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EARLY DETECTION OF PREGNANCY-ASSOCIATED SERUM PROTEINS
 USING ANTISERUM TO PLACENTAL ANTIGENS

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Abstract

Antisera against human placental proteins were developed in goats and rabbits, using immunoadjuvants and a prolonged injection schedule. The antisera were absorbed with normal serum proteins and then tested in immunodiffusion against normal and pregnancy sera. Two bands of precipitation due to pregnancy antigens were observed in pregnancy sera as early as 18 days after conception. Detection of these antigens has possibilities for application as an early pregnancy test.

Introduction

The possibility of using antiserum to placental extract to detect pregnancy at an early stage arose from an investigation directed toward determining whether placental and other fetal antigens are reexpressed in disease states, particularly cancer. Levels of existing serum proteins change in pregnancy, and new proteins appear that are produced by either the mother or the fetus. The occurrence of at least one and perhaps two serologically

specific components in pregnancy sera was demonstrated by Thornes (3) in 1958 by gel precipitation. In 1959, Smithies (4) showed the presence of a pregnancy-associated α -globulin in a region of the starch gel electropherogram which he called the "pregnancy zone." In 1960, Hirschfeld and Söderberg (5) found two "pregnancy precipitates" on immunoelectrophoresis of pregnancy sera, one in the fast α -globulin region, and the other in the slow α_2 -beta-globulin region. Since then, as many as four such pregnancy-associated plasma proteins have been detected.

Gall and Halbert (6) showed the sequential appearance of four pregnancy-associated plasma proteins that were studied as pregnancy progressed; Lin et al. (7) characterized these with respect to molecular weight, isoelectric point and electrophoretic mobility, pH and temperature stability, sedimentation behavior, solubility, and elution from DEAE cellulose. Possible enzyme activity was also investigated. Three of the proteins were routinely seen by immunoelectrophoresis. One of the proteins contained iron. None appeared in normal nonpregnancy plasma. The pregnancy samples tested were from subjects in the third trimester. The antisera used had been raised against pregnancy plasma and then absorbed exhaustively with nonpregnancy plasma. Bohn (8) used rabbit antihuman placenta antiserum absorbed with male serum to detect four pregnancy-associated plasma proteins. Only one was considered to be specific for pregnancy, while two could be detected sometimes in sera from nonpregnant female subjects, especially in those taking oral contraceptives.

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.

PREGNANCY-ASSOCIATED SERUM PROTEINS

Berne (9) used a quantitative radial immunodiffusion test to determine levels of a pregnancy-zone protein, termed by him α_2 -pregnoglobulin, also found in males and nonpregnant females. Users of oral contraceptive estrogen-progestin combinations showed levels reaching into the pregnancy range. First elevations of the α_2 -pregnoglobulin were detected at six weeks of pregnancy. The purpose of the present work was to determine how early in pregnancy any of the pregnancy-associated serum antigens could be detected.

Materials and Methods

Preparation of crude placental extract. Term placentas were obtained at delivery and chilled in 0.85% sodium chloride solution ^{for preservation}. Processing was begun immediately upon return to the laboratory. The placentas were washed for 1 hr in tap water to remove a maximum of blood elements and serum proteins. Fat was then cut away; ^{Why - 2 lines} ^(why? 1 line) 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, 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? (1 line)] The proteins of interest were precipi-

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containing 0.1% sodium azide, the solution was dialyzed against
 (what sort of buffer? ... critical ... 2 lines)
 this same buffer, and any material that precipitated was removed
 by centrifugation. (buffer was ... - 1/2 liter)

Any serum proteins left in the solution were removed by cycling it over an immunoabsorbent column of antibodies to normal
 (How ... ? What sort of ... 3 lines)
 human serum. 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 A₂₈₀ units (10) per ml without undesired precipitation occurring on storage, especially on freezing and thawing. ^A 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. ^[Now? what sort of ... - 2 lines] ~~This separation could be of interest for its own sake; in addition,~~ ^{Though not essential to the operation of the process,} some separation 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} ~~Efforts are under way~~ to modify the extraction procedure (for example, 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 serum ^{non-specific} proteins but still containing the relatively nonantigenic tissue proteins mentioned, were concentrated to about 15 A₂₈₀ 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 an equal volume of Freund's complete adjuvant ~~(1:1)~~, and the preparation was injected subcutaneously ^{to goats and rabbits,}

either in the hips or behind the neck (4 ml for goats, 1 ml for rabbits). ^[would be interesting to see if also work in mice]

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 immunodiffusion column} with

lyophilized, pooled serum obtained from normal human males (100 mg/ml of antiserum) to ^{remove non-specific antibodies.} ~~render it specific for placental antigen.~~

The unbound fraction contained pregnancy specific antibodies and normal animal protein. <sup>[Is any further treatment necessary or desirable for commercial use?]
 Results and Discussion Should the goat fraction be removed?</sup>

Gel precipitation was used to test the antiserum ^{Y4 pose} 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, ~~are shown in~~

Figure 1, ~~from~~ three women in their second pregnancy, in ~~Figure 2,~~ and ~~from~~ three women in their third pregnancy ^{showed positive reactions within 5 hours, with 2 to 4 bands clearly visible at the 40 mm.} in ~~Figure 3.~~ 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. A positive

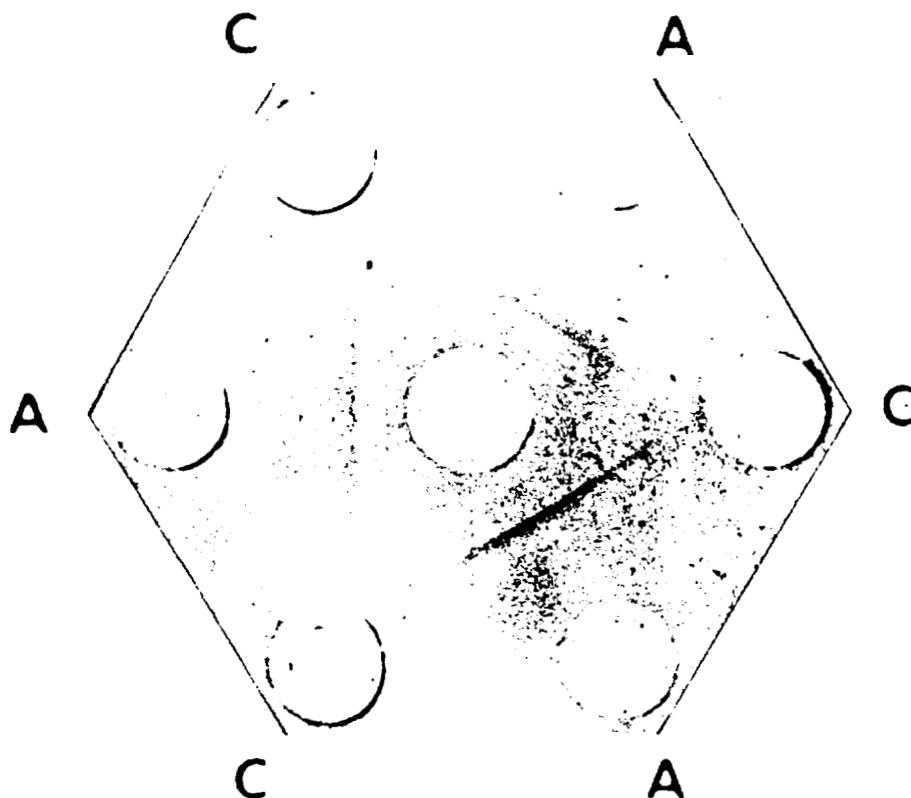


FIGURE 1

Detection of pregnancy-associated antigens in sera from women in the third trimester of their first pregnancy. Each of the A wells contains an individual serum. Control serum is in the C wells; the center well contains the absorbed antiplacental antigen antiserum. Time of reaction: 24 hr.

reaction was seen within 5 hr with all the third-trimester sera. These reactions improved with time, so that in 40 hr 2 to 4 bands of precipitation were observed for all samples. The four precipitation bands between a third-trimester pregnancy serum and the antiserum are unusually clear in Figure 4 (well marked 3P).

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, includ-

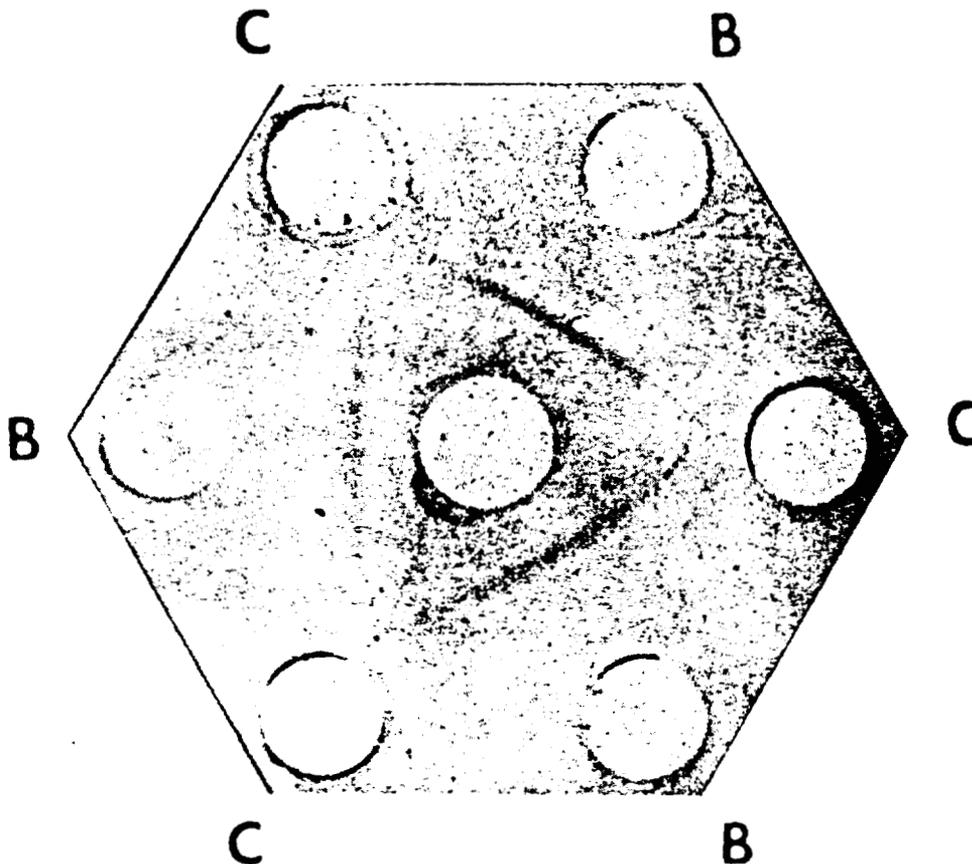


FIGURE 2

Detection of pregnancy-associated antigens in sera from women in the third trimester of their second pregnancy. Disposition of the wells and time of reaction as in Fig. 1.

ing those previously pregnant. ~~Thus far,~~ no reaction has been shown with sera from women taking estrogen-related contraceptives. (~~four cases tested~~).

In view of the positive reactions with third-trimester pregnancy sera, it was decided to see how early these reactions could be found in pregnancy sera. ^{In order to demonstrate the duration of pregnancy} A serum sample ~~was~~ was obtained from ~~early enough~~ ^{to be} 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~~ was obtained from a 25-

valuable for pregnancy test series runs were made on serum from women who suspected pregnancy

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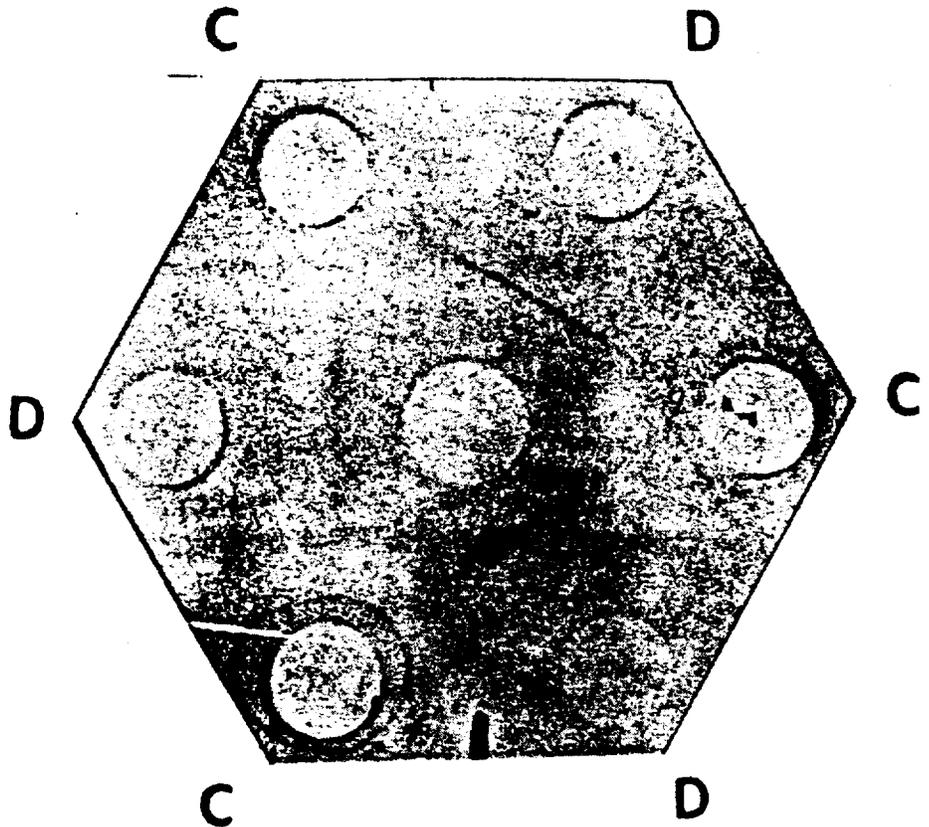


FIGURE 3

Detection of pregnancy-associated antigens in sera from women in the third trimester of their third pregnancy. Disposition of the wells and time of reaction as in Figs. 1 and 2.

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.

~~TH~~ Samples ~~M and J~~ were tested by double diffusion (~~Figures 4 and 5~~, respectively) 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 ~~M and J~~,

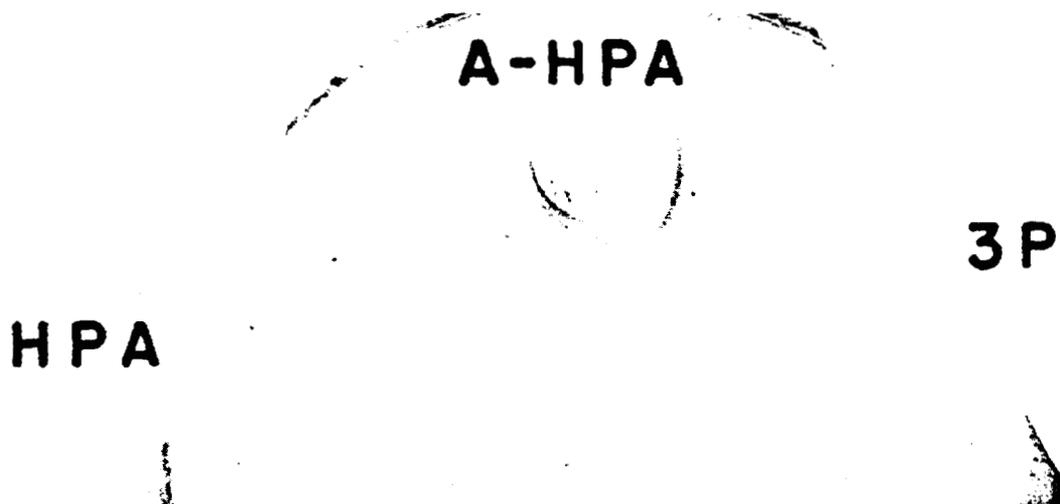


FIGURE 4

Comparison of antigens from early-pregnancy and third-trimester serum samples. Well 3P contained serum from a woman in the third trimester of her third pregnancy; well M contained serum from a woman 18-22 days pregnant; well HPA is human placental extract; A-HPA is the antiserum. Time of reaction: 16 hr.

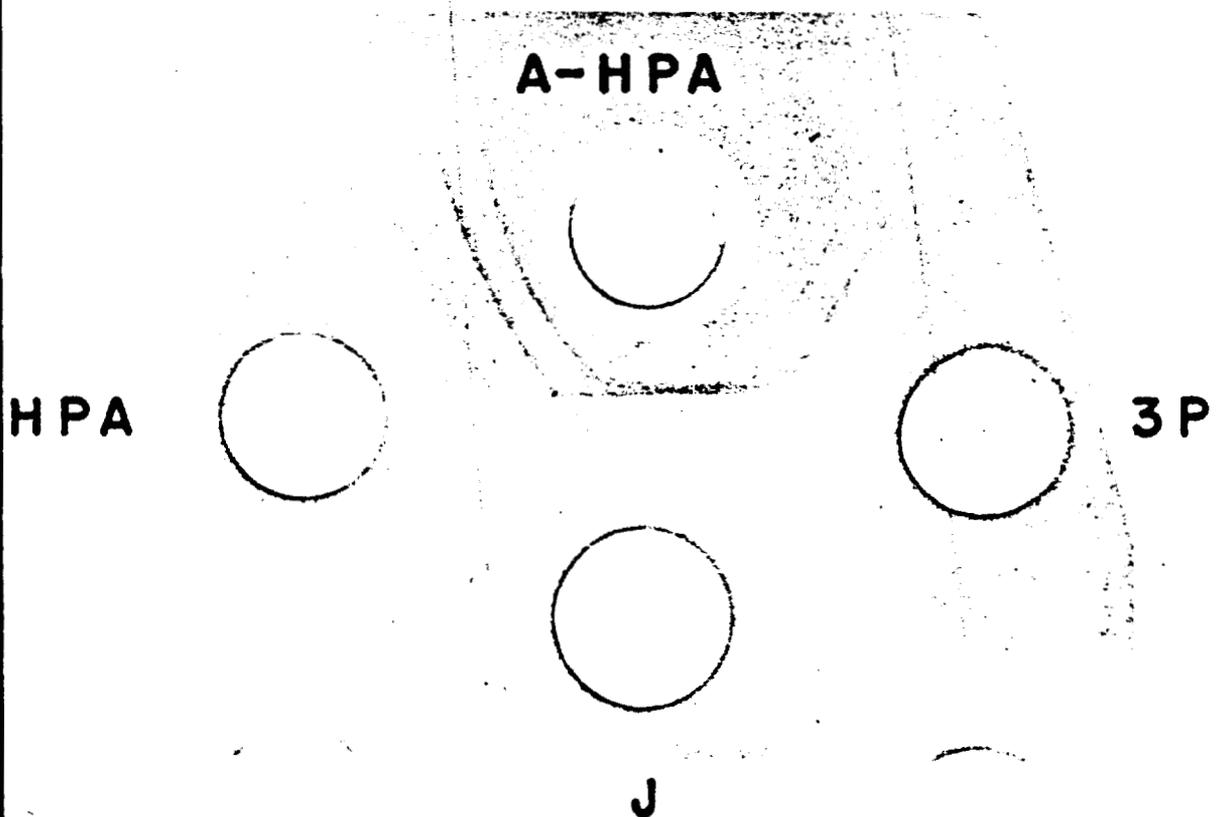


FIGURE 5

Same comparison as in Fig. 4, except that in this case the early-pregnancy sample (J) was a pregnancy serum taken 22 to 26 days after conception. Immunodiffusion for 16 hr.

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a precipitin band could be observed as early as 1 hr after the start of immunodiffusion. Three precipitin bands were observed for ^{the control} sample # within 24 hr after loading.

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 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.

Conclusions

We have demonstrated that antiserum against human placental antigens can be used to detect the appearance early in pregnancy of pregnancy-associated serum proteins. In the earliest sample tested, taken 18 to 22 days after conception, two such proteins were clearly detected. Another sample, taken 22 to 26 days after conception, gave indication of three pregnancy-associated antigens. Additional samples taken at even earlier stages of gestation should be tested to determine just how early such pregnancy-associated proteins can be detected. Further, samples from users of a wide variety of hormone-based contraceptives should be tested to

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ascertain if their use provokes the production of antigens that react with the antiserum. Finally, efforts should be made to characterize these early appearing pregnancy-associated proteins so that they can be compared with others described in the literature, and to determine whether they can occur in various diseases.

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10. An A₂₈₀ 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.
11. The Freund's complete adjuvant used was obtained commercially from Difco Laboratories. It contained in 10-ml ampules sterile light mineral oil plus emulsifier, and heat-killed Myco-bacterium butyricum (0.5 mg dry wt per ml).

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MUCH OF THIS IS FROM COURSEWORKS, SEE REF —

Prep. of a typical immunosorbent

Preparation of a Typical Immunosorbent

- 1) Protein to be bound to Sepharose 4B, known as the ligand, was concentrated in solution to 70 ml, and dialyzed against the "coupling" buffer; that is, the buffer in which the binding of ligand to Sepharose occurs.
- 2) By using a small calibrated column or syringe, 70 ml of Sepharose (same as volume of protein) based on column packing volume, were collected.
- 3) The 70 ml of Sepharose was washed with at least 20 volumes of 0.1 M phosphate, pH 6.5, but no azide, for fear of interference with binding of the amine groups.
- 4) For activating the Sepharose, 0.3 gm of cyanogen bromide per ml of Sepharose was used, or 21 gm total.
- 5) The 70 ml of Sepharose was mixed with an equal volume of coupling buffer in a 400 ml beaker, and the beaker with stirring bar was placed in an ice bath over a magnetic stirrer. It was preferable to use an ice bath to control the temperature rather than adding ice to the mixture during activation which results in dilution of activated sites.
- 6) THE slurry was titrated to a pH of 11.5 with 6M NaOH.
- 7) THE 21 gm OF cyanogen bromide was added to the Sepharose slurry, and as the reaction proceeded, the pH was maintained at 11.5 with NaOH and the temperature was maintained at $\approx 20^{\circ}\text{C}$.
- 8) The reaction was allowed to proceed until no more decrease in pH was noted, then ice was added to insure the end of the activation.
- 9) Quickly, the activated slurry was washed with ~~the~~ ^{1000 ml.} coupling buffer at 4°C on a Coarse Buchner funnel. (washing not to exceed 2 min)
- 10) The washed ~~any~~ ^{any} CNBr activated Sepharose was mixed with the ligand and the mixture was slowly tumbled overnight at ~~with~~ 4°C .

B) Basic Techniques of Preparation of Immunosorbent to be Packed Into Column

- 11) In cases where maximum ~~attachment~~ attachment of ligand to Sepharose is desired, the protein - Sepharose mixture may be mixed more than twenty-four hours. The unbound protein is washed off in coupling buffer (0.1 M Phos, 6.5, no azide), and washing is continued until background absorbance is reached.
- 12) To "soak up" any remaining sites, the immunosorbent is cumbled in 1M ethanolamine, ^{pH=8.0} for 2 hours.
- 13) The ethanolamine is washed off, and the immunosorbent is washed alternately with 0.10 M sodium acetate, pH 4.0, ~~and 0.1% azide~~, ^{sodium azide} 1.0 M sodium chloride, and 0.10 M sodium borate, pH 5.5, 0.1% sodium azide, and 1.0 M sodium chloride.
A WASH WITH GLYCINE-NACL, 0.1M AND pH 11.0, MAY ALSO BE INCLUDED HERE.
- 14) The immunosorbent is finally washed with 2.0 M sodium thiocyanate, 0.06 M sodium phosphate, pH=7.0, and 0.1% azide, followed by 0.1 M Phos, pH 7.0, 0.1% azide, until background absorbance is obtained. The immunosorbent is then ready for packing.

THESE ARE OPERATION NOTES, USED FOR
ROUTINE LAB FUNCTION & INSTRUCTION

1
ATTACHMENT

Isolation of Specific IgG from Antisera Utilizing
Immobilization of Antigen to Affinity Chromatographic Supports

2

One of the two methods of choice for isolation of IgG from antisera has been discussed; that is, the fractionation of antisera on DEAE cellulose. The great advantage of this method is the very mild chemical treatment to which IgG is subjected. A disadvantage is that homogeneous recovery of IgG is not assured and specific IgG is not recovered alone. The second method of choice for isolating IgG from antisera entails use of immunosorbents whereby the antigens of interest are immobilized on a support and the antisera is eluted over the immunosorbent.

A typical example of the isolation of the IgG, specific against human serum, from a rabbit antiserum to human serum, is shown in Figure ? . About 1850 A_{200} units of rabbit anti-human serum was applied to an immunosorbent column, 2.5 x 14 cm or approximately 70 ml, containing 700 mgs of normal serum attached by cyanogen bromide to Sepharose 4B. The bulk of the first peak was eluted with 0.1 M sodium phosphate buffer, pH 7.0, and 0.1% sodium azide. Complete and non-tailing elution of this "unbound material" (that is, not bound by antigen-antibody interaction) was achieved by adjusting the above buffer to 1.0 M sodium chloride. The antibodies bound to the whole serum immunosorbent were eluted with 2.0 M sodium thiocyanate, pH 6.6, and 0.1% azide. This fraction was dialyzed to 0.1 M PH_4S , 7.0, by an in-line dialyzer. About 1250 A_{200} units were recovered from the first peak and about 420 units were recovered from the second peak. In excess of 95% of the material applied to the column was eluted by all the various buffers.

In experiments in which the feed antisera was loaded in small discrete batches, onto the column, a mass of antibodies from antihuman serum at least equal to the mass of the serum proteins was bound, and later eluted with thiocyanate. Estimating the average molecular weight of serum at 100,000

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one can calculate that based on a antigen-antibody (IgG = 160,000 m.w.) singular molecular interaction, 500 mg of serum would bind maximally 800 mg of antibodies. Observed results were about 75% of this figure.

A possible disadvantage to this isolation of specific IgG by antigenic immobilization was observed to be that certain antigen-antibody affinities varied in strength, resulting in premature cleavage of these bonds in initial buffer or later in relatively mild thiocyanate or glycine buffers. Such an occurrence also has its positive aspects whereby gradient or step-increase elution could be used to fractionate the various antibodies bound to the immobilized serum proteins.

Peak 2 was regularly checked by immunoelectrophoresis against goat anti-rabbit serum for contaminating rabbit protein which might be nonspecifically bound to the immunosorbent. A typical example of this result is shown in Figure where pool 2 yielded the one expected precipitate band (IgG) against goat anti-rabbit antisera, but yielded the typical whole serum-antiserum pattern when reacted with normal serum. The latter pattern shows that ~~at least~~ ^{most} some of the IgG maintained its immunological activity following thiocyanate elution and dialysis. (effective dialysis was provided inline.)

The effect of the binding then dissolution of the bond by thiocyanate on the total reactivity of the IgG was investigated by simply reloading onto a normal serum-immunosorbent the IgG fraction previously eluted from the immunosorbent.

Results - CONTINUE TO NEXT PAGE

ADD COMMENT on How much IgG could be
Immune per Day

Caution must be taken to minimize time that the IgG are dissolved in the thiocyanate. Data now indicates that after exposure to the thiocyanate exceeding one hour irreversible loss of the immunological activity of the IgG occurs. The thiocyanate may be rapidly recovered from the IgG by in-line dialysis of the column effluent using Bio-Rad 80 Hollow Fiber Beaker Device, (exclusion limit 5000 mw). **(GIVE REFERENCE)**

In a series of experiments it has been shown that antibodies to normal human serum which have been "once-bound" to a normal serum immunosorbent, eluted in thiocyanate, and quickly dialyzed, will "rebind" to the same column at about 80-90% of initial binding quantity.

Production of antisera that is "balanced" against such a complex heterogeneous solution of proteins as whole serum is very difficult. A "balanced" antisera is one in which the antibodies exist in the same proportion of the IgG as their specific antigens comprised the antigenic mixture. For example, in whole serum, albumin may exist at levels of 35-55 mgs per ml. of serum, forming a percentage of about 50% of the serum protein. About 20% of the serum proteins is composed of IgG. The remaining portion of the serum is composed of some 50-60 odd other vital proteins, but obviously in small amounts. Thus, a balanced antisera would be one in which the IgG was approx. 50% antialbumin, 20% anti-IgG, etc. **OVER**

~~In fact, however, when antisera is raised against human serum in goats and rabbits, there seems to be considerable cross-recognition or partial recognition of several of the antigens from proteins by the hosts, particularly in the case of albumin. ^{Twitter,} The result is that the antisera is quite deficient in anti albumin (and others of course) so that only 1/4 of the theoretical amount is found. This result is shown in two IEP experiments.~~

Human serum and goat anti-human serum were mixed in proportions varying from serum - no antiserum, to 1/20 serum, 19/20 antiserum (a molar excess of at

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least 2 of antibody to antigen), precipitated overnight, and the supernatant applied to the wells of the agar plate. After electrophoresis, a high quality commercial anti-human serum antiserum was applied to the troughs. The pattern given in Plate 4 shows that in the last case of excess antibody virtually all antigens were subtracted, except IgG, albumin, and a few beta-globulins.

In the second experiment, IgG ~~XXXXXXXXXXXXXXXXXXXXXXXXXXXX~~ against human serum, isolated ^{using} ~~in~~ a human serum ~~of~~ immuno~~s~~orbent, was itself attached to Sepharose to produce an IgG, anti-human serum, immuno~~s~~orbent. Only 1 ml of human serum, 70 mg of protein, was applied to the column containing 300 mg of immobilized IgG. Even with this supposed excess of antibody, those antigens from the human serum that had not been sufficiently responded to by the hosts exceeded their available antibodies and ~~passed~~ ^{passed} unbound through the column. They are identified in plate 66 as IgG, albumin, and a few beta-globulins.

This experiment shows not only that the antisera is unbalanced, but also provides a simple solution to balancing it. From the first antisera raised, the IgG is isolated and made into an immuno~~s~~orbent.

The antigenic mixture is then eluted through this immuno~~s~~orbent, and the "unbalanced" antigens ~~pass~~ ^{pass} out unbound. They are concentrated and injected, either into ^{the} first animal or others, and the process is repeated.

In the future, antisera ~~in~~ ^{specific} for only one serum protein will be required for a variety of functions, among them being tests for genetic ~~variation~~ ^{variation}, production of "antisera minus one" preparation, and ⁱⁿ ~~as~~ addition for production of "balanced" antisera. Two monospecific antisera already in production are shown in Plate 91. The indicated 33c rabbit antiserum is monospecific for human serum albumin and 39B rabbit antiserum is monospecific for human serum transferrin.

ATTACHMENT 3

ANALYTICAL BIOCHEMISTRY 68, 371-393 (1975)

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Analytical Techniques for Cell Fractions

XX. Cyclic Affinity Chromatography:
Principles and Applications

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Principles and applications of an automatic cyclic affinity chromatography system, especially as applied to separation of proteins by immunosorption, are described. Use is made of columns of either immobilized antigens or antibodies to separate from a mixture, in a repetitive fashion, a desired protein or proteins by immunoadsorption followed by elution or to take from a mixture all proteins but the desired ones by allowing these latter to go through unadsorbed. The amplification provided by the cyclic use of the system and the biological amplification of hyperimmunization in achieving useful yields of desired proteins is discussed. Experience in the use of eluting solutions which do not cause damage to either the fixed or eluted proteins is presented. Representative separations done with the system are described, illustrating the different modes of use.

The objective of this paper is to outline general separation methods based on affinity chromatography which are applicable to the fractionation of complex mixtures and to the problem of isolating minor constituents including tumor-associated autoantigens from tissues, plasma, tissue-culture fluids, or urine. The methods depend on amplification provided by the cyclic use of affinity columns and on the inherent amplification of the immune systems of immunized experimental an-

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⁴ The Molecular Anatomy (MAN) Program was sponsored by the U.S. Atomic Energy Commission, the National Institute for Allergy and Infectious Diseases, the National Institute of General Medical Sciences, and the National Aeronautics and Space Administration.

⁵ Operated for the USAEC by the Nuclear Division of Union Carbide Corporation.

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imals. In addition to separations based on the selective adsorption of desired moieties, immunosubtraction offers the unique possibility of isolating labile proteins by contaminant subtraction, i.e., by binding all undesired proteins to a column while the desired protein passes through.

In the preceding paper (1) the instrumentation required for cyclic affinity chromatography was described. In this paper we present a review of different approaches to protein fractionation based on the use of stereo-specific adsorbents, together with methods of adsorption and elution. The elements of all the separations have been previously described but have not been integrated conceptually and experimentally. We have presented preliminary discussions of some of the work reported here (2,3).

SEPARATIONS METHODS

The use of affinity chromatography to purify antibodies or antigens once either is available in a pure state is well known (4). In addition columns of normal serum proteins have been used to subtract antibodies to them from antisera against hepatitis-associated antigen (5). Here we attempt to organize fractionation methods based on immunoadsorption in a systematic manner, with emphasis on the amplification.

Bootstrapping. We have used the term "bootstrapping" to describe a series of steps using both the amplification inherent in the immune systems of higher vertebrates and the amplification made possible by cyclic affinity chromatography. The use of this technique is illustrated diagrammatically in Fig. 1. In this and all subsequent diagrams Cyclum systems are illustrated diagrammatically as a column with two attached squares below, the first of which indicates a uv monitor attached to a strip-chart recorder, while the second and lower square indicates the distributor valve which passes unadsorbed protein (the "U" fraction) to a collecting vessel on the left and adsorbed and then eluted protein (or "A" fraction) to the vessel on the right. On all charts the unadsorbed peak ("U" fraction) is shaded for ease in identification.

Given a very small amount of a pure antigen, it may be used to produce an antiserum (Fig. 1, step 1), often of rather low titer. The IgG (immunoglobulin G) from this antiserum (step 2) is then isolated and used to prepare a first-generation affinity column. By prolonged recycling of this column, and by pooling and concentrating all the "A" fractions, sufficient antigen (step 3) may be prepared to allow preparation of much larger amounts of antisera (step 4) and a column of immobilized antigen. With this combination rather large amounts of *monospecific* IgG may be prepared (step 5). This in turn may be used to prepare a very large second-generation IgG column for larger-scale antigen production (steps 6 and 7). Many variations of this procedure are pos-

AUTOMATIC IMMUNOPRODUCTION

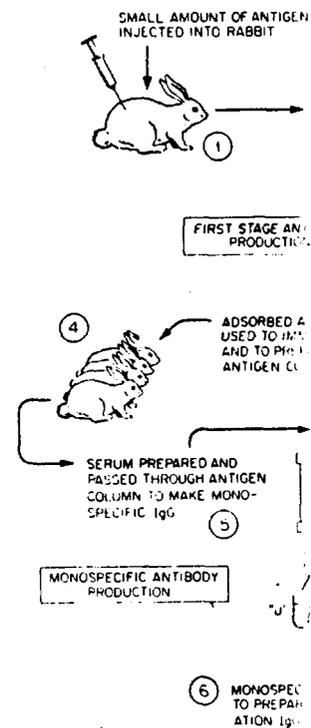


FIG. 1

sible; for example, one may elute from a complex, as subsequently, problems with require introduction of additional may also be required. least 100 should be possible.

All-but-one subtraction. required that are rapid and such antigens are often quite general method for isolating Fig. 2 and depends on the everything from a sample eluted.

Initially (Fig. 2, step 1) additional means and immobilize are hyperimmunized against techniques for producing balanced a later section.) The harvest immobilized starting antigen

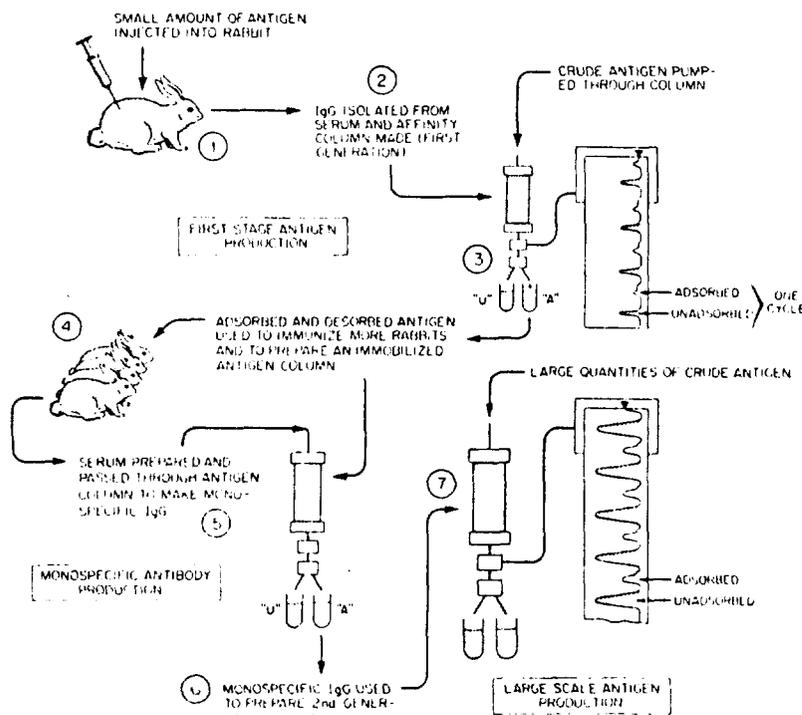


FIG. 1. Bootstrapping technique.

sible; for example, one may start with antisera or antigen or antibody eluted from a complex, as may occur in the kidney. As discussed subsequently, problems with contaminating antigen will occur and may require introduction of additional steps, and additional stages of amplification may also be required. To be really effective an amplification of at least 100 should be possible at each stage.

All-but-one subtraction. With very labile antigens methods are required that are rapid and involve minimum manipulation. Surprisingly such antigens are often quite stable if linked to an insoluble support. A general method for isolating labile antigens is shown diagrammatically in Fig. 2 and depends on the preparation of a column that will remove everything from a sample except the antigen desired.

Initially (Fig. 2, step 1) the antigen of interest is isolated by conventional means and immobilized (step 2). At the same time rabbits or goats are hyperimmunized against the starting antigen mixture (step 3). (Techniques for producing balanced antisera against mixtures are described in a later section.) The harvested serum (step 4) is passed over a column of immobilized starting antigen mixture (prepared in step 5) to prepare

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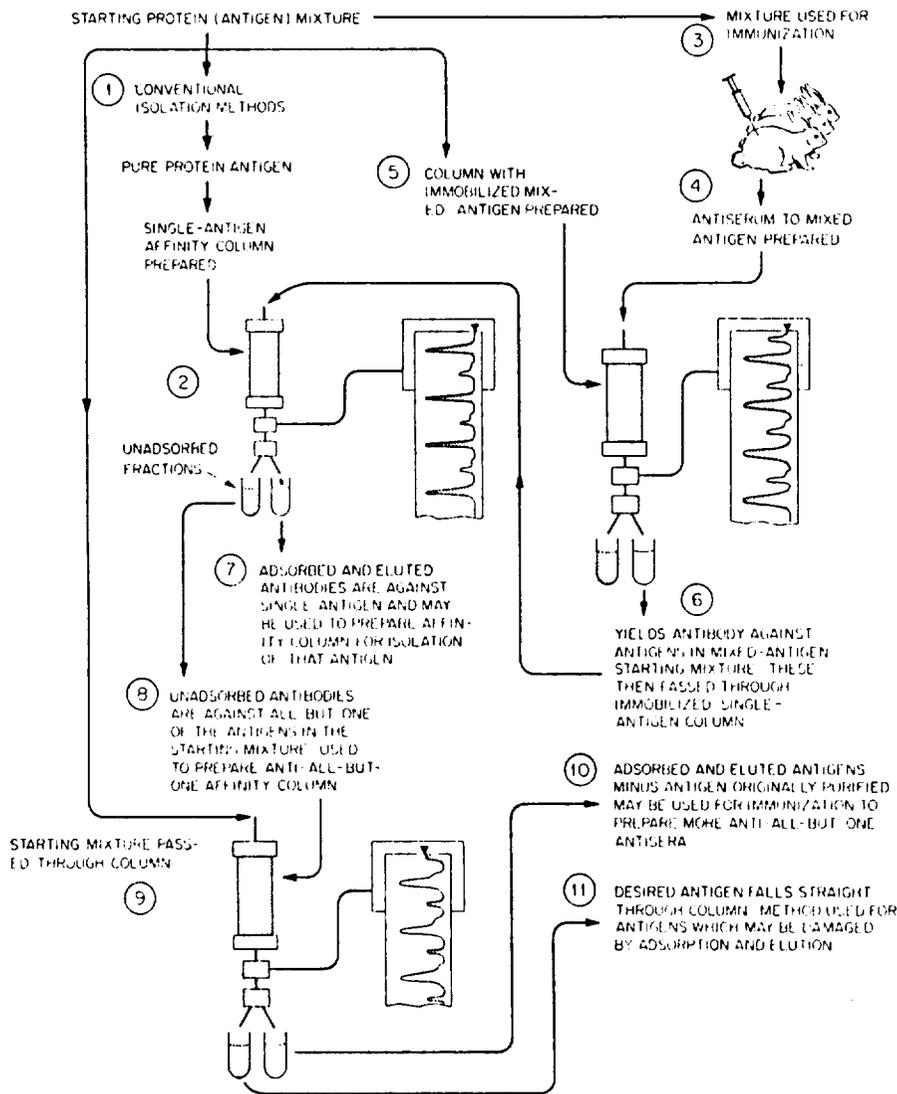


FIG. 2. All-but-one subtraction.

purified antibodies against it (step 6). When these antibodies in turn are passed over the single-antigen column, the antibodies against that antigen are adsorbed (step 7), while the unadsorbed antibodies pass through (step 8). The latter, which include antibodies against all but one of the antigens in the initial mixture, are now made into a much larger column (step 9). This may now be used for preparing the single antigen of interest by cyclic application of the starting mixture, in which case the

desired antigen falls directly after elution, may be used to preparation of more anti-all-

This general method will of serum lipoproteins and l. *Frontal separations.* Imm- metrically to all antigens in immunogenicity of different separation which is illustrat mixture (Fig. 3, step 1) and finity column (steps 2 and 3 pumped continuously through its length at different rates

STARTING ANTIGEN MIXTURE (FOR ILLUSTRATIVE PURPOSES OF THREE ANTIGENS ARE ASSUMED TO BE PRESENT)

4 MIXTURE DILUTED AND PUMPED CONTINUOUSLY THROUGH COLUMN

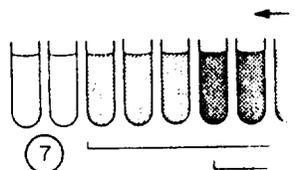
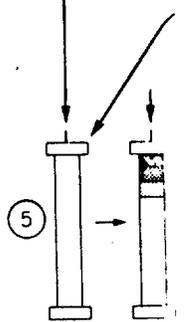
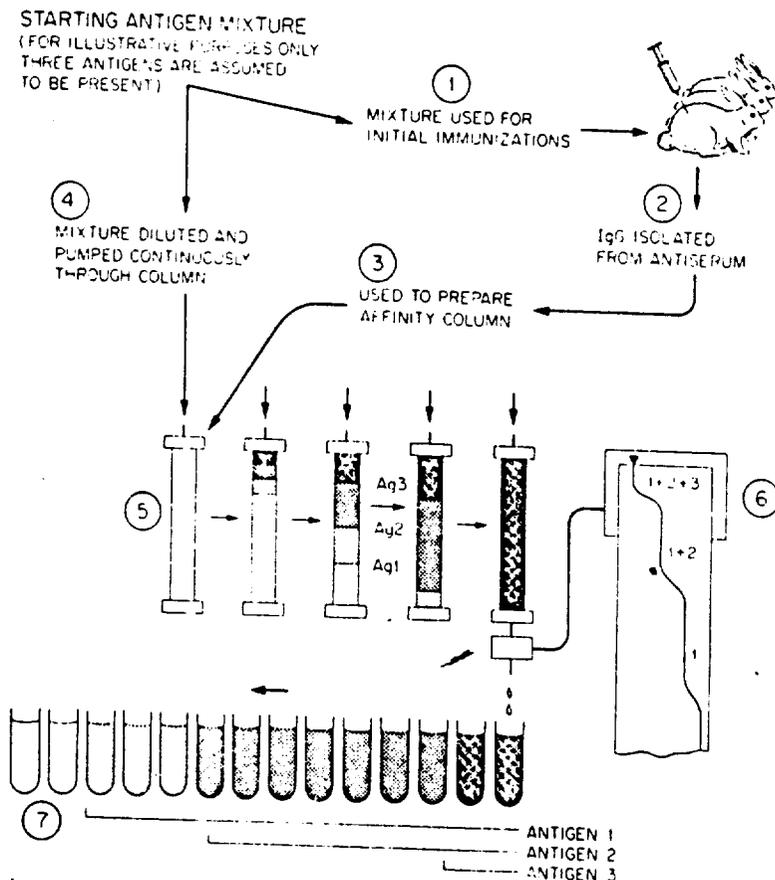


FIG. 3. Frontal separation.

desired antigen falls directly through the column. The adsorbed antigen, after elution, may be used to prepare even larger antigen columns for the preparation of more anti-all-but-one antiserum.

This general method will probably find greatest use in the preparation of serum lipoproteins and labile enzymes.

Frontal separations. Immune systems rarely if ever respond stoichiometrically to all antigens in a mixture used for immunization; in fact the immunogenicity of different proteins varies enormously. This simple fact may be made the basis of a general separations method termed *frontal separation* which is illustrated in Fig. 3. Antisera are prepared against a mixture (Fig. 3, step 1) and the IgG isolated and used to prepare an affinity column (steps 2 and 3). If a dilute solution of the starting antigen is pumped continuously through the column (step 4), it will saturate along its length at different rates for different antigens depending on the ratio



1075564 Fig. 3. Frontal separation of antigens on an unbleed antibody column.

between each antigen in solution and its corresponding antibody on the column (step 5). Quite obviously those antigens for which there are few or no antibodies will break out first. Those antigens for which an excess of antibody is present will break out last. It is evident that once a frontal analysis has been done, one may calculate an amount of the initial antigen mixture just sufficient to let a small amount of the least-bound antigen fall through, often in a high state of purity. By prolonged recycling (provided a sufficient amount of starting antigen is available) appreciable quantities of this initially emerging antigen may be isolated. The absorbance diagram indicates the degree of nonstoichiometry of the column (step 6) as does analysis of the collected fractions (step 7).

Once one antigen of a mixture has been isolated, an IgG column may be made to subtract it from the starting mixture. The subtracted mixture may then be used to prepare the second antigen to appear in a frontal separation. In this manner quite complex mixtures may, in theory, be resolved.

In practice it is even more convenient to add the antigen mixture to the column in known discrete identical volumes interspersed with buffer. Each may be collected and analyzed separately. The correct amount of mixture to add to allow certain antigens to break through and others to be retained may then be easily determined.

In bootstrapping and in all-but-one subtractions it is a great advantage to have balanced antisera. Frontal separations, in contrast, exploit the fact that almost none of them, as initially prepared, are balanced. We now consider the rather difficult problem of preparing balanced antisera.

Balanced subtraction and balanced antisera. There are many instances where columns are desired that will remove efficiently all the antigens from a mixture obtained from a normal source so that antigens of difference may be detected in a pathological one. In addition, balanced antisera are required for making efficient all-but-one subtractive columns.

Balanced antisera may be made by combining antisera against separate constituents in a mixture, provided these are available. More practical, but time-consuming approaches make use of hyperimmunization with complex mixtures—an approach long used in the preparation of antisera used for immunoelectrophoresis. However, the response of the animals to such immunization is rarely stoichiometric. Even if an antiserum is produced which precipitates all antigens in a mixture equally, such an antiserum would not necessarily make a balanced subtractive column. Large molecules such as α_2 -macroglobulin may bind many antibody molecules for each macroglobulin molecule during precipitation, while only one immobilized antibody molecule may be required to bind one macroglobulin molecule on a column. For this reason the only satis-

factory method of evaluation is to use it to make a column and test its characteristics.

It might be thought that to rise to a balanced or starting antigen mixture, however, again some antigens may be removed from the mixture. A number of antigenic sites may still be needed.

Two general procedures are used. First (Fig. 4A) the initial mixture (step 1), and then on a column of immobilized IgG thus isolated (step 2). The saturation profile is used to discover how unbalanced the mixture is used to isolate the antigen that breaks through first. The antigen is then recycled and used as an antigen for antiserum production (step 3). To prepare a second set of antisera, a second set of additional boosting antigens is used until the animals used on the column (step 10). In this manner major antigens (albumin) and antisera against albumin may be used to prepare batches of antiserum. The proper ratio to remove the proper ratio to remove the volume of serum. The full amount of serum is used to immunize animals using the antigen.

In the procedure shown in Fig. 4B, a column or antiserum is used to remove a given mixture. Instead of using a specific antigen in the sense of not being able to prepare a large specific antigen, either not bound or bound to a given amount of the starting mixture (step 5) and used to immobilize antibodies from these antigens (step 6) and used to immunize animals used to prepare a second set of antisera for which this set of antigens is isolated (step 9) and used

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factory method of evaluating an antiserum for this purpose is to actually use it to make a column and then determine its subtractive characteristics.

It might be thought that an unbalanced antiserum could easily give rise to a balanced one by passing it over a column of immobilized starting antigen mixture, and then eluting the bound antibodies. However, again some antigens bind much more antibody than is required to remove them from solution because of their size and because of the number of antigenic sites they bear. Thus adjustment of the proportions is still needed.

Two general procedures for doing this are outlined in Fig. 4. In the first (Fig. 4A) the initial step is the preparation of antisera against the mixture (step 1), and all of the antibodies thus produced are recovered on a column of immobilized antigen mixture (steps 2 and 3). The specific IgG thus isolated (step 4) is used to prepare an affinity column (step 5). The saturation profile of this column is then determined (steps 6 and 7) to discover how unbalanced it is and what sample volume should be used to isolate the antigens which quickly saturate the column (i.e., break through first). Those antigens are then isolated by repeating cycling and used as an antigen to boost the rabbits or goats used for antiserum production (steps 8 and 9). The antisera thus obtained are used to prepare a second set of columns which are in turn used to prepare additional boosting antigen (second-round antigen)—a process continued until the animals used are making an antiserum that will yield a balanced column (step 10). In many cases a mixture such as serum contains two major antigens (albumin and IgG) and many minor ones. Specific antisera against albumin and against IgG may be separately prepared and used to prepare batches of adsorbent which may then be mixed to give the proper ratio to remove both proteins completely from a known volume of serum. The fall-through from such columns may then be used to immunize animals using the scheme given in Fig. 4A.

In the procedure shown in Fig. 4B no attempt is made to prepare one column or antiserum that is completely balanced with reference to a given mixture. Instead specific anti-mixture antibody is prepared (specific in the sense of not containing inactive IgG) as in Fig. 4A and used to prepare a large specific-antibody column (steps 1-4). The antigens either not bound or bound in insufficient quantity to this column when a given amount of the starting antigen mixture is applied are then isolated (step 5) and used to immunize a second round of animals (step 6). Antibodies from these animals are in turn isolated (steps 7 and 8) and are used to prepare a second antibody column (step 10). Second-round antigens for which this second-round column lacks capacity are then isolated (step 9) and used to prepare third-round antigens (step 11). This

solution. For example, serum albumin as a contaminant in a preparation can be removed in the following way. Serum albumin is covalently linked to a column support, and an antiserum containing antibodies against serum albumin passed over it. The column is then well washed, and the preparation containing the albumin contaminant passed over it. The contaminating albumin is then bound noncovalently to the antibody which is noncovalently bound to the antigen which is covalently attached. During column regeneration both of the outer two sandwich components are removed. This method is convenient only for small-scale work, since the middle component (which may be either antigen or antibody) is lost.

In the second type of column, developed by one of us (6), the antigen (or antibody) covalently linked to the column support is used to remove its opposite number from solution, the column is then washed, and the two moieties (antigen and antibody) are then covalently linked together with a reagent such as glutaraldehyde. This *reverses* the column from an antigen to an antibody one (or vice versa) and the column may then be used to subtract the species originally covalently attached to the support.

This does not by any means exhaust the possibilities opened up by the combination of stereospecific adsorbents and the amplification inherent in rapid recycling and in the immune system itself. In theory, with three or four stages of amplification, factors of 10^6 appear feasible. If gel filtration can also be adapted to relatively rapid cycling so that only mixtures of a narrow molecular weight range need be dealt with, then the problems of immunosubtractive reagent preparation can be greatly simplified.

In the preceding paper the basic instrumentation required for cyclic column operation was described. Thus far in this paper we have been concerned with principles applicable to such systems. We now ask, can they in fact be made to work, and what are their limitations?

INITIAL EXPERIMENTAL SYSTEM

A very large number of variables must be considered in developing procedures, and the problem of optimizing separations is extremely difficult to approach initially. At the outset therefore we have made the following decisions, based in part on a long series of orienting experiments.

Elution. The initial problem is the general class of eluting agent to be used, and four were considered. The first class employs extremes of pH (7). This is effective for many purposes; however, in our hands columns were gradually degraded after continuous use and also presented the problem of pH reequilibration (generally a time-consuming procedure)

before each new sample destroyed by high or low pH that neutralizing solution effluent line, making exposures extremes quite brief. The which are not specifically efficiently universal for our substances such as urea which secondary and tertiary products that it gradually de substances eluted, is not which must be employed, tion of urea may gradu number of troublesome in.

The fourth class include anions, thiocyanate and lithium is one of the few have employed ammonium monium with lithium will

Temperature. Low temperature, and dialysis, possibly fore sought to operate all possible until a measurable is seen. Samples and colle

pH. All solutions have buffered with 0.1 M sodium forming all steps at a diff

Bacteriostasis. When pretended periods, it is essential solutions. Sodium azide, 0 protein samples it is converted drop per 10 ml.

Monitoring. Thiocyanate balance at 260 nm but have latter wavelength or at 280

Flow rates. We have in necessitated flow rates ran

* The word "chaotropic" was the effect of electrolytes on the ions for solubilization and dissociation studied by Dandliker *et al.* (9), to the effect of anions of the lyotropic

before each new sample is applied. In addition many antigens are destroyed by high or low pH. An advantage of pH elution, however, is that neutralizing solutions may be pumped directly into the column effluent line, making exposure of an adsorbed and eluted moiety to pH extremes quite brief. The second class of eluents includes strong salts which are not specifically chaotropic. This method does not appear sufficiently universal for our purposes. The third class encompasses substances such as urea which tend to break hydrogen bonds and to disrupt secondary and tertiary protein structure. Our experience with urea indicates that it gradually damages affinity columns, denatures many of the substances eluted, is not an efficient eluent, and, in the concentrations which must be employed, is not easy to get rid of. In addition isomerization of urea may gradually occur in concentrated solutions, and a number of troublesome impurities may be present.

The fourth class includes chaotropic ions.⁶ Of the commonly available anions, thiocyanate and trichloroacetate appear most effective, while lithium is one of the few useful cations. For introductory studies we have employed ammonium thiocyanate, and the effect of replacing ammonium with lithium will be reported later.

Temperature. Low temperature slows denaturation, adsorption, elution, and dialysis, possibly all to a comparable degree. We have therefore sought to operate all columns at room temperature and as rapidly as possible until a measurable advantage of working at lower temperatures is seen. Samples and collected fractions are refrigerated however.

pH. All solutions have been at or very close to pH 7.0 and have been buffered with 0.1 M sodium phosphate. The possible advantages of performing all steps at a different (but constant pH) require exploration.

Bacteriostasis. When protein separations systems are to be run for extended periods, it is essential to incorporate bacteriostatic agents in all solutions. Sodium azide, 0.1%, has been adopted as standard, and for protein samples it is convenient to make up a 20% solution and add one drop per 10 ml.

Monitoring. Thiocyanate and azide solutions have considerable absorbance at 260 nm but have very much less at 280 nm. Monitoring at the latter wavelength or at 285 or 290 nm is therefore used.

Flow rates. We have initially sought cycle times of 1-2 hr. This has necessitated flow rates ranging from 1 to 4 ml/cm²/min.

⁶The word "chaotropic" was coined by Hamaguchi and Geidusek in a study (8) of the effect of electrolytes on the stability of DNA secondary structure. Use of chaotropic ions for solubilization and dissociation of antigen-antibody complexes was extensively studied by Dandliker *et al.* (9), following earlier studies of Kleinschmidt and Boyer (10) on the effect of anions of the lyotropic series on antigen-antibody reactions.

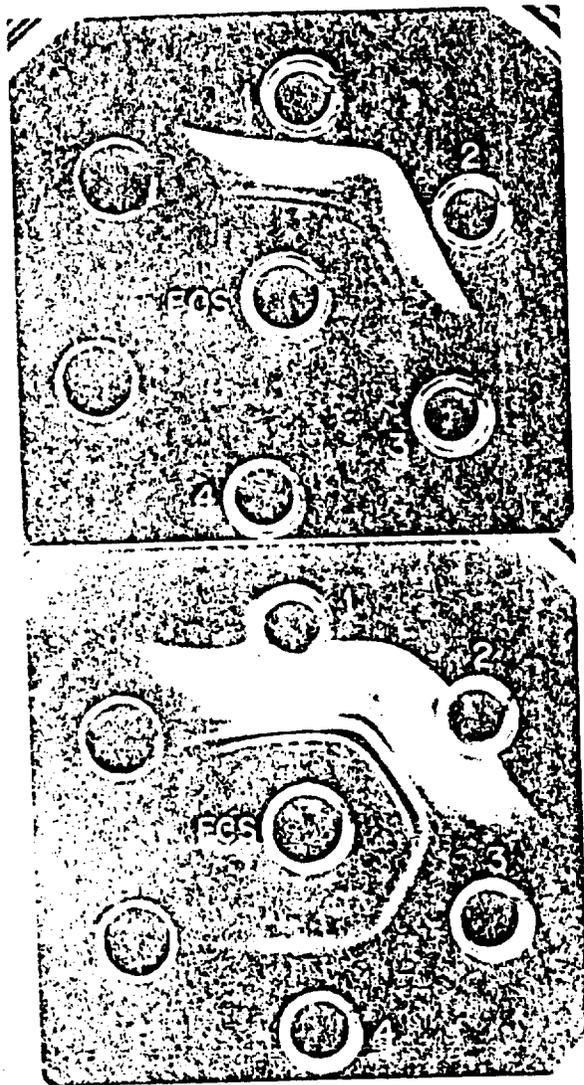


FIG. 7. Analysis of samples from the experiment shown in Fig. 6 to determine whether all antibodies had been removed from the starting serum. Unbound (U) and adsorbed and eluted (A) fractions were concentrated back to starting volume and examined by double diffusion on Ouchterlony plates. The center well contains fetal calf serum; well 1, starting rabbit anti-fetal calf serum; well 2, fraction A from Fig. 6; well 3, fraction U from Fig. 6; and well 4, fraction A from Fig. 8. Upper photograph at 24 hr, lower at 48 hr. Note that column failed to remove all of at least one antibody under the conditions employed.

adsorbed and eluted antibody by double diffusion analysis. It has been removed from the starting serum and is still functional. The results of the double diffusion analysis of the eluted antibodies were re-examined. These were eluted in active form. The concentration of antibodies was incompletely removed. The antigen concentration in the eluted antibodies was stable against an antigen which is not found in the starting serum. No analysis apply here. The completeness of re-



FIG. 8. Passage of unadsorbed antibodies were reconcentrated to starting volume and examined by double diffusion analysis. A very small amount of antibody was adsorbed. The 15 min long preceded sample application. Additional thiocyanate cycles were used. The results of the double diffusion analysis were the same as previous. The results are given in Fig. 7.

Isolation of Placental Antigens

The placental extract, isolated as described in Section , can be processed in two different ways to purify the unique antigens. The possibility of two different approaches will be true in all cases where abnormal proteins occur mixed with normal proteins. In the first method an immunosorbent is prepared, containing balanced antibodies specific against the normal proteins, and the sample is eluted through the immunosorbent so that the abnormal proteins are recovered in the unbound protein of the effluent. The second method starts once sufficient purity of the abnormal proteins is accomplished, so that antisera against them can be produced, the specific antibodies isolated, and an immunosorbent (second generation) consisting of only antibodies to the abnormal proteins can be produced. Subsequently the abnormal proteins can then be easily bound from a complex mixture.

An example of a sample absorbed with an immunosorbent of the first type is shown in Figure . About 40 A_{280} units of the placental extract were applied to a 1.5 x 15 cm column containing approx. 700 A_{280} units of a fair-quality IgG (anti-normal human serum) - cyanogen bromide activated-Sepharose immunosorbent. The first two unbound peaks were eluted with the starting buffer, 0.1 M sodium phosphate, pH 7.0, and 0.1% sodium azide. The first peak was determined to be primarily nucleic acids of large molecular weights. The second peak of 5 A_{280} units was considerably reduced in normal serum proteins and contained greater than five proteins unique to human placentas. The last peak contained normal serum proteins bound to the IgG, and required thiocyanate for elution. The column was then regenerated with the phosphate buffer. Using an automatic system such a column has been cycled through sample addition, elution, and regeneration 50 times to increase amount of working material.

A matter of considerable importance must be now mentioned; that is, what constitutes a "normal" or an "abnormal" protein? For that matter, what constitutes a "normal" or "abnormal" human? In the absence of an universally accepted answer, the problem must be approached by obtaining pools of many different "normal" individuals so that variants in certain "normal" proteins will not be mistaken to be the abnormal proteins being studied in a particular instance. Although as progress is made in comparing the various serum proteins among a population perhaps such a seemingly harmless disparity as a difference of one or two amino acids on a transferrin molecule will be found to be significant medically or pathologically.

As to our particular situation, care must be taken that the placental antigens, isolated as they are from a mixed pool of many different individuals, represent truly unique placental molecules and not individual protein variations. This is accomplished by absorbing the placental extract with an immunosorbent produced from IgG developed against "pooled" normal human sera (a "first-generation" column). Or, the placental extract may be first absorbed with an immunosorbent so that there is the possibility of 2 or 3 variant "normal" proteins still present, then an antiserum may be developed against this sample. The antiserum can be made monospecific for only placental proteins by absorption of the contaminating IgG that is anti-normal with an immunosorbent containing pooled normal human serum.

prior to injection

removal of contaminating antibodies after bleeding.

After extensive absorption, there should be an antiserum which is monospecific for only placental antigens. Most of the total IgG of this antiserum can be isolated ~~from~~^{using} a DEAE cellulose column and made into a "second-generation" column. Although ideally only the subset of the total IgG that is specific for the antigen is desired, at this point in the development either there is not enough of the antigens to isolate a

reasonable quantity of specific IgG via an immunosorbent, or the antiserum titer is too low for an efficient IgG recovery, or both cases may be partially true. It is the function of the "second-generation" column to allow production of sufficient quantities of antigen (tens to hundreds of milligrams) to provide both immobilized antigens and better antiserum from booster injections.

An example of a chromatograph of placental extract on a second generation column is shown in Figure 1. The column was made from the total IgG of an antiserum to partially pure placental antigens, the IgG having been isolated by DEAE and absorbed with only an individual human serum, as a pool of normal human sera was not available at the time.

The only active antibodies not specific for placental antigens remaining after this absorption would be those raised by whatever proteins of the pooled placental extract (representing a cross-section of material and fetal blood types) were different from or not included in the individual serum.

In healthy individuals, the most likely differences in proteins between maternal (or adult human) and fetal will be in the haptoglobins and transferrins.

AN EXAMPLE OF A CHROMATOGRAPH OF PLACENTAL EXTRACT ON A 2ND GEN. COLUMN IS SHOWN IN FIGURE 1. APPROX. 120 A₂₈₀ UNITS OF PROCESSED PLACENTAL EXTRACT WERE APPLIED TO A 2.5 x 15 cm COLUMN CONTAINING 1200 A₂₈₀ UNITS OF THE DESCRIBED IgG, INCLUDING ~~SOME~~ THE IgG ~~WHICH~~ SPECIFIC FOR PLACENTAL ANTIGENS. THE UNBOUND PROTEINS WERE ELUTED FROM THE IMMUNOSORBENT IN TWO PEAKS AND WERE NOT CHARACTERIZED. THE BOUND PROTEINS (ABOUT 12.0 A₂₈₀ UNITS) WERE ELUTED WITH 2.5 M THIOCYANATE BUFFER, pH 6.8, AND ARE BEING CHARACTERIZED AS FOLLOWS. IN PLATE 165 THE REACTION BY IMMUNOELECTROPHORESIS WAS INVESTIGATED FOR PLACENTAL ANTIGENS ISOLATED BY BOTH FIRST AND SECOND GENERATION COLUMNS. THE ANTISERUM (RAISED BY FIRST-TYPE ANTIGEN) HAD BEEN EXTENSIVELY ABSORBED WITH POOLED NORMAL HUMAN SERA TO MAKE IT SPECIFIC FOR PLACENTAL ANTIGENS. IT SHOWED NO REACTION WITH NORMAL SERA. HOWEVER, IT SHOWED AT LEAST 5 BANDS OF REACTION WITH PLACENTAL ANTIGENS OF BOTH FIRST AND SECOND GENERATION.

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EARLY DETECTION OF PREGNANCY-ASSOCIATED SERUM PROTEINS
USING ANTISERUM TO PLACENTAL ANTIGENS

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Abstract

Antisera against human placental proteins were developed in goats and rabbits, using immunoadjuvants and a prolonged injection schedule. The antisera were absorbed with normal serum proteins and then tested in immunodiffusion against normal and pregnancy sera. Two bands of precipitation due to pregnancy antigens were observed in pregnancy sera as early as 18 days after conception. Detection of these antigens has possibilities for application as an early pregnancy test.

Introduction

The possibility of using antiserum to placental extract to detect pregnancy at an early stage arose from an investigation directed toward determining whether placental and other fetal antigens are reexpressed in disease states, particularly cancer. Levels of existing serum proteins change in pregnancy, and new proteins appear that are produced by either the mother or the fetus. The occurrence of at least one and perhaps two serologically

specific components in pregnancy sera was demonstrated by Thornes (3) in 1958 by gel precipitation. In 1959, Smithies (4) showed the presence of a pregnancy-associated α -globulin in a region of the starch gel electropherogram which he called the "pregnancy zone." In 1960, Hirschfeld and Söderberg (5) found two "pregnancy precipitates" on immunoelectrophoresis of pregnancy sera, one in the fast α -globulin region, and the other in the slow α_2 -beta-globulin region. Since then, as many as four such pregnancy-associated plasma proteins have been detected.

Gall and Halbert (6) showed the sequential appearance of four pregnancy-associated plasma proteins that were studied as pregnancy progressed; Lin et al. (7) characterized these with respect to molecular weight, isoelectric point and electrophoretic mobility, pH and temperature stability, sedimentation behavior, solubility, and elution from DEAE cellulose. Possible enzyme activity was also investigated. Three of the proteins were routinely seen by immunoelectrophoresis. One of the proteins contained iron. None appeared in normal nonpregnancy plasma. The pregnancy samples tested were from subjects in the third trimester. The antisera used had been raised against pregnancy plasma and then absorbed exhaustively with nonpregnancy plasma. Bohn (8) used rabbit antihuman placenta antiserum absorbed with male serum to detect four pregnancy-associated plasma proteins. Only one was considered to be specific for pregnancy, while two could be detected sometimes in sera from nonpregnant female subjects, especially in those taking oral contraceptives.

Berne (9) used a quantitative radial immunodiffusion test to determine levels of a pregnancy-zone protein, termed by him α_2 -pregnoglobulin, also found in males and nonpregnant females. Users of oral contraceptive estrogen-progestin combinations showed levels reaching into the pregnancy range. First elevations of the α_2 -pregnoglobulin were detected at six weeks of pregnancy. The purpose of the present work was to determine how early in pregnancy any of the pregnancy-associated serum antigens could be detected.

Materials and Methods

Preparation of crude placental extract. Term placentas were obtained at delivery and chilled in 0.85% sodium chloride solution. Processing was begun immediately upon return to the laboratory. The placentas were washed for 1 hr in tap water to remove a maximum of blood elements and serum proteins. Fat was then cut away, 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, 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. Following centrifugation, the material was dissolved in a minimal amount of 0.1 M, pH 7, phosphate buffer

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containing 0.1% sodium azide, the solution was dialyzed against this same buffer, and any material that precipitated was removed by centrifugation.

Any serum proteins left in the solution were removed by cycling it over an immunoabsorbent column of antibodies to normal human serum. 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 (10) per ml without undesired precipitation occurring on storage, especially on freezing and thawing. 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. This separation could be of interest for its own sake; in addition, some separation 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. Efforts are under way to modify the extraction procedure (for example, mild acid extraction of diced placenta, without homogenization) in order to minimize the extraction of proteins that do not appear in the plasma.

Raising of antisera. The placental extracts, minus 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 an equal volume of Freund's complete adjuvant (11), and the preparation was injected subcutaneously, either in the hips or behind the neck (4 ml for goats, 1 ml for rabbits). 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. High-titer antiserum was obtained, usually in about 7 to 8 months. The antiserum was absorbed with lyophilized, pooled serum obtained from normal human males (100 mg/ml of antiserum) to render it specific for placental antigen.

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 are shown in Figure 1, from three women in their second pregnancy in Figure 2, and from three women in their third pregnancy in Figure 3. 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. A positive

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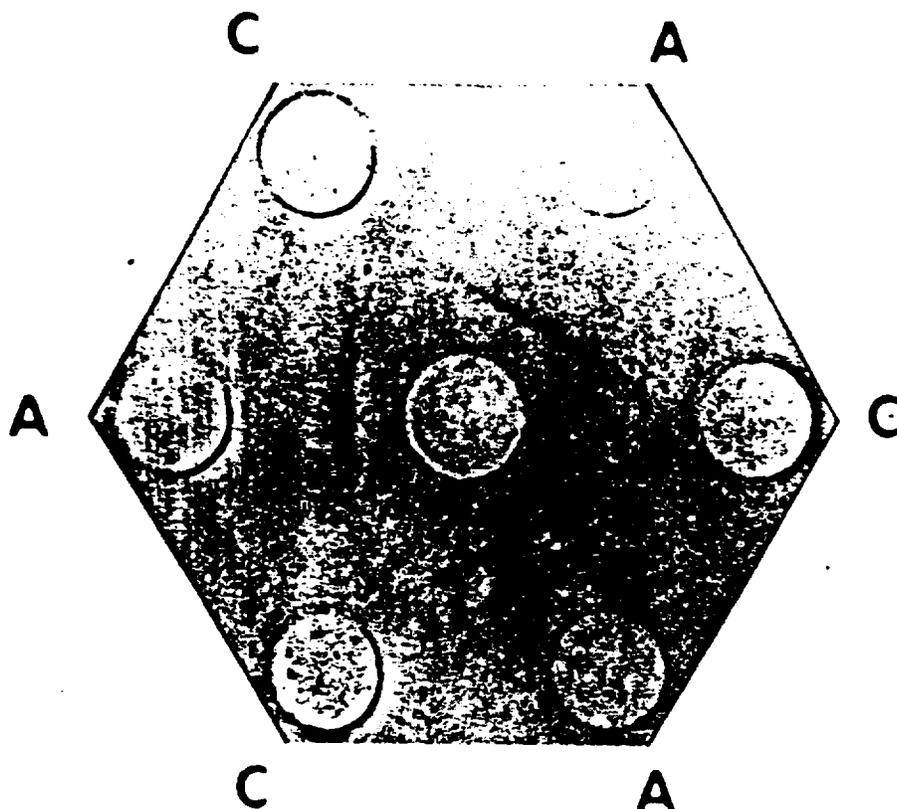


FIGURE 1

Detection of pregnancy-associated antigens in sera from women in the third trimester of their first pregnancy. Each of the A wells contains an individual serum. Control serum is in the C wells; the center well contains the absorbed antiplacental antigen antiserum. Time of reaction: 24 hr.

reaction was seen within 5 hr with all the third-trimester sera. These reactions improved with time, so that in 40 hr 2 to 4 bands of precipitation were observed for all samples. The four precipitin bands between a third-trimester pregnancy serum and the antiserum are unusually clear in Figure 4 (well marked 3P).

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, includ-

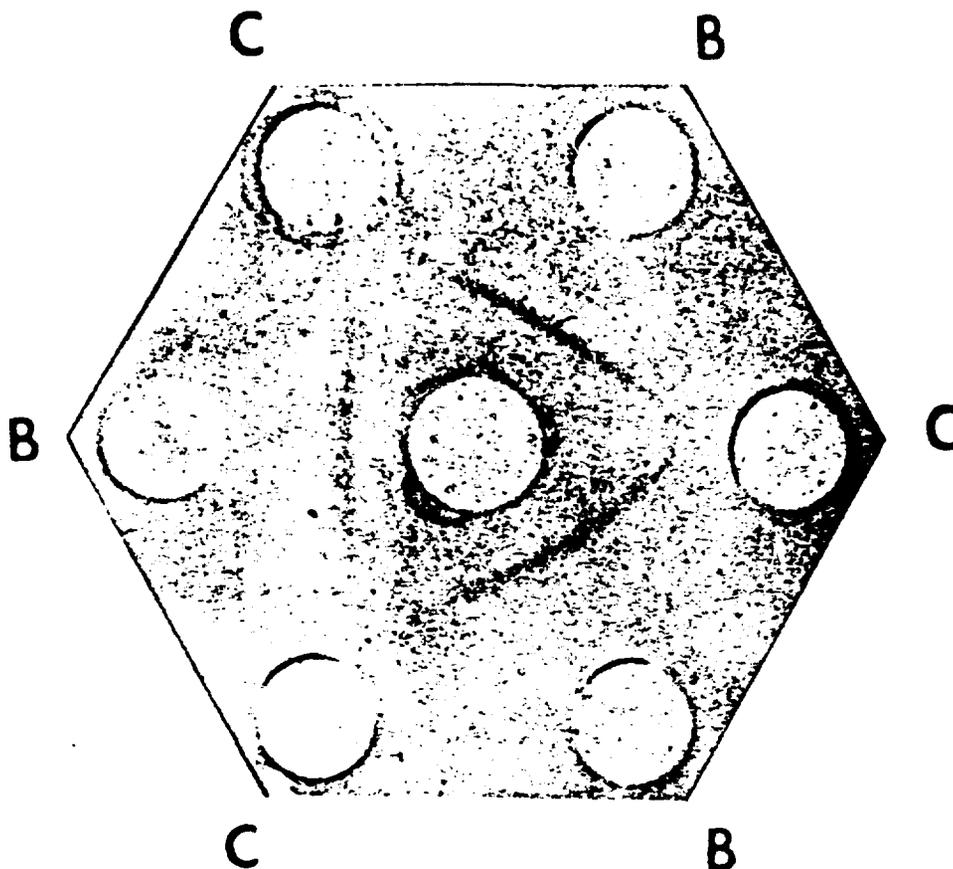


FIGURE 2

Detection of pregnancy-associated antigens in sera from women in the third trimester of their second pregnancy. Disposition of the wells and time of reaction as in Fig. 1.

ing those previously pregnant. Thus far, no reaction has been shown with sera from women taking estrogen-related contraceptives (four cases tested).

In view of the positive reactions with third-trimester pregnancy sera, it was decided to see how early these reactions could be found in pregnancy sera. A serum sample M 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 J was obtained from a 25-

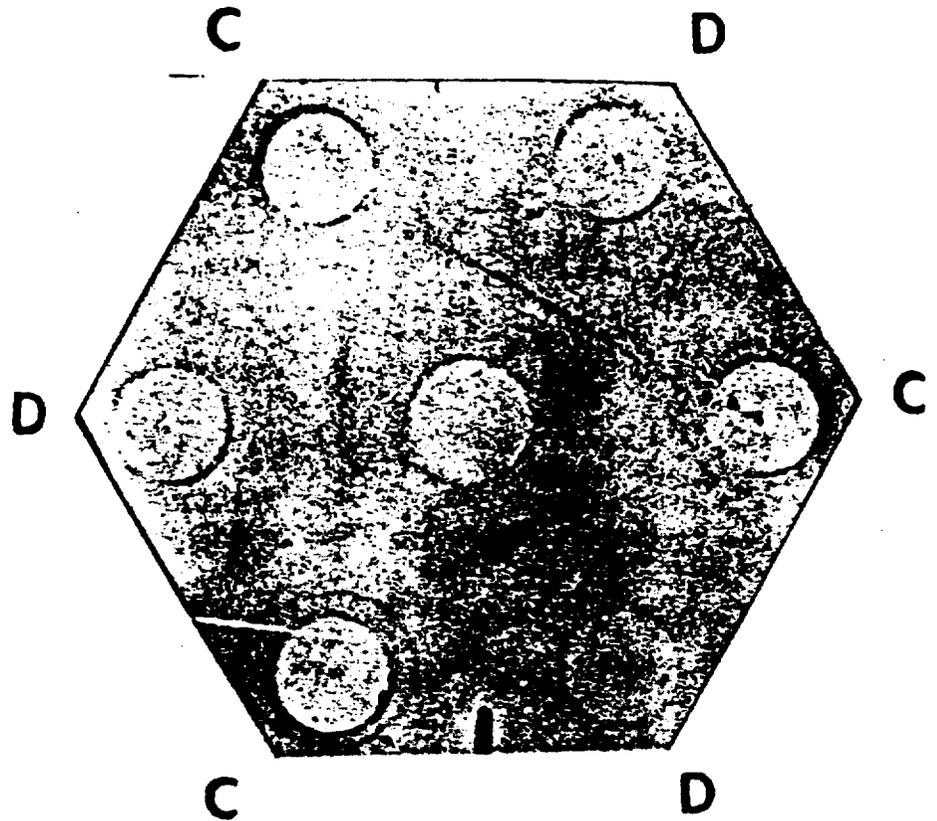


FIGURE 3

Detection of pregnancy-associated antigens in sera from women in the third trimester of their third pregnancy. Disposition of the wells and time of reaction as in Figs. 1 and 2.

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. Samples M and J were tested by double diffusion (Figures 4 and 5, respectively) 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 M and J,

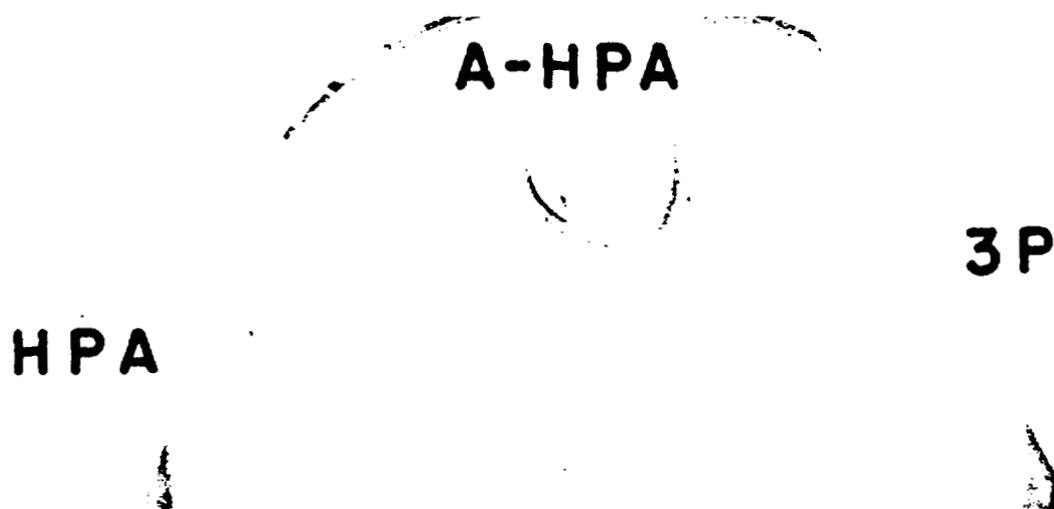


FIGURE 4

Comparison of antigens from early-pregnancy and third-trimester serum samples. Well 3P contained serum from a woman in the third trimester of her third pregnancy; well M contained serum from a woman 18-22 days pregnant; well HPA is human placental extract; A-HPA is the antiserum. Time of reaction: 16 hr.

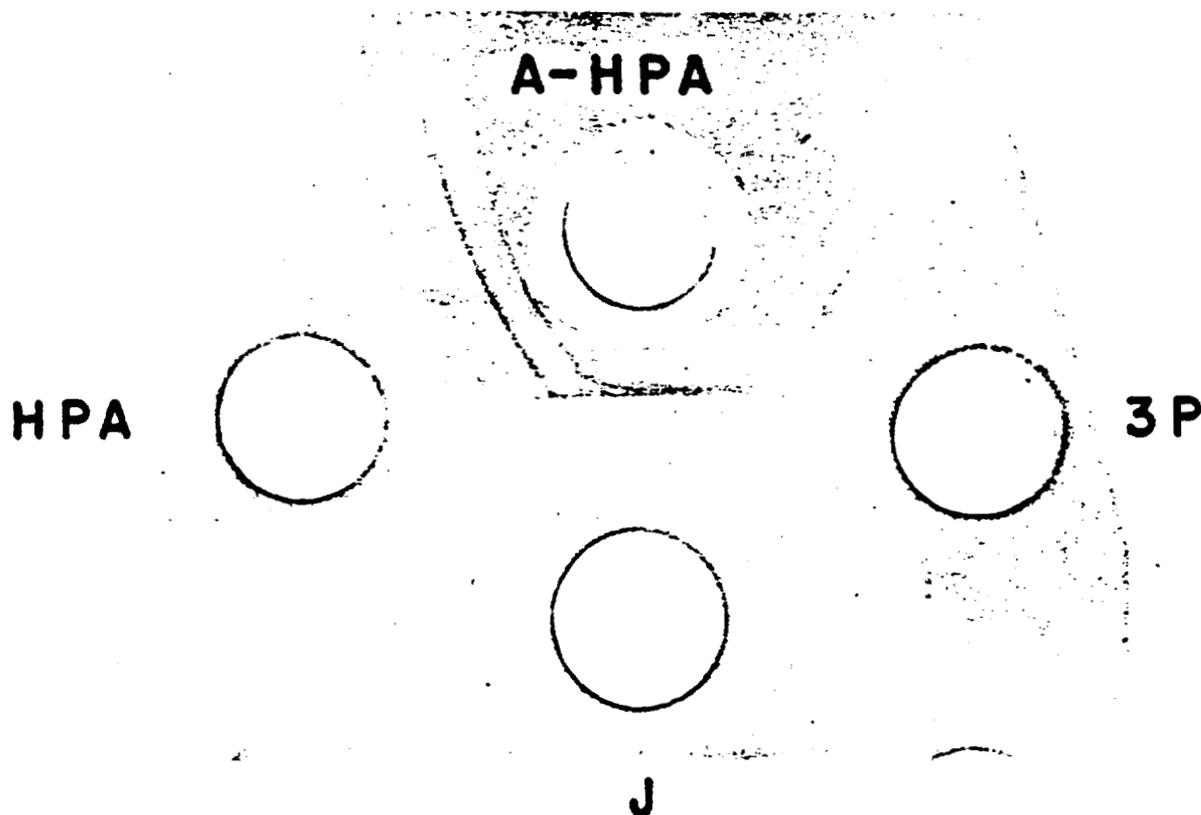


FIGURE 5

Same comparison as in Fig. 4, except that in this case the early-pregnancy sample (J) was a pregnancy serum taken 22 to 26 days after conception. Immunodiffusion for 16 hr.

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a precipitin band could be observed as early as 1 hr after the start of immunodiffusion. Three precipitin bands were observed for sample J within 24 hr after loading.

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 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.

Conclusions

We have demonstrated that antiserum against human placental antigens can be used to detect the appearance early in pregnancy of pregnancy-associated serum proteins. In the earliest sample tested, taken 18 to 22 days after conception, two such proteins were clearly detected. Another sample, taken 22 to 26 days after conception, gave indication of three pregnancy-associated antigens. Additional samples taken at even earlier stages of gestation should be tested to determine just how early such pregnancy-associated proteins can be detected. Further, samples from users of a wide variety of hormone-based contraceptives should be tested to

ascertain if their use provokes the production of antigens that react with the antiserum. Finally, efforts should be made to characterize these early appearing pregnancy-associated proteins so that they can be compared with others described in the literature, and to determine whether they can occur in various diseases.

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10. An A₂₈₀ 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.
11. The Freund's complete adjuvant used was obtained commercially from Difco Laboratories. It contained in 10-ml ampules sterile light mineral oil plus emulsifier, and heat-killed Myco-bacterium butyricum (0.5 mg dry wt per ml).

Several rabbit antisera specific against individual human serum proteins ^{were} processed on DEAE cellulose to remove their total IgG for study of immunosubtractive electrophoresis on density polyacrylamide gels ^{B. (man, Aditya, et al. immun)}
For example, 2.05 ml (approx. ⁹⁸ ~~81~~ ²⁵ ~~20~~ ²⁸⁰ units) of rabbit anti-human ^{hemopexin} were fractionated on a 2.5 x ²⁵ ~~30~~ cm column of DE32 yielding, as for all rabbit antisera, two early IgG peaks in 0.01 M phosphate buffer, as shown in Figure [?]

Also, 8 ml (approx. 1000 A₂₀₀ units) of dialyzed horse anti-whole human serum and 19.6 ml (approx. 1200 A₂₀₀ units) of dialyzed goat anti-whole human serum were fractionated on a 2.5 x 60 cm DE32 column, as shown in Figure [?]

. As previously noted, both horse and goat antisera yield two distinct IgG peaks when fractionated on DEAE. The chromatograph of 5 ml (approx. 330 A₂₀₀ units) of human pregnant sera on a 1.5 x 50 cm column is shown in Figure [?]

. A comparison of the chromatographs of the horse, human, and goats sera shows the most similarity for horse and human sera fractionation.

In order to recover the tailing 7 S γ_2 IgG from DEAE, it is necessary to increase the buffer concentration to 0.02 or 0.03 M phosphate, pH 8.0. If this remaining IgG contains 1 or 2 contaminating β -globulins, they may be removed by immunosubtraction.

PARAMETERS IN AFFINITY CHROM.

There are a number of chromatographic parameters to be considered in affinity chromatography. Among these are column temperature, ionic strength and pH of buffers and eluting solutions, flow rates, time of runs, column geometry, and column life. Attempts of varying sophistication have been made to investigate the above problems.

The immunosorbent columns are normally run at room temperature for which acceptable flow rates are obtained. When a column was run at 7°C, a marked increase in flow resistance was observed and all eluted peaks exhibited severe "tailing" problems. The increased flow resistance was such that practical limitations on gravity head made it impossible to achieve a flow rate comparable to that found for the same column at room temperature, so that a pump was necessary to reestablish this latter rate. (A Figure is available for temperature affect.) The existence of heightened flow resistance and increased tailing led to abandonment of low temperature operation.

Various buffers have been employed by investigation to dissociate the antigen-antibody bands found on immunosorbents. Weetal ^B utilized

0.05 M glycine-HCl-buffered 1% NaCl, pH 2.3, to remove human gamma globulins (HGG) that had been bound to rabbit anti-human gamma globulin immobilized on an activated glass support. Although the data was not clear, it seemed that as much as 30% of the HGG could not be reabsorbed when rerun over the immunosorbent. Chidlow and others (C) reported that 0.2 M glycine-HC (pH 2.2) removed almost all human serum albumin from immunosorbents consisting of anti-human serum albumins crosslinked by thiol complexes. No effort was made to determine the recovery of immunological activity. Cuatrecasas (C) used HCl to elute anti-porcine insulin from an insulin lysine Sepharose column. No destruction of antibody was detected with concentration of HCl as high as 3N, provided the fractions were neutralized immediately. Weintraub (C) employed 6M guanidine-HCl, pH 3.1, to dissociate human chorionic somato-mammotropin (HCS) from an immunosorbent of anti-HCS-sepharose. Unlabeled HCS was restored to full immunoreactivity even if it had been exposed to 6M guanidine for as long as 5 hours prior to dilution or dialysis against neutral buffer. Dandliker (C) reported that antigen-antibody complexes in solution are dissociated by thiocyanate, perchlorate, or iodide ions in the region of 1-4 M near neutrality. At lower pH ranges the reaction is even more rapid but the complications due to irreversible effects are increased. Recoveries of antibody activity for 2M thiocyanate and perchlorate ~~was~~ ^{were} 70-80% near neutrality.

We have experimented with several of the above schemes for breaking antigen-antibody bonds. It was found that 4M urea would dissociate only part of the antigen-antibody immunosorbent bonds, so that further material was eluted with 6M urea. However, the 6M urea resulted in considerable denaturation as evidenced by an insoluble cloudy ^{suspension} and white precipitates, even though it was immediately ~~diluted~~ ^{dialyzed}. No effort was made to ascertain the quantitative loss of immunoreactivity.. It was suspected that the urea might also slowly destroy the covalent structure of the immunosorbent.

The pH elution effect was investigated by using 0.03 M glycine-HCl, pH 3.0, to rupture antigen-antibody bonds. The effluent was neutralized immediately after elution, so that denaturation, on visual observation, seemed minimal. However, precipitates invariably formed during storage, although the IgG concentration was maintained at 1-10 mg/ml. It was suspected that a great deal of this precipitation might be due to antigen-antibody interaction due to undesired elution of not only antibody from the antigenic immunosorbent but also some of the antigen. This effect was confirmed by testing the dissolved precipitates in diffusion tests against solutions of antigen and antibody.

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A reaction was observed between the undesired antigens in the precipitate and its homologous antibody.

An additional observation concerning glycine-HCl was that it seemed to improve in quantitative elution of bound IgG when ionic strength was increased. This behavior indicated varying affinities for varying antibodies and offered possibility of gradient elution of antibodies from multiple antigenic immuno-adsorbents.

The eluent presently used to dissociate the antigen-antibody bonds is 2.5 M thiocyanate, 0.1 M phosphate, pH 7.0, 0.1% sodium azide. Ammonium and sodium thiocyanate have been utilized with equal effectiveness. Buffers with thiocyanate molarities below 1.0 do not give quantitative elution. ~~It was found that insignificant amounts~~

See also Anderson et al,
Attachment 3
p. 381

You may desire to obtain a copy
of the paper at ~~our~~ biology
library.

~~SECRET~~
~~CONFIDENTIAL~~ ??

an old progress report!

ATTACHMENT 7

Further progress has been achieved towards establishing a "factory" for the production of large quantities of immunoglobulins (including total IgG and specific subsets), cancer antigens and antigens particular to pregnant serum and placental extract, and monospecific antisera. Significant progress has resulted, particularly from the addition of immunoabsorbents to the normal arsenal of separation tools such as cellulose ion exchangers and Sephadex filtration media.

Using established techniques of ion exchange separation, a protein sample consisting of a phosphate extract of human placenta has been fractionated into greater than 10 major peaks on DEAE cellulose. Identification of all of these peaks has not yet been established. Apparently some of them contain serum proteins not totally eliminated from the placenta prior to extraction. Analysis of each peak, consisting of Ouchterlony and immunoelectrophoretic diffusion of the concentrated peaks against a rabbit antiserum to human pregnant serum, absorbed with whole human serum, has shown a number, at least 5, of molecular species quite well scattered over a phosphate buffer range of 0.02 M sodium phosphate to 0.10 M sodium phosphate with 0.5 M NaCl. The existence of such a separation range, as well as the different antigen locations observed on immunoelectrophoresis, indicates molecules with quite different charge densities and hence molecular structure. Evidence indicating a range of molecular weights is obtained from gel electrophoretic patterns of each peak. Furthermore, Friemel (1) has shown on G-200 Sephadex filtration that placental extract antigens fractionate over a range of molecular weights.

MORE ON THE ELEMENTS IS GIVEN ON FOLLOWING PNE. ESSENTIAL SOME ELEMENT SCHEDULE WAS FORMED FOR IgG PLACENTAS

The DEAE-cellulose colum provides such a good separation of the various antigens that it will be routinely used as a prefractionation step in a scheme

IMMUNOGLOBULIN SEPARATIONS USING IONIC EXCHANGE COLUMNS

The chromatographic separation procedure for 7 S gamma globulins (IgG) is basically simple, involving for the serum sample dialysis (usually) against the equilibrating solution of low ionic strength and high pH, application to the column, and elution with the equilibrating buffer. Most of the 7 S gamma globulins are the most cationic of the serum proteins, so they are eluted through the anion exchange column at virtually the velocity of the eluent, maintaining a very short finite equilibrium with the anion exchanger. This equilibrium may be further shortened by either increasing the ionic strength or lowering the pH of the eluent. However, both measures have the effect of decreasing the equilibrium times of other proteins having net charges similar to the more acidic 7 S gamma globulins resulting in possible contamination. Elution of other serum proteins following the IgG may be controlled to some extent by gradient slope and limit salt concentration.

In the normal chromatographic run, the equilibrium solution contains 0.01 M sodium-phosphate (pH 8.0), 0.001 M EDTA, and 0.02% toluene. After the first peaks containing the 7 S gamma globulins are eluted in this solution, the column is eluted with a five-column-volume concave gradient, varying between a starting solution of 0.01 M sodium-phosphate (pH 8.0), 0.001 M EDTA, 0.02% toluene, and a limit solution of 0.30 M sodium-phosphate (pH 8.0), 0.001 M EDTA, and 0.02% toluene. A series of step elutions can be followed by starting at 0.01 M sodium-phosphate (pH 8.0), etc., and increasing at suitable increments the buffer concentration towards the final concentration of 0.1 M sodium phosphate, pH 8.0, and 1.0 M sodium chloride. About 85% of the feed, based on absorbancy at 280 nm, is eluted with these two solutions. Remaining material, possibly lipoprotein, is very strongly attached to the column and requires for its elution a solution of 1.0 M NaCl, 0.10 M sodium phosphate, 0.001 M EDTA, 0.02% toluene, and 5% Tween 80 (a detergent). Columns thus treated have been used for as many as ~~five~~¹⁰ separations without showing deleterious effects of protein buildup.

The 7 S gamma globulins can also be isolated by multiple precipitations with ethanol or sulfate salts, followed by gel or anion exchange chromatography, but generally the recoveries are poorer, the globulins are more likely to be denatured, and impure, and the methods are more laborious than with multiple chromatography.

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The immunoglobulin classes IgA and IgM are difficult to isolate from sera on presently used anion exchange columns, both eluting in overlapping regions containing immunoglobulins and nonimmunoglobulins. The IgM can be isolated from sera by Sephadex G-200 chromatography and PeWikon block electrophoresis. The IgA are more easily obtained from colostrums than from sera, since IgA are the predominant globulins in colostrum while they are but minor (<10%) globulins in serum. Both IgA and IgM can be isolated by multiple precipitation followed by preparative electrophoresis, but again chromatography is preferred for reasons given for the IgG isolation.

The observations made during experiments and production runs with the various anion exchangers were combined with literature results to produce the recommended IgG separation methods listed in Table VI.F. .1. The recovery percentages stated are somewhat uncertain, since a routine screening test for determination of 7S gamma globulin content of each individual serum feed has not yet been established. The recovery values for human serum are the most certain, being based on the normal human serum IgG concentration of 18% of total serum proteins. Those sera from animals whose immunoglobulin systems have been challenged differently vary considerably in their immunoglobulin concentrations. Preliminary density scans of polyacrylamide gels have indicated that the horse and goat have the largest IgG concentrations, varying from 20 to 35%. Based on the recovery of A_{200} units, rabbit sera are estimated to vary from 10 to 20% and hamster sera from 5 to 10% in IgG concentrations.

The general trend of Table VI.F.1.1 indicates that for human and horse serum, with a properly prepared exchanger and an unhemolytic serum, there is very little difference in the chromatography of dialyzed and undialyzed serum. However, for such animals as the hamster and rabbit, there are considerable differences between chromatographs of dialyzed and undialyzed samples. There seems to be a group of proteins in these sera which have a high-ionic-strength charged "field" associated with them, causing them to move as a cohesive "slug" through the exchanger when the sera is undialyzed.

~~Technical Report for Molecular Anatomy Group~~

R. F. Hall

B. Experimental Animals - GOATS

1. Storage

Goats are usually procured by advertising in the local newspapers or contacting livestock dealers who purchase the animals at local auction markets or on the farms. Animals one year old or more are desirable because the immune system is fully developed at this time.

The animals are examined for evidence of disease and internal parasites upon arrival and placed in isolation for about three weeks to avoid introducing incipient disease to other animals already on hand. During this period anthelmintics are administered to eliminate internal parasites. A routine hematological examination is done to help determine the state of health before injections are begun.

Housing can be very simple. A shelter open on one side is all that is needed to give some protection from excessive sun and inclement weather. The animals may be confined inside a barn or allowed to run in a small outside lot or pasture.

Rations usually consist of good quality hay fed free choice daily along with a small amount of grain to help maintain condition.

2. Injection Schedules

Three kinds of injections are routinely used to immunize goats with various antigens. The subcutaneous injection utilizes an area of loose skin on the neck adjacent to a large lymph node, on the inside of the front leg between the thoracic area and the limb, or on the inside of the hind leg adjacent to the inguinal region.

Other areas on the animal's body may also be used successfully. The hair over the chosen area is clipped and the skin disinfected with alcohol or other standard skin antiseptic. A fold of skin is grasped with one's fingers and, using a sterile syringe and sharp 18-20 ga. needle, the antigen injected just beneath the skin.

The intramuscular injection uses a large muscle mass, usually in the posterior portion of the foreleg adjacent to the thoracic region, or in the posterior part of the hind leg midway between the hip joint and the stifle joint. Again, the hair is clipped from the area and disinfectant applied to the skin. The leg is grasped with one hand and the other hand thrusts the 18-20 ga., 1 inch needle attached to the syringe into the muscle mass to the full depth of the needle.

Intraperitoneal injection is the introduction of antigen into the peritoneal cavity which contains the gastrointestinal tract as well as other organs. The animal is restrained in a standing position and the hair clipped over the right sublumbar fossa just posterior to the last rib. The skin is disinfected as before and the 18-20 ga., 1 inch needle with syringe attached is inserted through the skin and muscle into the cavity. Gentle suction on the syringe should be applied to be sure the needle has not penetrated an intestine or other organ. The contents of the syringe are quickly expelled and the needle withdrawn.

In all these procedures the animal caretaker will restrain the animal, usually in a standing position, by using one hand to grasp the head and neck and the other hand to grasp the rump of the goat.

Injections are usually made at weekly intervals, at least in the beginning, in order to induce a rapid increase in the antibody level in the animal. After a desired level of antibodies is achieved it may be

necessary to inject less frequently to maintain a satisfactory level.

Adjuvants are materials which are added to the antigens to delay absorption of the antigen from the site of injection, thus allowing prolonged stimulation to the immune system. Preparations of water-in-oil (Freund) are commonly used as adjuvants and are somewhat irritating to animal tissues. There is usually some transitory, local pain to the animal when the injection is made and a localized swelling results from the presence of the adjuvant. The tissue reaction may progress to the formation of an abscess, but this happens rarely in goats.

The initial injection of antigen is incorporated in a volume of adjuvant and put into the test animal. Thereafter, the antigen is given subcutaneously or intraperitoneally without adjuvant. Intraperitoneal injections of antigen with adjuvant are commonly done, but the irritant nature of the adjuvant is likely to cause some local peritonitis and adhesions.

3. Bleeding Schedules

Blood can be collected from the goat frequently and in volumes sufficient to supply the needs of the investigator. Volumes of 200 ml. or more can be drawn at weekly intervals without harm to the goat.

Animals are fasted for 12 hours prior to bleeding to reduce the amount of fat and other substances undesirable in the processing of the serum. The animal is restrained by the attendant holding head and neck. The hair over the jugular furrow is clipped and disinfectant applied to site of venipuncture. Pressure posterior to site of bleeding causes the jugular vein to distend and a sharp 16 ga. needle is thrust into the vein. The blood is caught in a suitable container. If sterile

collections are desired a needle and syringe can be used to avoid contamination from hair and fingers.

Care should be exercised to avoid trauma to the perivascular tissues and formation of large hematomas in the jugular furrow. Sharp, small bore needles and exact location of the vein make bleeding a simple matter. If unable to obtain blood from one jugular vein, one can usually go to the contralateral vein and make satisfactory puncture.

B. Experimental Animals

The animals presently in use for the production of experimental and preparative-scale antisera are ^{NEW 21 MAR 40 W. 1112} rabbits and ^{usual Commercial} goats. For production of large quantities of antisera, goats are obviously preferable to rabbits, but they have the disadvantage that they require the injection of considerably more antigenic material to achieve a high quality antiserum.

Rabbits

1. Storage. -- The rabbits are maintained singly in cages with self-service feeders and water bottles.
2. Injection Schedules. -- For the first injection, the antigen is suspended in Freund's complete adjuvant about 1 hour prior to injection. The dispersal of the Freund's adjuvant is accomplished by sonification.

The antigen in Freund's complete adjuvant is injected either subcutaneously at two sites behind the rabbit's head or ~~intramuscularly~~ in each hip. For complex antigen mixtures, such as human serum, the first injection consists of about 60 milligrams of serum. This allows on the order of 0.5-5.0 mg of each minor serum constituent and of course a considerable amount of albumin and ~~transferrin~~^{F9G}. But these unusually large amounts of albumin and ~~transferrin~~^{F9G} are necessary to elicit an antibody response for these molecules which vary but little across animal lines and subsequently are not easily recognized as foreign antigens. For other purified antigens, the first injection usually consists of 1-5 mg.

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Subsequent boosts are given weekly by injecting ~~20~~ mg of the antigen, no adjuvant, subcutaneously at two sites in the rabbit's haunches. Addition of Freund's incomplete adjuvant to the antigen at these times will result in an increased antibody titer on the short term but one faces the possibility that knots, sores, and infection may result from this adjuvant.

Introduction and Historical Notes

What are adjuvants? As WHITE [1967 (2)] said, "the list of them reads like a medieval alchemist's shopping list." We would like first to give a clear and simple definition which yet takes into account the complexities of a field where many authors have used the same words for completely different concepts. RAMON (1926), whose primary goal was the enhancement of antibody synthesis against diphtheria or tetanus toxoid, called "adjuvant and immunity stimulating substances" products which, used in combination with specific antigen vaccines, enhance immunity levels above those that the vaccines are capable of developing when injected alone. Even at that early date, he concluded: "Si intéressant que soit ce procédé du point de vue pratique, il ne l'est pas moins du point de vue théorique, à cause des recherches qu'il peut susciter pour essayer de pénétrer le mécanisme intime, soit de l'augmentation d'antitoxine ainsi provoquée, soit de l'élaboration des antitoxines au sein de l'organisme". In other words, RAMON thought that adjuvants could be used as a tool to gain new insights into the mechanism of antibody response. FREUND (1956), without giving any definition, emphasized the different manifestations of adjuvant effect:

- (a) enhancement of antibody formation and alteration of sensitization to proteins;
- (b) sensitization to simple chemical compounds;
- (c) allergy.

Munoz (1964) defined an "adjuvant" as a substance that enhances the antibody response to antigen injected either simultaneously with it or within a period of time close to the injection of the antigen. This meaning is extended to all substances that enhance hypersensitivity reactions that are directly related to antibodies, or suspected to be associated with the antibody response. WHITE [1967 (1)] applied the term "adjuvant" (L. *adjuvare*: to help) only to substances which, when injected together with antigen (i) convert an apparently nonantigenic substance to an effective antigen, (ii) increase levels of circulating antibody, (iii) lead to the production of delayed hypersensitivity or to its increase, and (iv) lead to the production of certain disease states such as thyroiditis, aspermatogenesis, allergic encephalomyelitis, adrenitis or arthritis and iridocyclitis.

As we have seen from the different definitions, the manifestations of adjuvant effects are numerous, and to the antibody response, delayed hypersensitivity and allergic diseases we could add homograft reactions, some growth processes, inducement of plasmocytoma, ascites, and interferon synthesis.

Although an adjuvant may literally help the immune response, we feel compelled to discuss opposite effects too, since such very well-known adjuvants as Freund's adjuvant have been observed to lower the immune response (JANKOVIC, 1963). More recently extensive work has been done on phytohemagglutinin adjuvant activity; some authors have concluded that these heterogeneous substances exert an enhancing effect on the immune response [GAMBLE, 1966 (2); SINGHAL *et al.*, 1967],

while others have observed an opposite effect (MARKLEY *et al.*, 1967; JASIN and ZIPP, 1968).

Therefore the definition of an adjuvant must have two aspects. From a practical point of view, as pointed out by WHITE [1967 (1)], it refers to substances enhancing the immune response, whatever this may be.

From a theoretical point of view it should refer to any substance which acts on (i) the nonspecific part of the antigen, called "adjuvanticity" by DRESSER (1961), and (ii) the nonspecific activity of the cells involved in the immune response (mainly macrophages and lymphocytes) by enhancing cell multiplication or by stimulating cell transformation.

It is obviously difficult to make a clear distinction between such products as (i) phytohemagglutinin, which is able to enhance DNA, RNA and protein synthesis besides possessing blastogenic capacity; (ii) Freund's adjuvant, which stimulates hormonal secretions in addition to its activity on macrophages and lymphocytes; (iii) such hormones as somatotropin or folliculin, which enhance protein synthesis (including synthesis of globulin); and (iv) mitotic drugs, which will not be included as adjuvants. Thus we could define as adjuvants or immunity-stimulating substances *any product which acts (i) on a haptan or an antigen by enhancing its antigenic properties, or (ii) on the cells involved in the immune response (this being understood as including antibody synthesis, anaphylaxis, delayed hypersensitivity, allergic diseases, and graft reactions immunized).*

RAMON [1925 (1)] noticed a correlation in immunized horses between a local abscess at the site of antigen injection and a high level of antibodies; he demonstrated that it was possible to artificially increase diphtheria or tetanus antitoxin levels by adding substances such as bread crumbs, aleurone seeds, agar, tapioca, starch oil, lecithin.

SORDELLI and SERPA (1925) reported the antigenic value of the precipitate that occurs when diphtheria toxin and specific antitoxin are mixed together. HARTLEY (1952) and GLENNY (1926) pointed out that such a precipitate was antigenic and had a higher immunizing value than the supernatant liquid. Lipovaccines were discovered by LE MOIGNIC and PINOY [1916 (1,2)]. LEWIS and LOOMIS (1924) observed that antibody formation against various antigens was remarkably intense in guinea pigs which had received an injection of living virulent tubercle bacilli into the peritoneal cavity a few days before they were given antigens. These lipovaccines were also used by RAMON and ZOELLER (1927), WALSH and FRAZER (1934), COULAUD (1935), and SAENZ (1937).

With these observations as a foundation, FREUND (see FREUND *et al.*, 1937) started his classic experimentation on the production of delayed hypersensitivity and on antibody synthesis which showed that the two effects of the allergic irritability due to the tuberculous infection, namely the enhancement of antibody production and the alteration of sensitization, can be reproduced in the absence of tuberculosis (FREUND and MCDERMOTT, 1942). FREUND reviewed these observations in 1947 and 1956.

The powerful antibody-stimulating effect of Freund's adjuvant has not been equalled by any other adjuvant. This mixture has made it possible to produce antibodies even in animals considered poor producers, such as rats (HAVAS and ANDRE, 1955) or mice (ANACKER and MUNOZ, 1961; MUNOZ, 1963), and to induce auto-allergic diseases (KABAT *et al.*, 1946; MORGAN, 1946). The next step of the investi-

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