein (1) - uses blood from third trimester of pregnancy.

- prepared rabbit anti sera against third trimester human pregnancy plasma was prepared.

There is no suggestion of placental protein.

The only mention of placental material is in immunos on p 293, where Bohn's work is described. Again no suggestion was made to use placental material for the detection of pregnancy in early periods.

Bohn

Jankowsky

uses only one mouth placenta + pregnancy serum.

Like it clearly shows that the PAP's vary considerably in their occurrence in humans. It is by no means apparent that antiserum against some placental which are readily available, are expressed in the early stages in subsequent months to be detected

Draft
Uzzell/cad
11/12/76

His No.
3-3443

2:47,800

AGENT AND METHOD FOR THE EARLY DETECTION OF PREGNANCY
Inventor: David W. Holladay, Rt. 15, Roland Lane, Knoxville, Tennessee 37921

INSTRUCTIONS FOR DRAFT PATENT APPLICATION

Please provide the requested information, if possible. If a statement is incorrect, please replace it with a correct statement. I have tried to describe the invention as broadly as possible in order to obtain the broadest patent coverage. I apologize for the extent of the questions, but it is necessary that everything be explained as completely as possible to prevent the invention from being limited to the particular reagents or process steps used. Feel free to make any changes, deletions, etc. which you deem advisable. Also, please point out any additional surprising or unpredictable results which were observed.

ABSTRACT OF THE DISCLOSURE

An agent capable of detecting the early stages of pregnancy in humans is provided by preparing an antiserum to proteins isolated from pregnancy-associated material such as placentas. By conventional techniques of detecting antigen-antibody reactions, the antiserum is used to detect the presence of pregnancy-specific proteins in women at the early stages of pregnancy.

Background of the Invention

This invention was made in the course of, or under, a contract with the Energy Research and Development Administration. It relates in general to pregnancy detection and, more specifically, to an agent and method for detecting pregnancy in women during the very early stages. Pregnancy has been detected as early as 18 days after conception according to this invention.

1075900

There has long been a need for a simple, reliable test which is capable of detecting pregnancy within a short time after conception. For example, such a test would be of great value for those women with an ancestral history of genetic disorders so that preventive therapy could be initiated as soon as possible. Those women working in potentially toxic or radioactive environments or taking potentially harmful medication also have a need for early pregnancy information.

THE FDA is now issuing warnings to women who become pregnant while fitted with IUDs. If the IUD is not removed, there is a greatly enhanced possibility of fetal or spontaneous abortion.

In the prior art pregnancy has been detected by a variety of well-known methods. Most of the prior art methods are unreliable when administered sooner than sixty days after conception. One method which has been shown effective for early pregnancy detection is described by B.B. Saxena et al. in the article "Radioreceptor Assay of Human Chorionic Gonadotropin: Detection of Early Pregnancy" Science, Vol. 8 pp. 793FF. (1974). This method is rather complex and requires the use of radioactive substances, and, therefore, might be unsuitable for routine clinical use. The present invention involves the use of host-produced antibodies to detect the presence of pregnancy-specific proteins in the sera of women in the early stages of pregnancy. The occurrence of pregnancy-specific components in sera from pregnant women was first demonstrated by Thornes and reported in an article by MacLaren et al. in Am. J. Obstet & Gynecol. 78:939(1959). Smithies in Adv. Protein Chem. 14 65,(1959) observed the presence of a pregnancy-associated alpha-globulin. Hirschfeld and Soderberg in Nature 187:332(1960) found two precipitates on immunoelectrophoresis of pregnancy sera. Gall and Halbert, Int. Arch. Allergy Appl. Immunol. 42:503(1972); and Lin et al., Am. J. Obstet. Gynecol. 118:223(1974) observed and characterized four sequential pregnancy-associated

plasma proteins found in the third trimester of pregnancy. Antisera had been raised against pregnancy plasma and then absorbed exhaustively with non-pregnancy plasma. Bohn in Arch. Gynaekol. 210:440(1971) used rabbit antihuman placenta antiserum absorbed with male serum to detect four pregnancy-associated plasma proteins. Only one was considered pregnancy-specific and two could sometimes be detected in sera from non-pregnant female subjects, especially those taking oral contraceptives. Berne, in Clin. Chem (Winston Salem, N.C.) 19:657, Abstr. 093, 1973 found pregnancy zone protein in women six weeks pregnant. The protein was also found in men and non-pregnant women. While the prior art was aware of the presence of proteins associated with pregnancy, there was no awareness of proteins which were specific to pregnancy and which could also be detectable sufficiently early in the gestation period to be useful as a pregnancy test.

Summary of the Invention

It is an object of this invention to provide an agent capable of detecting the early stages of pregnancy in humans.

It is a further object to provide an agent capable of detecting early pregnancy by simple and well-known clinical operations.

It is a further object to provide a method for the detection of pregnancy which requires no radioactive material or complex procedures. ✓

It is a further object to provide an agent and method for detecting pregnancy which is effective less than thirty days after conception. ✓

These and other objects as will be apparent are provided according to this invention by a pregnancy detection agent comprising an antibody to a pregnancy-specific protein isolated from non-specific antibodies and prepared by the method comprising:

1075902

- a) first providing a mixture of proteins occurring in pregnancy-associated material and containing an antigenic pregnancy-specific protein;
- b) isolating said antigenic pregnancy-specific protein from non-specific antigenic proteins;
- c) innoculating a host animal with said isolated pregnancy-specific antigenic protein to cause said host animal to raise antibodies to said pregnancy-specific antigenic protein;
- d) harvesting serum proteins from said innoculated host animal, said serum proteins containing antibodies to said pregnancy-specific antigenic protein, and
- e) isolating said antibodies to said pregnancy-specific antigenic protein from antibodies to non-specific human proteins.

The preferred pregnancy-associated material ^{For obtaining the proteins} is placentas. More concentrated pregnancy-specific antibodies are obtained by separating pregnancy-specific antibodies from the remaining animal serum proteins and/or antibodies.

A method of detecting the existence of pregnancy in humans according to this invention comprises contacting serum or urine from a patient with an antibody to a pregnancy-specific protein isolated from non-specific antibodies, and observing whether an antibody/antigen reaction occurs, the occurrence of said reaction being indicative of pregnancy. It is possible that other body fluids such as lymphatic fluid might contain pregnancy-specific proteins according to this invention, however urine and serum are most suitable for routine clinical testing.

Detailed Description

Very good

An important aspect of this invention is the discovery that structures and substances produced during the course of pregnancy in women contain proteins which are also present in detectable quantities in the very early stages of pregnancy. Another important aspect is the discovery that at least some of these pregnancy-associated proteins are both highly specific to pregnancy and sufficiently antigenic to raise antibodies in host animals which can then be isolated and used to detect the presence of the proteins in pregnant women relatively soon after conception, by standard immunological techniques.

For purposes of this invention, the terms specific and non-specific relate to pregnancy-specific and non-pregnancy-specific. A non-specific protein is one which is not specific to pregnancy. A pregnancy-specific antibody is an antibody to a pregnancy-specific protein. A non-specific antibody is an antibody to a non-specific protein. The term "antigenic" protein refers to a protein capable of producing detectable antibodies in a host animal. For purposes of this invention, a non-antigenic protein is one which raises no more than miniscule amounts of antibody which would not significantly interfere with the detection of pregnancy-specific proteins by pregnancy-specific antibodies.

The first step in preparing the pregnancy detection agent of this invention is to prepare a mixture of proteins occurring in pregnancy-associated materials such as placentas, umbilici, amniotic fluid, fetal tissue, etc. Placentas are preferred because of their ready availability. The separation of proteins from pregnancy-associated material need not be quantitative. The mixture of proteins can be prepared from the pregnancy-associated material by a number of well-known

biochemical techniques for concentrating proteins from biological tissues or fluids. This protein mixture will contain antigenic pregnancy-specific protein. While there are normally several antigenic pregnancy-specific proteins present in pregnancy-associated material, only one need be present for purposes of this invention. For example, pregnancy-associated tissues can be diced and homogenized in a stabilizing medium to minimize precipitation and agglomeration of proteins. Suitable stabilizing media include ^{pH 7} phosphate buffer, or a mild acid such as 0.1 M perchloric acid. The homogenized suspension is then separated such as by centrifugation, filtration, etc. to provide a supernatant liquid. Proteins can be separated from the supernatant by adding a precipitant. Suitable precipitants include ammonium sulfate, sodium sulfate, or other salting agents as are known in the art. The precipitated proteins are separated from the solution e.g., by centrifugation and redissolved in a buffer solution containing a bacteriostat or bacteriocide; for example, 0.1% sodium azide to prevent growth of unwanted bacteria. Other suitable reagents include merthiolate, ^{Novoforn, burmal} toluene, etc. The protein solution can then be dialyzed against the same buffer to remove the traces of the precipitant. Dialysis can be carried out with any of the well-known dialysis membranes such as viscose dialysis tubing sold by Union Carbide Corporation under the trade name VISKING. The protein solution is separated from precipitated material to provide a relatively pure solution of proteins.

The next step in preparing the agent of this invention is to isolate antigenic pregnancy-specific protein from non-specific antigenic proteins. Preferably, this isolation should be complete, however, small amounts of antigenic non-specific proteins can be tolerated with the specific proteins since subsequent processing is

capable of removing non-specific antibodies. It has been found that the removal of most or all of the antigenic non-specific proteins from the mixture of proteins prior to injection into a host animal will yield a substantially higher titer antisera in the host. When non-specific antigenic proteins are removed prior to inoculation, the host animal's immune response is directed toward the pregnancy-specific protein. When non-specific antigenic proteins are not removed prior to inoculation, the normal human proteins are likely to shield out the response to the pregnancy-specific proteins. [Question - Would usable or separable quantities of pregnancy-specific antibody still be

produced?] INJECTION WITH A CRUDE MIXTURE OF PLACENTAL ANTIGENS AND HUMAN SERUM PROTEINS RESULTED IN ANTISERA WITH A VERY WEAK TITER AGAINST PLACENTAL ANTIGENS & PREGNANCY SERA. FOR EXAMPLE, WHEN THE ANTISERA TO CRUDE PLACENTAL EXTRACT WAS COMPARED TO ANTISERA TO A PARTIALLY PURIFIED PLACENTAL EXTRACT, WITH EACH TESTED AGAINST EARLY PREGNANT SERA, THE ANTISERA TO PARTIALLY OR HIGHLY PURIFIED PLACENTAL EXTRACT REACTED TO GIVE MULTIPLE BANDS OF PRECIPITATION AT MUCH EARLIER TIMES THAN FOUND FOR ANTISERA TO CRUDE EXTRACT. SUMMARILY, THE WHOLE CRUDE ANTISERA WOULD PROBABLY NOT BE ~~USEFUL~~ ^{RELIABLE} ^{DEPENDABLE} IN CLINICAL TESTS.

There are at least two methods by which antigenic pregnancy-specific proteins can be isolated from non-specific antigenic human proteins. In one instance, the mixture can be contacted with antibodies raised against human serum containing no pregnancy-specific proteins, hereinafter termed anti-normal human serum. Suitable serum can be collected and pooled from a large sample of males and non-pregnant females, ^{and used to raise host-produced antibodies.} The antibodies to this pooled serum will react with non-specific antigenic proteins in the mixture, and the reaction products can be separated from the mixture, thereby providing a mixture of pregnancy-specific proteins and non-antigenic, non-specific proteins. For purposes of this invention, such non-antigenic proteins can be regarded as non-interfering material. Alternatively, the protein

mixture obtained from the pregnancy associated material can be contacted with antibodies raised against pregnancy-specific proteins, whereby only antigenic pregnancy-specific proteins react with the antibodies and are recoverable from the mixture. This method will provide an innoculent more concentrated in pregnancy-specific protein. *sp?* *Good!*

The preferred method of carrying out antibody/antigen reactions for isolating the pregnancy-specific proteins from antigenic non-specific proteins is by passing the protein mixture through an immuno-absorbent column *(prepared with) probably better then labeled. indigins antibodies are* loaded with appropriate antibodies, e.g. anti-normal human serum or anti-pregnancy-specific proteins. *invariant post of column supply* When the column is *prepared* loaded with *(reacts with normal serum proteins)* anti-normal human serum, the unbound fraction con-

tains pregnancy-specific proteins isolated from antigenic non-specific protein and ~~the pregnancy-specific proteins~~ *contaminating normal serum proteins are bound to the column and thus separated from pregnancy specific proteins. If the column is prepared from* the pregnancy specific proteins are bound to the column and may be eluted with *capable of separating antibody/antigen complexes* a suitable eluent such as a 2.5 M sodium thiocyanate solution. *The normal proteins pass through unbound.*

The next step in preparing the agent of this invention is to inoculate a host animal with the isolated pregnancy-specific antigenic proteins to cause the host animal to raise antibodies to the pregnancy-specific proteins. All that is required prior to inoculation is that the proteins be in a solution capable of injection. The solution from the immuno-^{ad}sorbent step can be concentrated and directly injected [Question - Should thiocyanate be first removed

from the eluate?] *yes!* *If the pregnancy specific proteins were isolated by being bound to immobilized anti-pregnancy specific antibodies, then they would have to be eluted with thiocyanate. Then, to assure that they were in a native-type structure the chaotropic thiocyanate was always removed by dialysis as soon as station possible after elution of the column. The Cyclim System Accomplished Dialysis Automatically.* Of course, antibodies could be raised less efficiently against dilute solutions.

Suitable hosts are virtually any warm-blooded animals such as goats, cattle, horses.

rabbits, chickens, etc. Goats are preferred because they have an extensive and multi-varied antibody response, normally superior to other animals. Of course, it can be readily appreciated that the terms "antigenic" and "non-antigenic" refer to the particular host animals being used since an antigenic protein to ^{one}~~some~~ animal may be non-antigenic to others. No warm-blooded animal has proven incapable of producing antigens to at least one pregnancy-specific protein. As is customary in the raising of antibodies, conventional adjuvants can be employed to accelerate antibody-production. The injection schedule can be expected to vary for different types of hosts and it is well within the skill of those acquainted with the manufacture of vaccines, etc. to work out a suitable injection schedule. The same injection schedule will be appropriate for producing anti-normal human serum.

The next step in preparing the agent of this invention is harvesting serum proteins from the inoculated host animal. After an inoculation schedule (e.g., weekly) of several months, the host animals are bled and their ^{Sera}plasma harvested.

~~Serum~~ ^{Serum} (note, ~~serum~~ see book) The ~~plasma~~ is separable from whole blood by conventional means such as centrifugation. ^{intra cloning}

~~The plasma~~ ^{The} serum, contains host serum proteins comprising antibodies to at least one of the pregnancy-specific proteins. ^{See book for serum/plasma}

The next step in preparing the agent of this invention is isolating antibodies to the pregnancy-specific proteins from antibodies to non-specific human proteins.

Even if the proteins injected into the host were completely free of antigenic non-specific human proteins, it is necessary that this isolation be performed to remove traces of antibodies to non-specific human proteins which were native to the host or which were raised against marginally non-antigenic human proteins. [Any other

reason for this step?] ^{We} No, simply desire to have pure antibodies to pregnancy-specific proteins

the fluid part of blood in its natural condition is called plasma. the ~~the~~ blood cells have been spun down by centrifugation. In the whole blood, the plasma forms about 55% of the volume, the corpuscles (red cells or erythrocytes, white cells-leucocytes or lymphocytes, and ~~the~~ granulocytes) form ~ 45%.

the fluid part of the blood after it has gone through the process of clotting or coagulation is called serum.

While the composition of these 2 fluids is similar, serum lacks certain of the substances which have been depleted in the clotting process; ^{e.g.} It lacks fibrinogen but contains excess thrombin.

Plasma is obtained by centrifuging freshly drawn blood. Serum is obtained when the blood is permitted to clot and stand for about 24 hours, so that the clot shrinks and serum is squeezed out.

We generally harvest the serum, although there is little substantive difference in the 2 fluids

The preferred method of carrying out this isolation step is by passing the host serum through an immuno^{ad}-absorbent column loaded with normal human serum proteins. The material which is not adsorbed on the column is thus a mixture containing an antibody to a pregnancy-specific protein isolated from non-specific antibodies, and as such is now an agent capable of detecting the presence of pregnancy-associated proteins in women, in accordance with this invention. It is preferred that this column be loaded with serum proteins obtained from a large sample of males and non-pregnant females, having all of the blood types. Females taking estrogen-based oral contraceptives ^{Good} should be included in the sample, if possible. The serum of course is typically collected as plasma separated from whole blood and lyophilized or freeze-dried for storage. This pooled ^{sera} serum is also preferred for injection into a host to produce anti-normal human serum as used for carrying out this adsorption of non-specific antigenic human proteins from the original protein mixture. The anti-normal human serum can be prepared by any of a number of well-known serological methods. The preferred method is to make balanced antisera which contains sufficient antibodies to remove all of the non-specific antigenic proteins in a protein mixture. The procedure for carrying out immunoadsorption chromatography and for preparing a balanced antisera is described in N.G. Anderson, et al, "Cyclic Affinity Chromatography: Principles and Applications", Analytical Biochemistry 68, 371-393 (1975), which is herein incorporated by reference.

It is generally desirable that pregnancy-specific antibodies be further isolated from some or all of the animal serum proteins. This can be accomplished by passing the animal protein mixture, either before or after pregnancy-specific antibodies are isolated from antibodies to non-specific proteins, through a conventional column containing diethylaminoethyl cellulose ~~and~~ beads. Such beads are capable of adsorbing immunoglobulin from the remainder of the animal's proteins. [Is there anything one should know about bead size, porosity, composition, etc.?] Please provide the reference for insert 7, describing DEAE chromatography.

THE MOLECULAR ANATOMY PROGRAM FIRST SEMI-ANNUAL
PROGRESS REPORT, MAR 1 to AUG 31, 1970, ORNL-4733
pp 384-394, There are 2 or 3 major suppliers of DEAE
OUR BEST DEAE WAS OBTAINED FROM W & R BALSTON LTD MAIDSTONE KENT
ENGLAND. THEIR SALES DIV in THE US. WAS REEVE ANGEL, CLIFTON N.J.
07014 ph. 201 773-5500. Over for use into.

Also, if desired, antibodies to pregnancy-specific proteins can be further separated from other animal antibodies by passing the antiserum through an ^{ad} immunosorbent column loaded with pregnancy-specific proteins. The pregnancy-specific antibodies are adsorbed thereon and can be eluted with a solution capable of dissociating antigen/antibody compounds. ^{lexes} An example of such a solution is a chaotropic ion solution such as a thiocyanate or trichloroacetate solution. ^{Ammonium} ~~Ammonium~~ and sodium salts are preferred. The preferred antibody eluent is a 2.5 M sodium thiocyanate, 0.1 M sodium phosphate pH 7.0, 0.1% sodium azide (bacteriostat) solution. Below 1.0 M thiocyanate does not give quantitative elution. It can be readily seen that this adsorption/elution step is capable of effecting the isolation of pregnancy-specific antibodies from non-specific human proteins as well as from animal protein.

1075911

WHATMAN HAS TWO SERIES OF DEAE SUPPORTS.

THE DEAE 22-23 SERIES HAS BUNDLES OF CELLULOSE MICROFIBERS IN THE SIZE RANGE OF 40 - 300 MICRONS.

THE DEAE 32-52 SERIES HAS "MICROGRANULES" OF CELLULOSE PARTICLES WITH AN AVERAGE DIAMETER AS EQUIVALENT SPHERES OF ~ 40 MICRONS, DISTRIBUTION FROM 20 - 60 MICRONS.

IT IS CLAIMED THAT THE DE 32-52 SERIES GIVES BETTER RESOLUTION / SEPARATION THAN THE DE 22-23 SERIES. HOWEVER THE DE 22-23 SERIES ALLOWS FASTER FLOW RATES.

WE USED BOTH SERIES WITH SIMILAR SUCCESSFUL RESULTS.

IN GENERAL WE FOUND WHATMAN DEAE SUPERIOR TO ^{THAT OF} OTHER SUPPLIERS. THERE HAS BEEN SOME DEGREE OF PULP QUALITY CONTROL ~~IN~~ THE PRODUCTION PROCESSES OF VARIOUS SUPPLIERS.

The immunoabsorbent columns useful for preparing the agent of this invention can be prepared by conventional techniques. What is necessary is that the column contain the desired antibodies or antigens immobilized so that they are reactive with their corresponding antigen or antibody. The immunoabsorbent columns therefore provide both reaction contact between antigen and antibody as well as separation of unreacted components which exit the column as the unbound fraction. Such immunoabsorbent columns are well-known in the art of serology. For purposes of illustration, the following example is provided for the preparation of an immunoabsorbent. The method is suitable for binding any desired protein to the packing material. Those skilled in the art can prepare similarly effective immunoabsorbent columns by a variety of different methods.

Example I

Protein to be bound (either antigen, antibody, or non-antigenic) is concentrated in aqueous solution to 70 ml. and dialyzed against the coupling buffer to provide a protein solution in coupling buffer. Coupling buffer is the buffer in which the binding reaction ultimately occurs. Examples of suitable coupling buffers are

(give pH also) *You have several questions here and following concerning the matrix, coupling solution, activating agent, etc for immunoabsorbents. I am attaching additional information in this entire procedure.*

Seventy ml (a like volume as the protein) of Sepharase 4B is washed with at least 20 volumes of 0.1 M phosphate pH 6.5. Sepharase 4B is (chemical formula, physical characteristics,) and is available from

[Is there any non-trademarked material which could be used?]

[Is there anything critical about the form of Sepharase 4B?]

The washed Sepharase is mixed with an equal volume of coupling buffer in a 400 ml. beaker with magnetic stirrer, and in an ice bath to form a slurry. The slurry was

titrated to a pH of 11.5 with 6M NaOH. The cooled slurry is added 21 g. of cyanogen bromide (0.3 g/ml. Sepharose) and the pH was maintained at 11.5 with NaOH and the temperature was maintained at about 20°C. Under these conditions the cyanogen bromide reacts with the Sepharose to cause activation. The reaction was allowed to proceed until no more decrease in pH was noted, to ensure the end of the activation. The activated slurry was washed with 4°C coupling buffer on a coarse Buchner Funnel. This washing must proceed within 2 minutes from the completion of activation. Why? - *Must be done immediately after activation*

The washed CnBr-activated Sepharose 4B was mixed with the solution of protein to be bound and the mixture is slowly tumbled overnight at 4°C. Additional mixing (i.e. over 24 hrs.) would normally provide more extensive attachment of proteins. What other activation agents would be useful?

Space or chemical cross-linkers such as glutaraldehyde

After mixing, the unbound protein is washed off in coupling buffer

(0.1 M 6.5 pH phosphate) and washing is continued until no background

absorbance is detected in the wash water. After this washing, the

loaded Sepharose is tumbled in 1M ethanolamine pH 10 for 2 hours

to load any remaining sites on the resin. The ethanolamine is washed off

with water = 0.1M phosphate buffer and the loaded Sepharose is washed alternately with 0.10 M

sodium acetate, pH 4, 0.1% sodium azide to prevent bacterial growth, 1.0 M

sodium chloride, 0.1 M sodium borate pH 8.5, 0.1% sodium azide and 1.0 M

sodium chloride. An additional wash with 0.1M glycine NaOH (pH 11.0) can also

be included. The ^{purpose} purpose of these washing steps ~~are~~ are:

is not to be used as a test

EST

The protein-loaded Sepharose is finally washed with 2.0M sodium thiocyanate, 0.06 M sodium phosphate, pH 7.0 and 0.1% sodium azide, followed by 0.1M sodium phosphate pH 7.0 and 0.1% sodium azide until background absorbance is obtained. The protein is now firmly bound to the Sepharose to provide an immunosorbent particle ready for use as column packing.

The pregnancy detection method of this invention comprises contacting serum from the patient with antibodies to pregnancy-specific proteins isolated from non-specific antibodies. Since the existence of the antigenic pregnancy specific-proteins has herein been demonstrated, the source of the protein is not an essential feature of the pregnancy test. The desired antibodies can be prepared by the methods disclosed herein, or it is conceivable that at least one of the pregnancy-specific proteins can be identified and synthesized, thereby avoiding the necessity of separating the pregnancy-specific protein from antigenic non-specific human proteins. The synthetic protein could then be used directly to raise antibodies useful for detecting pregnancy.

All that is necessary for this pregnancy detection method is that serum from a patient be contacted with an antibody raised against a pregnancy-specific protein and the results observed to detect signs of antibody/antigen reaction. The occurrence of the reaction of course, indicates pregnancy. It can be readily appreciated that any of the several methods for detecting antigen/antibody reaction is suitable for detecting pregnancy according to my method. Examples of such methods are gel precipitation, gel electropherograms, immunoelectrophoresis, cross-

immunoelectrophoresis, and immunodiffusion.

The primary utility of the pregnancy detection method of this invention is for detecting pregnancy within about 2 months of conception. After 2 months, pregnancy is reliably detectable by well-known external symptoms. The method of this invention is particularly useful for detecting pregnancy within one month of conception, when other tests are inconclusive. It is likely that it will prove effective within one week of conception.

In order to demonstrate a preferred method for preparing the pregnancy detection agent of this invention and the operability of the agent for detecting pregnancy, a detailed example is presented. It will be apparent that substantial variation from the procedures described therein can be made without departing from the spirit and scope of the invention.

Example

Preparation of crude placental extract . Term placentas were obtained at delivery and chilled in 0.85% sodium chloride solution for preservation. The placentas were washed for 1 hr. in tap water to remove a maximum of blood elements and serum proteins. While the mother's or infant's serum would likely contain traces of pregnancy-specific proteins, the placenta contains substantially more of those proteins. Fat was cut away since it is superfluous and non-antigenic, and the placentas were cut into pieces about 1 cm on a side and frozen. Subsequently, the frozen material was homogenized in three volumes of 0.1 M, pH 7, phosphate buffer. The homogenate was centrifuged at 17,000 x g (acceleration of gravity) for 10 min. in an angle-head centrifuge, the supernatant was collected, and the pellet was rehomogenized in two volumes of the same buffer, and a second centrifugation at 17,000 x g for 10 min. was done. The proteins of interest

precipitated from the pooled supernatants by adding solid ammonium sulfate to 55% saturation and separated by centrifugation. Some of the unprecipitated proteins have since been shown to be pregnancy-specific and can also be recovered if desired. Following centrifugation, the material was dissolved in a minimal amount of 0.1 M, pH 7, phosphate buffer containing 0.1% sodium azide, and the solution was dialyzed against this same buffer, using viscose dialysis tubing such as is sold by Union Carbide Corporation. Any material that precipitated was removed by centrifugation. The precipitate was mostly large macromolecules representing the connective tissue of the placenta. Any serum proteins left in the solution were removed by cycling it over an immunoadsorbent column loaded with anti-normal human serum. The cycling was carried out automatically by an apparatus as disclosed in Anderson, et al., Analytical Biochemistry 66, 159-174

(1975). [Was the bound fraction also eluted between cycles? With what?]

YES. OTHERWISE THE ANTI-NORMAL SERUM ANTIBODIES WOULD HAVE BEEN BLIND OR COVERED WITH THEIR SPECIFIC NORMAL SERUM ANTIGENS AND THUS COULD NOT HAVE REACTED WITH THE NEXT SAMPLE. THEY WERE ELUTED WITH AMMONIUM α POTASSIUM TRIOXALATE, THE COLUMN WAS REEQUILIBRATED WITH PHOSPHATE BUFFER, AND ANOTHER PLACENTAL EXTRACT SAMPLE WAS LOADED.

The unbound fraction emerging from this treatment was still a complex mixture, containing at least 20 different proteins, as judged by gel electrophoresis. The solution could not be concentrated beyond 20 A_{280} units per ml without undesired precipitation occurring on storage, especially on freezing and thawing. An A_{280} unit is the quantity of protein per ml of water or buffer giving an absorbance of 1 at 280 nm in a cell with a light path of 1 cm. Apparently, the bulk of the material that precipitated was placental tissue proteins, mostly acid in nature. If desired, this material could be separated into several subfractions by chromatography over

diethylaminoethyl cellulose. Though not essential to the operability of the process, some separations of placental tissue proteins that appear in the serum from those that do not could be effected to provide a more concentrated agent. Only 10 to 15 of the proteins in the mixture have proved to be sufficiently antigenic to ^{result} result in detectable antibody production to them. It is likely possible to modify the extraction procedure (for example, mild acid extraction of diced placenta, e.g., with 0.1 M perchloric acid, without homogenization) in order to minimize the extraction of proteins that do not appear in the plasma; however, this is unnecessary for the operability of the detection method.

The placental extracts, minus non-specific serum proteins but still containing the relatively non-antigenic tissue proteins mentioned, were concentrated to about 15 A_{280} units per ml for injection purposes. An estimate of the antigen content was 0.1 to 1 mg/ml, based on intensity of reaction in immunodiffusion. The extract was mixed with equal volume of Freund's complete adjuvant, and the preparation was injected subcutaneously into goats and rabbits, either in the hips or behind the neck (4 ml for goats, 1 ml for rabbits). Freund's complete adjuvant is commercially available from DIFCO Laboratories (Do you know the address?) ^{Yes, But you could obtain from anyone in biology division} and contains sterile light mineral oil plus emulsifier and heat killed Mycobacterium ^{there are several other suppliers, I believe.} butyricum (0.5 mg. dry weight per ml).

Intravenous injection should normally be avoided due to the increased likelihood of anaphylactic shock and sudden death of the host. Thereafter, the same amounts of extract without adjuvant were injected subcutaneously at weekly intervals. Every fourth week, injection was done with antigen plus adjuvant. First bleeding was at 5 weeks (40 ml for rabbits and 150 ml for goats) and was biweekly thereafter to

test for antibody production. High-titer antiserum was obtained, usually in about 7 to 8 months. The antiserum was absorbed on a conventional immunoadsorbent column loaded with lyophilized, pooled serum obtained from normal human males (100 mg/ml of antiserum) to remove non-specific antibodies. The unbound fraction contained pregnancy-specific antibodies and normal animal protein, isolated from antibodies to non-specific human protein. If desired, i.e., for commercial preparation, the bulk of the animal protein can be removed by DEAE cellulose chromatography, in which the unbound fraction can be passed through a DEAE cellulose column whereby the antibodies load the column and the bulk animal protein passes through.

Gel precipitation was used to test the antiserum-to-placental-antigens for the presence of antigens that appeared in the sera of pregnant women. The antiserum was first tested against sera from women in the third trimester of pregnancy. Tests against the sera from three different women in their first pregnancy, three women in their second pregnancy, and three women in their third pregnancy showed positive reactions within 5 hours with 2 to 4 bands clearly visible after 40 hours. The control for all three plates was from a 28 year old woman who had had one successful pregnancy 5 years previously, and who since that time had been taking an estrogen-based birth control pill. No reaction was noted at any time with the control.

The antiserum gave negative tests against a succession of batches of pooled normal human male sera. It was positive against all of a series of 40 samples of third-trimester pregnancy sera. It was negative against the sera of ten non-pregnant women, including those previously pregnant. Of four cases tested, no

reaction has been shown with sera from women taking estrogen-related contraceptives. In order to demonstrate the detection of pregnancy early enough to be valuable for a pregnancy test, several runs were made on sera from women who suspected pregnancy. A serum sample was obtained from a 29 year-old woman 6 days after she had failed to begin her menstrual period. A good estimate of the time duration since conception was 18 to 22 days. A serum sample was obtained from a 25 year-old woman 10 days after she had failed to begin her menstrual period, giving a time duration of 22 to 26 days since conception. The samples were tested by double diffusion against antiserum to placental antigens. Placental extract and serum from a woman in the third trimester of her third pregnancy were used as reference.

After 16 hr. one sharp band and one faint band of precipitation were observed in both samples, and the two bands merged or showed identity with bands from both placental extract and the third-trimester pregnancy serum sample. For both samples, a precipitin band could be observed as early as 1 hr. after the start of immunodiffusion. Three precipitin bands were observed for the second sample within 24 hr. after loading. Improved immunodiffusion techniques would likely permit a decision on pregnancy on the same day the blood sample is withdrawn.

Another early pregnancy serum sample, taken at 28 to 32 days after conception, was tested against the antiserum and gave two precipitin bands within 16 hr. Pregnancy was confirmed at 8 weeks of gestation by routine examination for all three of the women. The human placental antigen preparation gave at least ten distinguishable precipitin bands with the antiserum. The precipitin bands that are the strongest for very early pregnancy samples show identity with the weakest bands from

samples from third-trimester pregnancies. This indicates that the antigens involved reach a concentration peak early in pregnancy, and then slowly decrease during gestation. It is possible that some early appearing antigens may not be detectable in the second and third trimester.

While the particular pregnancy-specific antigenic proteins utilized for this invention have not yet been characterized, their identification is not essential to manufacturing the agent of this invention or to performing the pregnancy test of this invention.

Recent advances in cancer research have intimated that certain proteins present in pregnancy-associated material such as placentas, are re-expressed in the early stages of cancer. This is consistent to the idea that the formation of the fetus is physiologically similar to the formation of tumor cells in cancer patients. For a description of the observed relationship between fetal and placental proteins see, for example, Ruoslahti, et al, *Int. J. Cancer*: 7 218-225 (1971), Thompson, et al, *Medical Sciences* 64, 164-6 (1969), Gold, *Prog. Exp. Tumor Res.* 14: 43-58, and *Am. Assn. Cancer Res. Abstracts* 490, 568, 584, 842 (1974). Accordingly, a possible utility for the pregnancy detection agent of this invention is the early detection of at least some forms of cancer.

What is claimed is:

1. A pregnancy detection agent comprising antibodies to a pregnancy-specific protein isolated from non-specific antibodies and prepared by the method comprising:

- a) first providing a mixture of proteins occurring in pregnancy-associated material and containing an antigenic pregnancy-specific protein;
- b) isolating said antigenic-pregnancy-specific protein from non-specific antigenic proteins;
- c) innoculating a host animal with said isolated pregnancy-specific antigenic protein to cause said host animal to raise antibodies to said pregnancy-specific antigenic protein;
- d) harvesting serum proteins from said innoculated host animal; and
- d) isolating antibodies to said pregnancy-specific proteins from antibodies to non-specific human proteins.

2. The agent of claim 1 in which said proteins occurring in pregnancy-associated material are recovered from placentas.

3. The agent of claim 1 wherein said method further comprises isolating antibodies to said pregnancy-specific proteins from non-antibody host animal serum proteins.

4. A method for detecting the existence of pregnancy in humans comprising contacting serum or urine from a patient with an antibody to a pregnancy-specific protein isolated from non-specific antibodies, and observing whether an antibody/antigen reaction occurs, the occurrence of said reaction being indicative of pregnancy.

5. The method of claim 4 wherein said serum or urine is withdrawn from said patient within about two months of conception.
6. The method of claim 4 wherein said serum or urine is withdrawn from said patient within about one month of conception.
7. A method of detecting the existence of pregnancy in humans comprising contacting serum or urine from a patient with the agent of claim 1 and observing whether an antibody/antigen reaction occurs, the occurrence of said reaction being indicative of pregnancy.
8. The method of claim 7 wherein said serum is withdrawn from said patient within about two months of conception.
9. The method of claim 7 wherein said serum is withdrawn from said patient within about one month of conception.
10. The method of claim 7 wherein said proteins occurring in pregnancy-associated material are recovered from placentas.

Professional Bibliography of
John E. CATON

1. Studies of the Spectra of Copper Dimethylglyoxime, Nickel Dimethylglyoxime, and Nickel Ethylmethylglyoxime, Talanta **13**, 967 (1966). J. E. Caton and C. V. Banks.
2. Hydrogen Bonding in Some Copper (II) and Nickel (II) vic-Dioximes, Inorg. Chem. **6**, 1670 (1967). J. E. Caton and C. V. Banks.
3. Separation of Escherichia coli Ribosomal Ribonucleic Acids by Reversed Phase Chromatography, Biochemistry **10**, 1890 (1971). B. Z. Egan, J. E. Caton, A. D. Kelmers.
4. Electrophoresis of Ribonucleic Acids on Polyacrylamide Gel Gradients, Anal. Biochem. **42**, 14 (1971). J. E. Caton and G. Goldstein.

1075924

5. Variable-Speed Drive for the Mechanical Stage of a Microscope Used as the Optical Component in a Microdensitometer, Anal. Biochem. **46**, 232 (1972). J. E. Caton, D. D. Willis, and N. G. Anderson.
6. Protein Purification by Immunoabsorption, Enzyme Engineering **2**, (1974). J. W. Holleman, D. W. Holladay, J. E. Caton, and N. G. Anderson
7. The Nature of Polyhedral Inclusion Bodies of *Thiobacillus neopolitanus*, Proc. Electron Microscopy Soc. Am., 30th Ann. Mtg. Baton Rouge, Louisiana: Claitors Publishing Division, 1972, p. 316. F. L. Ball, J. M. Shively, J. P. Breillatt, J. E. Caton, and D. H. Brown.
8. Immunosubtractive Electrophoresis on Gradient Polyacrylamide Gels, Anal. Lett., **7**, 483 (1974). J. E. Caton, D. W. Holladay, and N. G. Anderson.
9. Searching for Human Tumor Antigens, Cancer Research **34**, 2066 (1974). N. G. Anderson, D. W. Holladay, J. E. Caton, E. L. Candler, P. J. Dierlam, J. W. Eveleigh, F. L. Ball, J. W. Holleman, J. P. Breillatt, and J. H. Coggin, Jr.
10. Analytical Techniques for Cell Fractions. XIX. The Cyclum: An automatic System for Cyclic Chromatography, Anal. Biochem. **66**, 159 (1975). N. G. Anderson, D. D. Willis, D. W. Holladay, J. E. Caton, J. W. Holleman, J. W. Eveleigh, J. E. Attrill, F. L. Ball, and N. L. Anderson.
11. Analytical Techniques for Cell Fractions. XX. Cyclic Affinity Chromatography: Principles and Applications, Anal. Biochem. **68**, 371 (1975). N. G. Anderson, D. D. Willis, D. W. Holladay, J. E. Caton, J. W. Holleman, J. W. Eveleigh, J. E. Attrill, F. L. Ball, and N. L. Anderson.
12. Electrophoretic Methods for Detecting Differences in Seed Proteins of Soybean Varieties and Induced Mutants, Agr. Food Chem. **22**, 1043 (1974). K. L. Lowry, J. E. Caton, and D. E. Foard.
13. Control and Maximization of Tobacco Smoke Dose in Chronic Animal Studies, Proc. Tobacco Smoke Inhalation Workshop on Experimental Methods in Smoking and Health Research, June 19-21, 1974: DHEW Publication No. (NIH) 75-906, p. 17. P. Nettesheim, M. R. Guerin, J. Kendrick, I. Rubin, J. Stokely, D. Creasia, W. Maddox, and J. E. Caton.
14. Early Detection of Pregnancy-Associated Serum Proteins Using Antiserum to Placental Antigens, Immunological Communications **5**, 1 (1976). D. W. Holladay, J. E. Caton, F. L. Ball, J. W. Holleman, and N. G. Anderson.
15. Quantitative Exposure of Respiratory Airway Epithelium to 7,12-Dimethylbenz(a)anthracene, submitted to Cancer Research. R. A. Griesemer, P. Nettesheim, D. H. Martin, and J. E. Caton.

16. Evolution of Preneoplastic and Neoplastic Lesions in Grafted Rat Tracheas Continuously Exposed to Benz(a)pyrene, submitted to Cancer Res. P. Nettesheim, R. A. Griesemer, D. H. Martin, and J. E. Caton.

Published Abstracts:

1. Comparison and Evaluation of Separations in Polyacrylamide Gel Electrophoresis. 160th ACS Natl. Mtg., Chicago, Illinois, September 14-18, 1970, Anal 018. J. E. Caton and N. G. Anderson.
2. Use of Reversed Phase Chromatography for Separating E. coli Ribosomal RNA's and Other Selected RNA's. 160th ACS Natl. Mtg., Chicago, Illinois, September 14-18, 1970, Anal 056. B. Z. Egan, J. E. Caton, and A. D. Kelmers.
3. Separation of E. coli Ribosomal RNA's and Mammalian RNA's by Reversed Phase Chromatography. Federation Proc. 29, 671 Abs (1970). B. Z. Egan, J. E. Caton, and A. D. Kelmers.
4. Isolation of Tumor-Associated Macromolecules Using Immunosubtraction. Federation Proc. 32, 961 (1973). N. G. Anderson, D. W. Holladay, J. E. Caton, and L. B. Peterson.
5. Immunosubtractive Electrophoresis on Gradient Polyacrylamide Gels. 165th ACS Natl. Mtg., Dallas, Texas, April 8-13, 1973, Anal 31. J. E. Caton, D. W. Holladay, and N. G. Anderson.
6. Affinity Chromatography of Human Serum Using Antisera Against "All But One" Human Serum Proteins, 165th ACS Natl. Mtg., Dallas, Texas, April 8-13, 1973, Anal 32. J. E. Attrill, J. E. Caton, D. W. Holladay, and N. G. Anderson.
7. Casting Cylindrical Polyacrylamide Gels in a Spinning Rotor. Federation Proc. (1974). J. E. Caton, N. G. Anderson, J. N. Brantley, and C. A. Hahs.
8. Ribulose-1,5-diphosphate Carboxylase from Thiobacillus neapolitanus. Federation Proc., (1974). J. M. Shively, J. P. Breillatt, and J. E. Caton.
9. Depletion of Constituents in Contained Smoke Aerosols for Inhalation Exposure Dosimetry. Abstracts, 29th Tobacco Chemists' Research Conference, October 8-10, 1975, Abst. No. 50. J. E. Caton, J. R. Stokely, and M. R. Guerin.

Presentations of Research Studies:

1. Comparison and Evaluation of Separations in Polyacrylamide Gel Electrophoresis. 160th Natl. ACS Mtg., Chicago, Illinois, Sept. 14-18, 1970. J. E. Caton.

2. Immun subtraction Electrophoresis on Gradient Polyacrylamide Gels. 165th Natl. ACS Mtg., Dallas, Texas, April 8-13, 1973. J. E. Caton.
3. Casting Cylindrical Polyacrylamide Gels in a Spinning Rotor. Biochemistry/Biophysics 1974 Meeting, Minneapolis, Minnesota, June 2-7, 1974, J. E. Caton.
4. Exposure Chemistry of Noninvasive Dosimetry of Individual Smoke Constituents During Inhalation Bioassay. Tobacco Working Group Meeting, February 18-19, 1975, Bethesda, Maryland. J. E. Caton.
5. Dosimetry Measurements for Inhalation Studies. ORAU Training School on Biological Considerations of Environmental Pollutants, August 19, 1975, Oak Ridge, Tennessee. J. E. Caton.
6. Depletion of Constituents in Contained Smoke Aerosols for Inhalation Exposure Dosimetry. 29th Tobacco Chemists' Research Conference, October 8-10, 1975, College Park, Maryland. J. E. Caton.
7. Cigarette Smoke Dosimetry in Mice, Contractor's Meeting, Council for Tobacco Research, USA. New Orleans, Louisiana, April 13, 1976. J. E. Caton.

Lectures and Seminars:

1. The Potential of Analytical Polyacrylamide Gel Electrophoresis. ORAU Traveling Lecture presented at:
 - a. Clemson University, Clemson, South Carolina, February 1, 1972, (Dept. of Plant Pathology and Biochemistry).
 - b. University of Miami, Coral Gables, Florida, April 24, 1972, (Dept. of Chemistry).
 - c. Florida Atlantic University, Boca Raton, Florida, April 25, 1972, (Dept. of Chemistry).
 - d. University of Mississippi, Jackson, Mississippi, November 13, 1972, (School of Medicine).
 - e. University of West Florida, Pensacola, Florida, February 14, 1973, (Dept. of Chemistry).
 - f. University of Oklahoma, Norman, Oklahoma, December 4, 1974, (Dept. of Chemistry).
2. Analytical Electrophoresis. ORAU Traveling Lecture presented at:
 - a. University of Puerto Rico, Rio Piedras, Puerto Rico, April 27, 1972, (Dept. of Chemistry).

- b. Langston University, Langston, Oklahoma, December 5, 1974, (Dept. of Physical Science).
 - c. Agnes Scott College, Decatur, Georgia, February 26, 1975, (Dept. of Chemistry).
 - d. Christian Brothers College, Memphis, Tennessee, February 18, 1976, (Dept. of Biology).
3. Immunological Reagents in Analytical Chemistry. ORAU Traveling Lecture presented at:
 - a. Maryville College, Maryville, Tennessee, January 21, 1975, (Dept. of Chemistry).
 - b. Tougaloo College, Tougaloo, Mississippi, February 17, 1976, (Natural Sciences Division).
 4. Electrophoresis. (General Lecture) Medical Technology Class, St. Mary's Hospital, Knoxville, Tennessee, January 18, 1972.
 5. Electrophoresis as an Analytical Tool, Analytical Specialities Meeting, Union Carbide Corporation, Oak Ridge, Tennessee, March, 1972.

Patent:

1. Rotor for Centrifugal Testing of Electrophoresis Gel. United States Patent No. 3,927,826; December 23, 1975. N. G. Anderson and J. E. Caton.

1075928

GLASS

Enzymes immobilized on inorganic carriers

Reuse of enzymes is possible if they are attached to water-insoluble matrices. Covalent attachment to inorganic carriers improves structural and operational stability as well as boosting resistance to microbial attack

by Howard H. Weetall
Research and Development Laboratories, Corning Glass Works

A technology considered a laboratory curiosity only five years ago has blossomed into what may be one of the major technological advances in decades.

It all began half a century ago when Nelson and Griffin first adsorbed the enzyme invertase to animal charcoal and found that the "immobilized enzyme" still retained biological activity (1). Today, this technology is at a point where a Japanese company using insolubilized enzymes is commercially preparing optically active amino acids.

Conventionally, enzymes have been used only once in commercial processes before being either inactivated or removed from the product. The substitution of immobilized or bound enzymes would permit reuse of the enzymes, since recovery is simplified and processing can be accomplished on a continuous basis, reducing enzyme and labor costs.

There are several methods by which enzymes have been immobilized on water-insoluble carriers. The simplest is adsorption. Enzymes have been adsorbed on inorganic matrices like glass (2), bentonite, mineral salts (3) and charcoal (4). Other methods include bonding to ion-exchange resins, cross-linking, adsorption followed by cross-linking, entrapment in a polymer lattice and covalent attachment (4,5).

Until recently all enzymes attached to water-insoluble matrices, except for the adsorbed enzymes, were attached to organic polymers. Inherent in the use of organic polymers are several problems that have discouraged the commercialization of this technology. These problems include:

1. Susceptibility to pH and solvent conditions—many polymers change configuration under differing conditions, thus creating changes in flow rates if used in columns.
2. Susceptibility to enzymic attack—many poly-

mers are susceptible to attack by microorganisms or enzymes.

3. Operational stability—many enzymes immobilized on organic polymers have poor stability under operating conditions.

4. Particle size and condition—many matrices are of extremely small particle size and gelatinous in nature.

A method of covalently attaching enzymes to inorganic materials has been developed in the laboratories of Corning Glass Works. The materials range from glass to stainless steel.

Enzymes attached to the inorganic carriers have many advantages over enzymes attached to organic polymers. These include:

1. Structural stability—the inorganic materials are not susceptible to pH and solvent conditions. Therefore, they will not change size or configuration during usage.

2. The inorganic matrices are not susceptible to microbial attack.

3. Inorganic materials can easily be shaped permitting a wide variety of configurations.

4. Enzymes coupled to inorganic materials appear to have greater operational stability.

At Corning, enzymes were attached to the inorganic carriers via silane coupling agents (6-11). Most studies were carried out using porous 96 per cent silica glass particles having 40 square meters per gram surface area and 550 Å pore diameter. Particle sizes ranged from 40 to 80 mesh. The reaction between the porous glass and the silane is schematically shown in Fig. 1. There is a wide variety of silanes available having different organic functional groups. Any one of these may be used for coupling enzymes to inorganic materials (Fig. 2).

Two derivatives were used for the majority of the studies, the alkylamine-glass shown in Fig. 1 and the anhydride in Fig. 2.

The enzymes were generally coupled to the amine derivative through one of the coupling methods shown in Fig. 3. Although other approaches were tried, they were not studied in detail. By utilizing other silanes, however, one could couple enzymes by any desired method.

The enzymes insolubilized on porous glass were characterized by classical methods for parameters such as pH profile, kinetics and activation energy.

pH profiles differ

Every enzyme shows optimal activity at some pH value. Enzymes insolubilized on inorganic carriers also give maximal activity at some pH value. However, the profiles for the soluble and insoluble enzymes were not always the same. Figures 4 and 5 show the pH profiles of two protein hydrolyzing enzymes. Notice the difference between the native and insolubilized enzymes in Fig. 4. In the case of the pepsin, however, a difference in figures is noted only between a crude native and crude insolubilized derivative. How can these differences be explained?

Ephrium Katchalski has shown that when an enzyme is covalently attached to a charged matrix, a microenvironment is created. A charged matrix can accumulate oppositely charged ions causing a concentration of these ions at the carrier's surface. Thus the pH at the carrier surface is different from that in the external solution. The pH shift observed with the insolubilized papain is an excellent example of this phenomenon. Although the external pH is 7.0, the microenvironment of the enzyme is at 6.0, the optimal pH for the enzyme.

The pepsin situation is probably similar except that the observed difference between the insolubilized crude enzyme and the soluble material is most likely due to the attached nonenzymatic material which creates a microenvironment unlike that in which the insolubilized crystalline enzyme is found. The glass complicates the picture even more because of the presence of both free amine groups and silanol residues. The amines, however, near neutral pH values will not be protonated. Also, many will be coupled to the enzymes, leaving mostly silanol residues having a negative charge (Fig. 6).

The effect of varying carriers is graphically illustrated by comparing a maleic anhydride-ethylene copolymerized alkaline phosphatase with the same enzyme insolubilized on glass (Fig. 7). The maleic

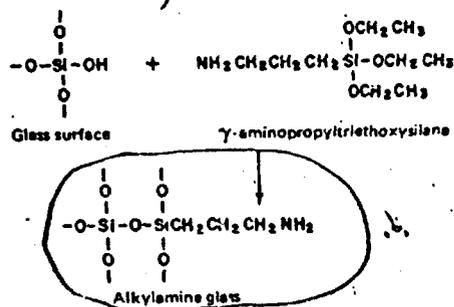


Fig. 1. Schematic representation of reaction between porous glass and γ -aminopropyltriethoxysilane.

anhydride copolymer shows a pH optimum on the acid side of the native enzyme, while the glass shows a large shift to the alkaline region. The effect a carrier has on the apparent pH optimum of an enzyme suggests the possibility of tailoring a carrier specifically to permit an enzyme to operate efficiently at an apparent pH not optimal for that enzyme. This may have applicability in a variety of areas where the enzyme cannot presently be utilized. Insolubilized enzymes may also be capable of operating in the presence of relatively high concentrations of organic sol-

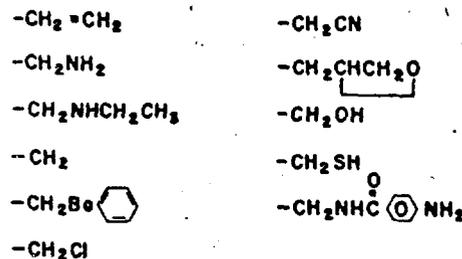


Fig. 2. Functional groups available on silane coupling agents. These functional groups or derivatives prepared from these groups can be covalently attached to enzymes by most known coupling methods.

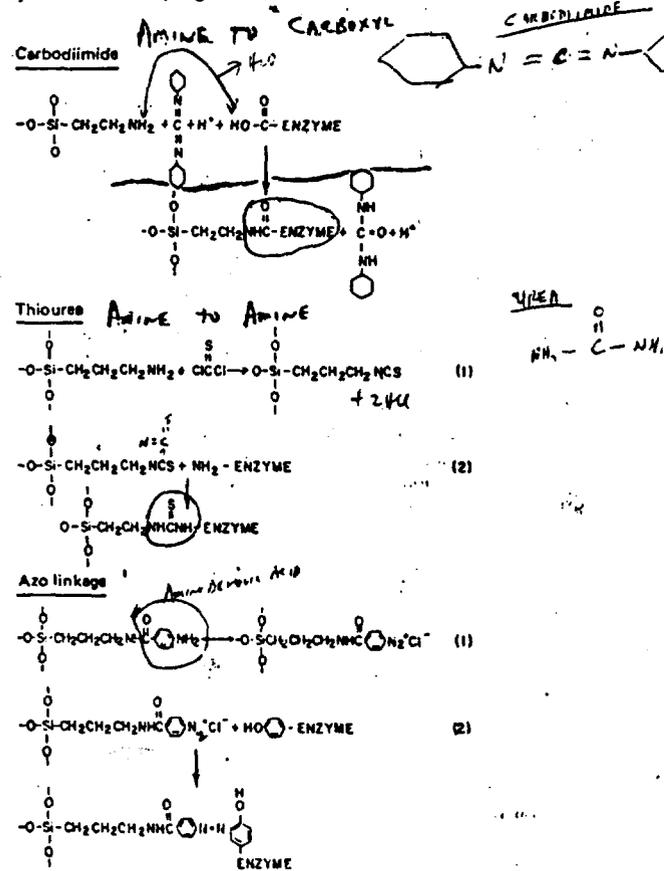


Fig. 3. Methods most commonly employed for preparing enzymes covalently coupled to inorganic carriers.

Immobilization of Biomaterial Supports

A number of methods are available for attaching biologically active materials to Biomaterial Supports. The particular method used may affect the activity of the immobilized product. The simplest method is adsorption onto the porous supports in a single step reaction involving hydrogen and ionic bonding. This technique works well except where bonding at the active site on a biologically active protein molecule is involved.

Biologically active materials can also be chemically coupled to the Supports. Proper selection of the coupling method can reduce or eliminate loss of activity that may result from reactions with the active site. Chemical coupling methods that have been widely used include Schiff base reaction, azo linkage, amide linkage, and thiourea linkage. Several selected procedures for preparing intermediates and for coupling are given below. However, the number of immobilizing reactions available is limited only by the imagination of the researcher. The reactions involved in immobilizing biological materials are carried out with standard laboratory equipment.

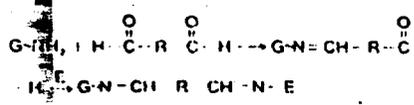
Reactions should not be stirred with magnetic stirring bars, as this can grind the glass.

Adsorption NOTE: CAUTION!

To one g of GZO Biomaterial Support add five ml of a solution containing approximately 10 to 50 mg of active protein per ml of a suitable buffer. The adsorption is usually done in an ice bath, but can be carried out at any temperature at which the active protein is stable. The adsorption is facilitated by shaking or tumbling the reaction mixture for two hours. Ideally, the adsorption is carried out in a fluidized bed for approximately two hours. The protein solution is decanted; then the glass is washed once with water, once with 0.05 M NaCl solution, and several more times with water to remove loosely bound protein. The product is stored under buffer solution at room temperature until used.

Chemical Coupling

1. Carbonyl Intermediate and Schiff Base Coupling

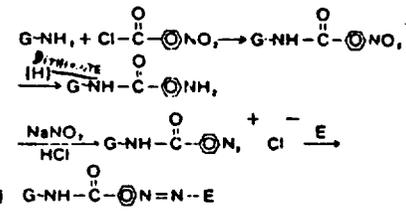


To one g of the GAO glass add 10 ml of a 2% aqueous glutaraldehyde solution

(This reaction can be run in an acidic or neutral solution). Place reaction mixture in a vacuum desiccator and evacuate with an aspirator to remove air and gas bubbles from the pores. Allow the reaction to proceed for approximately 30 minutes at reduced pressure and room temperature, then continue at atmospheric pressure for 30 to 60 minutes. The glutaraldehyde solution is decanted and the glass washed at least three times with water. The material is now ready for the protein coupling step.

The coupling is usually done in an ice bath, but can be carried out at any temperature at which the active protein is stable. A protein solution in suitable buffer is added to the glass derivative so that the liquid just covers the glass. Generally 50 to 100 mg of protein per g of glass is more than sufficient for coupling. The mixture is stirred and evacuated for approximately 30 minutes, then continued at atmospheric pressure for another 30 minutes. The product is washed three times with distilled water or buffer and then stored under buffer or as a wet cake in the refrigerator.

2. Aromatic Amine Intermediate, Diazotization, and Azo Coupling



To one g of the GAO glass add 10 ml of a chloroform solution containing 100 mg of p-nitrobenzoyl chloride and 50 mg of triethylamine. The reaction mixture is refluxed for one hour; the solution is then decanted and the glass washed three times with chloroform. The glass can be air dried or heated for 30 minutes at 80° C to remove the chloroform.

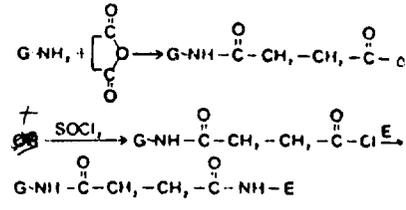
The nitrated glass is reduced by adding 10 ml of a 1% aqueous sodium dithionite solution and refluxing for 30 to 60 minutes. The reaction solution is decanted and the aromatic amine product washed three times with water.

The aryl amine glass is diazotized by adding 10 ml of 2N HCl to the glass and cooling the reaction mixture in an ice bath. To this mixture is added 0.25 g of sodium nitrite; then the reaction mixture is evacuated for approximately 30 minutes to remove air and gas bubbles from the pores. The reaction product is decanted and washed with cold water, a 1% solution of cold sulfamic acid, and two more times with cold water.

The diazotized product is coupled with protein by adding a solution containing 50 to 100 mg active protein in sufficient

buffer (pH 7 or greater) to cover the glass. The reaction is continued for 60 minutes in an ice bath; then the solution is decanted and the product washed three times with water. The immobilized protein can be stored under buffer or as a wet cake in the refrigerator.

3. Carboxyl Intermediate and Amide Coupling

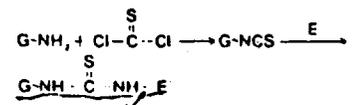


To one g of the GAO amine glass add 10 ml of a 1% aqueous succinic anhydride solution and adjust the pH of the reaction mixture to 6 with sodium hydroxide. Maintain the pH between 6-7 until no further adjustment is needed, then allow an additional hour of reaction time at room temperature. Wash exhaustively with water.

To the carboxyl glass add 10 ml of a 10% solution of thionyl chloride in dry chloroform and reflux for four hours. Decant the reaction solution and wash the glass three times with chloroform. Dry the product for 30 minutes in a vacuum oven and use for coupling shortly after drying.

The acid chloride product is coupled with protein by adding a solution containing 50 to 100 mg active protein in sufficient pH 8-9 buffer to cover the glass. Maintain the pH with sodium hydroxide for approximately 60 minutes, then decant and wash at least three times with water. The immobilized protein can be stored under buffer or as a wet cake in the refrigerator.

4. Isothiocyanate Intermediate and Thiourea Coupling



To one g of the GAO amine glass add 50 ml of a 10% solution of thiophosgene in chloroform and reflux for four hours. Decant and wash the glass three times with chloroform. Dry the product for 30 minutes in a vacuum oven and use for coupling shortly after drying.

The isothiocyanate product is coupled with protein by adding a solution containing 50 to 100 mg active protein in sufficient pH 9-10 buffer to cover the glass. Place the reaction mixture in a vacuum desiccator and evacuate with an aspirator to remove air and gas bubbles. Allow the reaction to proceed for 30 minutes at reduced pressure and room

Prior (Lit)

A) Fetus

Hb C is generally detectable about the 24th day of the gestation period. - 10th day after ovulation, but 24th day after last ovulation

5000 IU on 24 hrs

Peak ^{usually during} exceeds 10 days in duration.

Normally only 4000-11000 IU

col 7

32

col 2 - antibodies to Hb C as processed in animals

Teaches that Hb direct precipitin reaction is

not sufficiently sensitive to detect in unconv. cases, low levels of Hb C

Teaches precipitin test is old

- Teaches that direct precipitin test has led to so-called indirect tests.

Resolves indirect test - agglutination

Obj is to discuss a direct test based upon immunochromatography principles including gel diffusion

col 3 - circumvent Hb C on adjacent selected in unconv. cases.

col 3-20-28 discusses precipitin test

col 3-33-36 precipitin tests can be carried

out in a medium through which

antigen + antibody can diffuse in aqueous soln.

Teaches makes anti-Hb S by immunization animal with mixture of Hb S + Hb A

Teaches that non-specific Hb antibodies are present & should be removed by adsorption from normal Hb serum

col 4 (top) When highly pure Hb C is used to produce antigens, it is not necessary to remove non-specific antibodies

Figure 4

HCG antigen

Pregnant - Non pregnant women serum

Results as expected with APK - a control serum

serum was tested with APK

particular sera reserved for pregnancy

id of sera adjusted to 4-7

Non pregnancy sera which reacted with HCG antigen

were used to show non-specific antibodies - centrifuged

Several times until it tested negative

Test results showed adsorbed serum was much

more specific

more specific

I suggest that a higher purified human suspension

would give more accurate results & greater specificity

suggested using

When detection is that free HCG is not used. HCG is not purified as suggested

Figure 5

Teaches that HPL was continually up to birth

Teaches, p145, that radial immunodiffusion method

is a sensitive reaction method.

No data on the early serum - lots of charts

in Figure Five.

Only teaches that it is antigenic

Figure 7 - alpha-fetoprotein - not related to preg. test

Figure 11 - Teaches only that Placental tissue proteins

are present present in pregnancy & fetal serum

The antigen was checked by double antigen against

placental tissue & pregnancy - No teaching of time

Ref. V - Teaches that a specific material is present in placental

serum

In re Nelson 126 PA 253

In re Nelson and Shalica 1960 CCPA
251

In re Berkowitz 40 CCPA 642 (1970)

245

A specification need not contain a working example
of the invention otherwise disclosed in such a manner
that one skilled in the art ^{is} able to practice it
without undue amount of ⁱⁿ ~~it~~

Ex

In re August + Embler 190 PA 214

To require such content

"Appellants have broadly disclosed a class of catalytic
complexes which we deem to be part of their
invention. But in this disclosure, the public may have been
deprived of the knowledge of appellants' own. In this art, the
performance of trial runs using different catalysts is
reasonable, even if the end result is uncertain,
there is no reason to

Not
1974

it appears that persons skilled in the art
particularly, the art is in preparation that some of the

In re Doyle CCPA 179 PA 227 1973

When an examiner has advised a reasonable
basis for questions, the acquisition of a disclosure,
applicant has the burden of supplying adequate
information from which the examiner as well as the
court and the court could base a finding of whether
challenge is proper. Ordinarily that requires factual
proof at least what would be required now that was
actually done in carrying out the invention, material
particularly within the scope of applicant's present

Review of applicant

Translucens

Jankovsky.

One page

Pregnancy test

- in adv to prepare high titre antibodies

add human a

- first pregnancy month placenta

of human a animal

- removal of interfering - serum

in globulin fraction heated 50°C for 1 min

lyophilized

for carrying out the reaction, antibodies

is mixed with

both urine and blood

mixed on a slide.

Blood serum

Blood supplement blood from human - 2 to 2

in urine - permits complete

visible agglutination

In slight pans often the slide

can be mixed twice with urine a
blood.

Summary

- has been tested and diagnosis

of antibodies on slide.

second article Brock

He is concerned

immunology

immunogenetics of placental

4 aspects

a) - placenta as an immunological
barrier

b) Immunogenetics

c) Immunological competence

d) Immunology of trophoblast tumours

Present investigation is concerned
with (b) of placenta, esp. of trophoblast.

Referred discussion

Knowledge of solid antigens can
yield understanding

*)

Research was undertaken to detect
antigens in placenta - purify them

From leucocytes of antipine

Materials + methods

1) prepared from human placenta (~ term
probably) - .15m PO₂ buffer pH 7.4 +
centrifugation

2) ammonium sulfate fractionation -
stepwise addition of 10% 50% up
to various fractionations

precipitates were centrifuged separately
use Sorenson buffer.

After centrifugation, ppts were
dialyzed. Polyethylene glycol
gel-chromatography

Sephadex +

DEAE

.5M HCl

pH 8 → .5M Tris HCl.

Immuno diffusion + immunoelectrophoresis

Fractions were tested by Ouchterlony

+ absorbed antiplacenta serum
+ absorbed antipregnancy serum.

Absorbed antiplacenta + antipregnancy
were obtained by also immunization
with plac. ext or preg serum
and reacting. absorption with
Human Serum.

Results

a) Slighter NH_4SCN addition showed that
antigens detectable with a Ass ~~essentially~~

a Ass - antipregnancy
a Api - antiplacenta

ppt at - saturation degree of 0.04 → .5

only a small amount of additional material

records with Aas

Ochsen with Aapi shows ppn lines •
plurality of ppn lines

obtained 4 peaks
from elution curve

minimum chemical analysis

1st peak - 2 antigen Aapi Aas
2nd peak - 1 antigen Aapi
1 Aas

Ochsen test AAs - low concentration a
second antigen obtained
at high concentration
at peak 3

3rd peak 2 antigen Aapi
no with Aas

On basis of Ochsen test, after fractionated
it could be assumed that these antigens
were called out to a low % at a
saturation degree of .4

Peak 4 contains no ppn Aapi }
Aas } 1

Antigen detectable in 3rd peak can
be isolated by ion exchange.

On basis of Ochsen + Electrophoresis, can
be identified as a fraction of pregnancy
zone - of Hoffman.

moves in α_2 region. Ppt ~~at~~ with AAPI
AAS.

Second antigen in peak 3 ^{of Hoffman} - remains
in β range AAPI
AAS.

Also contained in pregnancy serum
umbilical & amniotic fluid.

Antigen in peak 2 also moves in β
range

AAPI
AAS

Both

Detectable antigens ppm with AAPI of
3rd peak do not ppt with AAS
signify they are fetal or placental specific
one was identified as umbilical cord
serum antigen #4 of Hoffman
~~2nd detectable with AAS~~

Insomuch as useful as a clinical diagnosis
of a disturbed pregnancy is left to
speculation

Hoffmann I

- said human placenta is suitable for obtaining
antigen and mono-specific immune sera
against various - typical antigens.

p 4 - no teaching of early immune

p 79 - antibodies but

No teaching that a plurality of antigens
can be used for the detection of pregnancy
during early periods.

Hoffmann II

Scap 7 lines are suitable detected by the
use of absorbed anti-placenta immune serum.
from placental extract

- also present in sera of pregnant women.

p 1 scap pregnancy serum is not available in
sufficient quantities for production of immune
serum, while placenta after birth can be easily
procured for obtaining antigen.

Purpose is to test suitability of absorbed
anti-placenta immune serum for detection +
correlation pregnancy induced serum antigen.

a) Removing protein from placenta

b) inserted into placental extract into serum

p 5 - after immunization, a precipitating

immune serum is obtained which reacts both
with pregnancy serum + placenta extract.

After absorption of anti-placenta immune serum with
human serum, antibodies directed against serum proteins are
removed.

over the

no time.

p 7. In reaction of pregnancy serum, at least 2 types are
obtained which are immunologically identical.
no test as used early on serum.