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Immunoenzyme Assay for Human Prostatic Acid Phosphatase (40690)

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Determination of circulating acid phosphatase (EC 3.1.3.2) has been a useful procedure, primarily in the assessment of metastatic carcinoma of the prostate (1, 2). Since the demonstration of the distinct antigenic specificity of human prostatic acid phosphatase (PAP) by Shulman and his co-workers (3), several immunochemical assay methods for this enzyme have been introduced and found to be superior to the enzymatic activity assay method for the diagnosis of prostatic cancer (4–10). During the elaboration of a radioimmunoassay based on the Farr method (11), we realized that it is possible to measure the enzyme activity (as well as the radioactivity) of ^{125}I-labeled PAP in ammonium sulfate-precipitated antigen–antibody complexes. This approach is comparable to the histochemical demonstration of enzyme activity in the immune complexes in radial immunodiffusion (5) and immunoelectrophoresis (4, 9, 10). This modified Farr technique (11), which we call immunoenzyme assay (IEA), has an advantage over radioimmunoassays (6–8) in its simplicity and speed. Counterimmunoelectrophoresis (9, 10) is basically a qualitative method while IEA is quantitative. In a preliminary screening of clinical serum samples for the PAP determination, excellent correlation was demonstrated between IEA, double-antibody radioimmunoassay, and counterimmunoelectrophoresis.

Materials and methods. Chemicals. Saturated ammonium sulfate [$(NH_4)_2SO_4$] was prepared in double-distilled water as a stock solution and stored at 4° with crystals remaining in the flask to prevent the solution from becoming supersaturated. Disodium *p*-nitrophenylphosphate was obtained from Calbiochem (San Diego, Calif. 92112). All of the assay tubes (10 × 75 mm, glass) were siliconized to reduce the nonspecific binding of acid phosphatases. Up to 8% of the enzyme binds to the glass wall without siliconization. Siliclad (Clay Adams, Parsippany, N.J. 04054) was used for the siliconization of glass tubes according to the instructions of the manufacturer.

Serum specimens. Normal human sera were obtained from volunteers among the laboratory staff and medical students. Over 200 normal sera were collected from apparently healthy male and female volunteers. Some clinical serum specimens (70) which showed normal range of serum acid phosphatase values by routine spectrophotometric assay at clinical laboratories were obtained from the Departments of Obstetrics and Gynecology and Oncology. The sera of prostatic carcinoma patients (50 males; age 57–72, average age 56) were provided by the Department of Urology, Wayne State University–Detroit Medical Center. Sera were stored frozen at −20° until they were assayed. Those human materials were obtained with informed consent; the WSU Human Experimentation Committee approved this study.

Standard prostatic acid phosphatase. The prostatic acid phosphatase was purified from pooled human semen. Methods of purification and physicochemical characterization for this enzyme have been described previously (12). The PAP preparation used in this study was free from any extraneous proteins judged by immunochemical criteria, was homogeneous in terms of molecular weight (M_r 100,000), and had specific enzyme activity of 500 units/mg protein. One unit of enzyme catalyzed the hydrolysis of 1 μmol of *p*-nitrophenylphosphate per minute. Concentrations of purified enzyme solutions were determined by absorbance measurement at 280 nm, assuming $E^{1\%}_{1\,cm} = 14.4$ (13). Kinetic parameters of purified PAP have been described previously (12).

Anti-prostatic acid phosphatase sera (anti-

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PAP). Preparation and the specificity of anti-PAP sera have been reported previously (14, 15). A batch of rabbit anti-PAP serum, R14, was used in this study and the concentration of specific antibodies were estimated by quantitative precipitin and radioimmunoassay at 100 μg antibody N/ml.

Immunoenzyme assay (IEA). For testing either plasma or serum specimens, standard PAP solutions were prepared in a batch of normal human female serum of which the absence of PAP was established by the radioimmunoassay method. Serial dilutions of purified PAP were made to yield 250, 125, 62.5, 31.2. 15.6, and 7.8 ng PAP/ml. These PAP standard solutions were distributed in siliconized assay tubes in a volume of 100 μl per tube and stored frozen until use. Tubes containing 100 μl of PAP-free female serum served as the control.

Rabbit anti-PAP serum, R14, and a batch of unimmunized normal rabbit serum, NR6, were diluted 10-fold in phosphate-buffered saline (PBS), pH 7.2. To one of the triplicate assay tubes containing 100 μl of either standard PAP or clinical serum samples, 100 μl of diluted NR6 was added. This series of tubes served as the control group. To two of the triplicate assay tubes, 100 μl of diluted anti-PAP serum, R14, was added and this series of tubes served as the assay group.

These tubes were then incubated at 4° for 60 min and 100 μl of cold 100% saturation of $(NH_4)_2SO_4$ solution (767 g/liter) was added to each tube to precipitate the antigen–antibody complexes. After an additional 60-min incubation at 4°, the tubes were centrifuged at 4° for 15 min at 1500*g* to sediment the antigen–antibody complexes.

After removing the supernatants, the pellets were dissolved in 100 μl of 0.1 *M* acetate or citrate buffer, pH 5.0, at room temperature by vortex mixing. In most of the assays, the pellet dissolved completely to yield a clear solution. These dissolved immune complexes were warmed for 2 min at 37° in a heating block or water bath. The enzyme assay was initiated by the addition of 50 μl of 40 m*M* *p*-nitrophenylphosphate to each tube, and the enzyme reaction was allowed to proceed for 4 min at 37°. The enzyme reaction was stopped by the addition of 0.7 ml of 0.2 *M* NaOH. It is imperative to follow the rigid time course for each assay tube to obtain reproducible accurate assay data. The resulting *p*-nitrophenol concentration was determined by the absorbance at 410 nm. A standard correlation curve was established by plotting the absorbance (410 nm) at each endpoint against the mass of PAP in each corresponding tube.

Other biochemical and immunological assay. Serum acid phosphatase of each sample was assayed at the clinical laboratory before they were referred to this laboratory. Clinical laboratories at Wayne State University–Detroit Medical Center adopted the automated procedure of Roy *et al.* (16) for the measurement of serum acid phosphatase. In this laboratory, each clinical sample was assayed for PAP by a double antibody radioimmunoassay and a counterimmunoelectrophoresis and the details of the assay procedures were reported previously (8, 10).

Results and discussion. In a previous investigation, we confirmed the immunologic specificity of the PAP-anti-PAP reaction (14, 15). Human acid phosphatases remain soluble at 33% saturation of $(NH_4)_2SO_4$ (15), whereas PAP-anti-PAP immune complexes are quantitatively precipitated at this concentration of $(NH_4)_2SO_4$ (Fig. 1). Coprecipitation of unbound PAP and of nonprostatic acid phosphatases at 33% saturation of $(NH_4)_2SO_4$ was 7–10%. The primary antigen–antibody interaction reaches nearly 80% of the equilibrium level within the first hour of incubation (Fig. 2). Therefore, a short incubation (1-hr) period was chosen for all of the following experiments. Other investigators (11, 17, 18) have demonstrated that antibody does not release, exchange, or bind appreciable amounts of antigen after the addition of $(NH_4)_2SO_4$. However, the concentrations of antibody are critical for the quantitative recovery of immune complexes in this technique (Fig. 3). Under the conditions of antibody excess, over 80% of antigen was recovered as immune complexes during 1-hr incubation at 4°. Therefore a 10-fold dilution of anti-PAP was used in the standard assay.

In the standard assay method, immune complexes are precipitated by 33% saturation of $(NH_4)_2SO_4$ and the amount of PAP in the complexes are estimated by the average velocity of enzymatic activity. In separate ex-

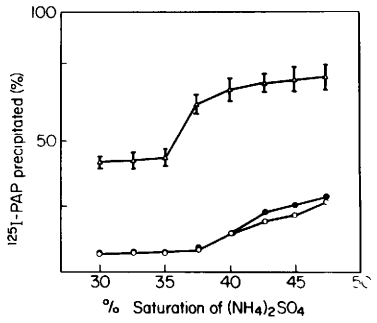

FIG. 1. Salting out of PAP by $(NH_4)_2SO_4$ in the presence of immunoglobulins. A series of tubes containing 100 μl of ^{125}I-labeled PAP (10 ng/ml) were incubated with equal volume of diluted rabbit sera (1:500), either anti-PAP serum, R14 (△), anti-human-serum-albumin serum (●), or normal serum, NR1 (○) for 1 hr at 4°. At the end of incubation, 100 μl of $(NH_4)_2SO_4$ was added to the final concentration of $(NH_4)_2SO_4$ of 1.33–1.9 *M* which was equivalent to the 30–47.5% saturation of $(NH_4)_2SO_4$. After centrifugation, the radioactivity of the pellet was counted and the amount of ^{125}I-labeled PAP precipitated was expressed as the fraction of the total ^{125}I-labeled PAP added. Each point with bars represents the average of five determinations and the ± 2 SD range.

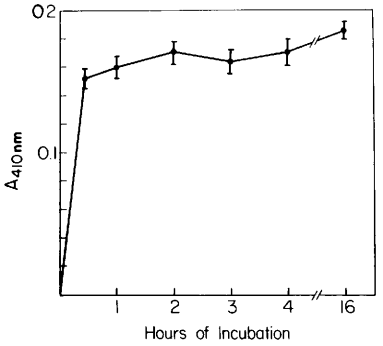

FIG. 2. The time course of the binding of PAP to anti-PAP antibodies. One hundred microliters of PAP (30 ng/ml) and anti-PAP serum, R14, (1:10) were incubated at 4° for various periods, the resulting antigen–antibody complexes were precipitated by $(NH_4)_2SO_4$, and the amounts of PAP in the complexes was estimated by enzyme assay. Each point with bars represents the average of five determinations and the ± 2 SD range.

periments, the catalytic activity of PAP was found to be unaffected either by $(NH_4)_2SO_4$ (up to 2.0 *M*) or by the presence of antibodies. The residual amounts of $(NH_4)_2SO_4$ carried through the precipitation and resuspension of antigen–antibody complexes may not affect the enzymatic activity of PAP. Figure 4 illustrates the correlation between the PAP concentration and its enzymatic activity. Twenty consecutive runs of standard PAP dilutions yielded linear correlation curves with identical slope and intercepts. The overall performance of this technique is shown in Table I.

Critical conditions in this assay are: (a) temperature, (b) substrate concentration, and (c) background enzymatic activity in the normal rabbit control tubes. All of the assay tubes were equilibrated to minimize the effects of temperature, and the time intervals for the addition and mixing of substrate were kept identical from tube to tube. Probably these steps would limit the handling of many

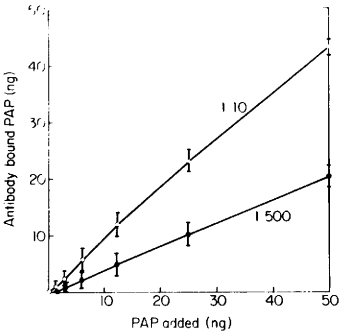

FIG. 3. Effect of anti-PAP antibody concentration in the PAP-anti-PAP interactions. To a series of tubes containing 100 μl of various concentrations of PAP (0–500 ng/ml), an equal volume of diluted (1:10 or 1:500) R14 serum was added, the immune complexes were precipitated by $(NH_4)_2SO_4$ at the end of incubation, and the enzyme activity of each pellet was determined. The masses of recovered PAP were calculated from the enzyme activity and the specific enzyme activity of the original PAP. Each point with bars represents the average of six determinations and the ± 2 SD range.

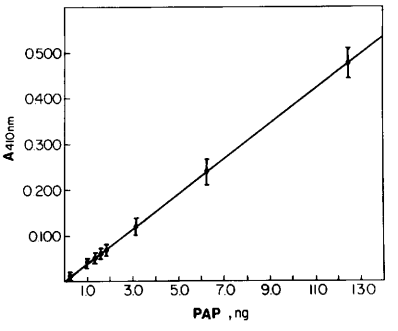

FIG. 4. Standard curve for IEA for PAP. Each point with bars represents the average of 20 independent determinations and the ± 2 SD range. The least squares slope is 0.0368. Details of the assay method are described under Materials and Methods.

TABLE I. ASSAY PERFORMANCE OF IEA FOR PAP

1. Sensitivity: Lowest detectable dose—4 ng/ml
2. Reproducibility: Variation within repeated estimates in different assays

Sample	No. of assays	PAP (ng/ml)[a]	CV (%)
L	20	23 ± 7.0	12.5
P	20	38 ± 4.5	4.1
G	20	124 ± 7.0	2.4

3. Accuracy: Closeness of measurements to the true value

Sample	Std. PAP added (ng PAP)	No. of assays	Recovery (%)[a]	CV (%)[b]
NH	3	24	94 ± 11.8	4.5
NH	6	24	98 ± 10.3	6.6

[a] Mean ± standard deviation (SD).
[b] Coefficient of variation (CV).

samples at one time unless the system could be automated. As described in the previous papers (12, 14, 15), the K_m of PAP isozymes for *p*-nitrophenylphosphate range from 4.1 to 6.0×10^{-4} *M*. Therefore, approximately 20-fold K_m was chosen as the substrate concentration, and the initial velocities of the enzymatic (0.5 to 25 ng) activity showed linearity under this substrate concentration.

The complication of this assay was a high background enzymatic activity associated with certain control serum samples. High background enzymatic activity was found with two types of specimens. Specimens with high plasma protein concentration seemed to give rise to slight turbidity after resuspension of the immune complexes after the $(NH_4)_2SO_4$ incubation and this turbidity interfered with the absorbance reading. Specimens containing a high concentration of acid phosphatases tend to give rise to a significant amount of residual acid phosphatase to the $(NH_4)_2SO_4$ precipitates of normal rabbit γ-globulins and caused high absorbance reading of the control tubes. The former complication could be solved by clarification of assay tubes by a brief centrifugation. The latter complication was corrected by the repeated assays with variously diluted samples and/or by washing the antigen–antibody complexes in the 33% saturation of $(NH_4)_2SO_4$. The hemolytic or lipemic sera did not affect the results of the IEA method.

In other experiments, we tried a second antibody method (goat anti-rabbit IgG antibodies) to precipitate soluble immune complexes to compare the quantitative recovery of immune complexes. The second antibody method gave comparable results. However, the method required a longer incubation period and was more expensive.

In order to investigate the correlation of PAP values between the IEA method and the double-antibody RIA, we assayed 40 normal male sera (age 48–66) and 25 clinical serum samples of female patients (Departments of Obstetrics and Gynecology and Oncology; age 35–55) for serum PAP levels by both methods. The normal serum PAP values were 10.5 ± 6.5 ng/ml (range 2.5 to 68 ng/ml) for males and 3.5 ± 2.8 ng/ml (range 2.5 to 9.5 ng/ml) for females. These values are comparable to the normal value (16.0 ± 8.0 ng/ml; range 2.5 to 48 ng/ml) we have reported for 162 normal adult males (8). The normal serum PAP values were 10.0 ± 4.4 ng/ml (range 0.00 to 37 ng/ml) for males and 10.5 ± 4.5 ng/ml (range 0.00 to 18 ng/ml) for females by the IEA method. Therefore, the normal values of serum PAP for males and females by the IEA method are comparable to those obtained by the double-antibody RIA. Fifty serum specimens from patients diagnosed as having prostatic carcinoma in various clinical stages were assayed for PAP by the double-antibody RIA and by the IEA method. With few exceptions (7 out of 50), assay values correlated closely (Fig. 5). It is not clear at this moment whether the discrep-

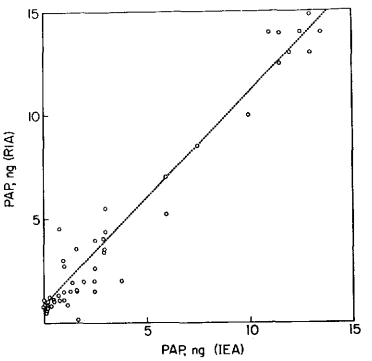

FIG. 5. Unknowns in serum specimens of prostatic cancer patients. Comparison between the IEA and the double-antibody RIA. The equation of the regression line is $y = 1.02x + 0.82$ (y = the double-antibody RIA of Choe *et al.* (8), x = the IEA method, $r = 0.9993$, $n = 50$, $p < 0.0025$).

ancies in these seven cases reflect the occurrence of variant of PAP synthesized by tumor cells or suggest differential inactivation of various PAP isozyme subpopulations by each antiserum. Theoretically, RIA can measure proteins which are immunologically cross-reactive with PAP but devoid of any enzyme activity. Traditionally, enzyme levels are determined as activity rather than as protein. Activity measurement is proportional to the enzyme protein only when the kinetic parameters of enzyme molecules are identical. It is not established yet that kinetic parameters of acid phosphatases from various sources, including neoplastic tissue, are identical. Actually their K_m and V_{max} could be widely different. Therefore, it is possible that the two immunoassay values may not correspond to each other in all cases.

IEA offers several advantages over the RIA method in terms of safety, simplicity, and cost. Problems inherent in the RIA method are the short shelf life of radioisotope-labeled antigen, relatively lengthy assay steps, spillage of radioactive reagents, and cost of assay. On the other hand, the IEA method has a few unique problems which will limit its general application. When a large number of samples were assayed manually for enzymatic activity, experimental errors are magnified by improper temperature equilibration, mixing of substrate, and inconsistency of incubation period. Automation may increase the precision of the assay. Furthermore, there is a possibility that some of the antibodies might block the catalytic site of the enzyme molecule and so could not be used in the technique described.

Summary. Human sera or plasma containing acid phosphatases were mixed with rabbit antiserum specific for prostatic acid phosphatase (PAP) and the resulting primary immune complexes were precipitated by ammonium sulfate. The amount of specifically precipitated PAP was estimated directly by its enzyme activity. Using this assay, as little as 10 ng/ml of the PAP could be determined. Excellent correlation of this assay method with other immunoassays of PAP was demonstrated.

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