through zero and all correlation coefficients exceeded 0.97. Error was less than ± 5.0%. The lines were reproducible; calibration lines from individual runs done under similar conditions on different days were identical. An example of the measurements and variation of results is shown in figure 2. These results show that even under the marginal conditions of low luminal pressure and low vascular constriction (i.e. when the concern is poor electrode contact), reliable, repeatable calibrations can be achieved if care is exercised.

This in situ apparatus was designed to be applied to an isolated canine gracilis vein at the end of an acute experiment using the gracilis to study skeletal muscle ischemia. Although the animal can survive since only a small aliquot of blood is required to fill the system, the vein is destroyed because of a need to cannulate both ends. Therefore, this system is not suited to chronic work or repeated calibrations in the same location. Another potential problem is the sedimentation of red cells in the system, producing inhomogeneity of hematocrit at the probe. However, flow through the small reservoir is great enough to ensure adequate mixing, and this difficulty was not encountered.

In conclusion, the apparatus described employs a low volume, constant pressure reservoir to provide a range of flow rates in an in situ gracilis vein at low luminal pressures. This has allowed repeatable, reliable calibration of our electromagnetic flow equipment for use on gracilis veins in vivo. The apparatus is simple in design, inexpensive, and can be used on virtually any small vessel in situ.

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Enzyme immunometric assay for the determination of pregnancy associated plasma protein A (PAPP-A) with the antigen as solid phase (conjoint IEMA)

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Summary. Purified pregnancy associated plasma protein A (PAPP-A) can be effectively bound to polystyrene microtitre plates. This immobilized antigen competes with the added serum PAPP-A of unknown concentration for the limited amount of peroxidase-labeled monospecific anti-PAPP-A antibody incubated simultaneously. The sensitivity is 0.1 WHO unit/ml and non-specific binding is 1.0%.
Key words. Serum, pregnancy; placental proteins; enzyme immunoassay.

Pregnancy associated plasma protein A (PAPP-A) is one of the new generation of placental proteins. This large glycoprotein (M_r 730,000) was first described by Lin et al.[2] and has been purified[3]. Its concentration in the maternal circulation increases as pregnancy advances[4]. In order to elucidate the function of this protein, various assays have been developed, beginning with a modified Laurell immunoelectrophoresis[5]. The low sensitivity of this method restricted its use to measurements in late pregnancy. Later radioimmunoassays (using labeled PAPP-A)[6-8] and immunoenzymometric assays (ELISA, using labeled antibody)[9,10] have been developed to increase the sensitivity. The above ELISA's are two-site (sandwich) immunoassays where immunoglobulin is bound to the solid phase. Here the efficient adsorption of the antigen itself (PAPP-A) to polystyrene plates and the determination of PAPP-A by competition of the latter with the solid phase for a limited amount of peroxidase-labelled antibody is described (Conjoint immunoenzymometric assay, CIEMA).

Materials and methods. PAPP-A was purified from a pool of late pregnancy (32–36 weeks) serum as described elsewhere[3], but the ConA-Sepharose and negative affinity chromatography steps were replaced by chromatography on heparin-Sepharose (Pharmacia, Uppsala, Sweden) as reported by Davey et al.[11]. Eluted PAPP-A was pooled and stored at −20 or −70 °C. The activity of this fraction was measured by immunoelectrophoresis[5] and its protein content determined[12]. Polystyrene microtitre plates (96 wells, Kontron Ltd, St. Albans, England) were coated with this PAPP-A fraction at various dilutions (see results) in sodium carbonate buffer, 0.05 M, pH 9.2. Incubation was 24 h at room temperature. Blocking of remaining sites and the subsequent washes were performed as described[10]. The assay itself was run by incubating the test serum or standard (0.1 ml) together with the diluted enzyme-antibody conjugate (see results) in sodium phosphate, 0.01 M, pH 7.0; NaCl, 0.14 M; Tween-80, 0.05% v/v; normal donkey serum, 10% v/v (0.1 ml) in a PAPP-A-coated well. The absence of cross-reaction with various other proteins has been demonstrated for this conjugate[10]. It is important that the standard or test serum is added to the well containing immobilized PAPP-A before the addition of the conjugate, or that the 2 solutions are added to the well immediately after mixing in a separate tube. This is in order not to favor the soluble over the insoluble PAPP-A to be encountererd first by the antibody. The incubation was 4 h at 37 °C. The wells were then washed with 4 × 0.3 ml of 0.9% NaCl containing Tween-80 (0.1% v/v). Then the amount of peroxidase bound in each well was determined with orthophenylene-diamine as chromogen at pH 5.0 as described earlier in detail[10]. The absorption was measured in an automatic microplate reader (Kontron SLT-210, 486.1 nm). Nonspecific binding was determined in wells not coated with PAPP-A, and zero values were obtained by using 0.9% NaCl or a pool of male serum as test sample in coated wells. All measurements throughout were performed in triplicate.

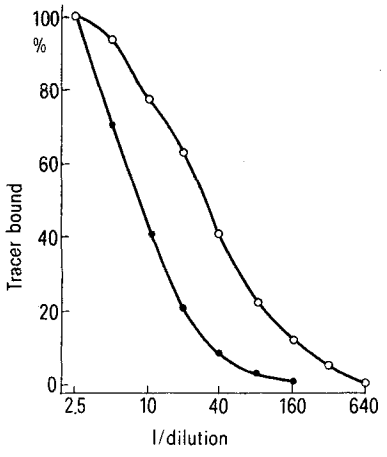

Figure 1. Enzyme-antibody conjugate dilution curve. The wells (0.2 ml capacity) were coated with PAPP-A at 640 ng (○) and 25.6 ng (●) of PAPP-A per well. Serial 2-fold dilutions of conjugate between 1:2.5 and 1:640 were used.

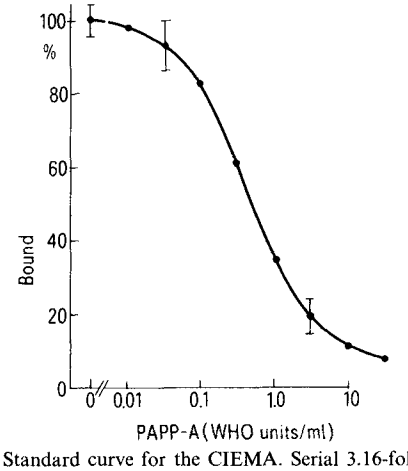

Figure 2. Standard curve for the CIEMA. Serial 3.16-fold dilutions of standardized pregnancy serum between 0.01 and 31.6 WHO units/ml were used. Conjugate dilution was 1:40, and the initial coating was 256 ng PAPP-A per well.

Results. A binder dilution curve was established by adding the conjugate at increasing serial 1:2 dilutions (from 1:2.5 to 1:640) to PAPP-A-coated wells in the absence of free PAPP-A. The curves for 2 concentrations of solid-phase PAPP-A are shown in figure 1. At the highest concentration of conjugate (1:2.5), the optical absorptions were 12.2 ± 0.3 OD for 640 ng and 0.072 ± 0.006 OD for 25.6 ng of solid-phase PAPP-A per well. In order to find an optimal compromise between the requirement of about 50% binding, the convenience of OD-values between 0.1 and 1.0, and the sensitivity of the assay required, a conjugate dilution of 1:40 (i.e. 2.5 µl/well) and coating concentration (solid-phase) of 256 ng PAPP-A per well (1.28 µg/ml) were selected.

Standards were made up using a pool of late pregnancy serum previously standardized against the WHO preparation. The highest standard was made up to 31.6 WHO units/ml in male serum and then serially 1:3.16 ($\sqrt{10}$) diluted into male serum down to the lowest standard at 0.01 WHO units/ml. One hundred µl of these standards were incubated with 100 µl of enzyme-antibody conjugate (diluted 1:40). The resulting standard curve is shown in figure 2.

Two patients in early pregnancy were followed between 5 and 18 weeks from the last menstrual period. Their serum was used without dilution (100 µl). Figure 3 shows the increasing inhibition observed with advancing pregnancy. Male serum alone (instead of pregnancy serum) gave a reading of 1.64 ± 0.07 OD. The same in a well not coated with PAPP-A gave 0.017 ± 0.001 OD, i.e. non-specific binding was 1.03%.

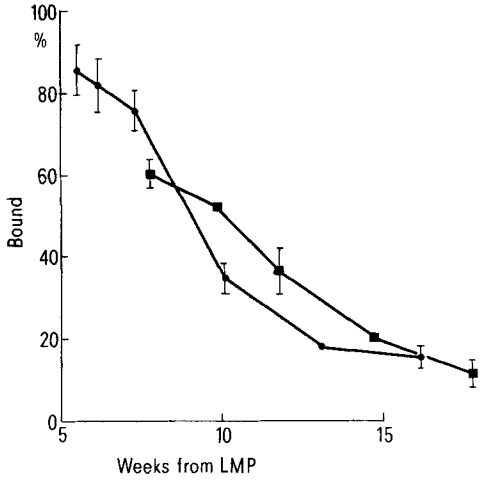

Figure 3. Increasing inhibition with advancing pregnancy. Each curve represents a patient. For conditions, see figure 2.

Discussion. This conjoint immunoenzymometric assay (CIEMA) was found to have a sensitivity around 0.03 WHO units/ml which is similar to the characteristics of the two-site IEMA (ELISA). However, the simultaneous incubation of all immunoreactants shortens the total assay time considerably and involves fewer manipulations. This is counterbalanced by the disadvantage that biological fluids might contain enzyme inhibitors and proteolytic enzymes affecting the conjugate in simultaneous incubation. Under our conditions (4 h at 37°C), no significant serum-dependent modification of peroxidase activity could be detected. Nevertheless, some sera might contain abnormal factors under pathological conditions. The CIEMA requires only 1 antigenic determinant per antigen molecule. This makes it the method of choice when, in contrary to the PAPP-A described here, small analyte molecules are measured. It then comes down to the question whether it is possible to immobilize the small antigen molecule to the solid phase without losing antigenic activity, and covalent coupling might be necessary. The CIEMA can be run with a monoclonal antibody. This gives a higher specificity to the method but limits possible increases in sensitivity since the antibody, as in the immunoassays involving labeled analyte, is not used in excess. Binding therefore depends on the affinity of the antibody which is often weak in the case of monoclonals. Absorbed monospecific polyclonal antibodies are usually satisfactory, but in practice it depends whether sensitivity or specificity is the predominant requirement. For large molecules like PAPP-A, it depends on several circumstances whether a CIEMA or a two-site IEMA should be used. Both assays can easily be run by the same laboratory since the same equipment and the same enzyme-antibody conjugate can be used in both systems.

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Announcements

Corrections

J.M. Lipman, B.J. Hicks and *L. Sokoloff:* Rabbit chondrocytes are binucleate in auricular but not articular cartilage, Experientia *40* (1984) 553-554. The following table was inadvertently omitted.

Binucleate chondroctytes in relation to age

Age (months)	% Binucleate Articular	Auricular
4	0.0	13.5
5	n.d.	24.2
18	0.25	21.0
48	0.25	n.d.

n.d., not determined; n = 400 cells.

L.C.J. Young and J. Horn: Pathological changes in the heterologous phase of antibasement membrane antibody mediated disease in the rat, Experientia *40* (1984) 579-581. The following table was inadvertently omitted.

Summary of time course binding of heterologous antibodies to various target organs as detected by direct immunofluorescence and immunoperoxidase techniques

Type of antibody given	Time after antibody administration 0.5–48 h Anti-GBM	Anti-ABM	Anti-SBM	2 days–4 weeks Anti-GBM	Anti-ABM	Anti-SBM	10 weeks Anti-GBM	Anti-ABM	Anti-SBM
Target organs									
GBM	++++	++++	+++	++++	++	++	+++	+	+
ABM	++	++	+	++	–	–	–	–	–
SBM	–	–	–	–	–	–	–	–	–
CBM	–	–	–	–	–	–	–	–	–

GBM, glomerular basement membrane; ABM, alveolar basement membrane; SBM, salivary basement membrane; CBM, choroid plexus basement membrane; + to ++++, increasing intensity of antibody binding; * Results of direct immunofluorescence are identical to those obtained by immunoperoxidase technique.

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