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A NEW ASSAY FOR PROSTATIC ACID PHOSPHATASE IN SERUM

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Inasmuch as very low levels of prostatic acid phosphatase in serum must be detected in order to diagnose prostatic carcinoma before extensive metastases have developed, it is necessary that the method of analysis be accurate and relatively specific for prostatic acid phosphatase. One factor that contributes greatly to the inaccuracy of most assays for acid phosphatase in serum is the large nonenzymatic control that results from preformed chromogenic substances in the serum or substrate. Most of these methods also suffer from the fact that they measure total acid phosphatase in serum instead of only that portion arising from prostatic tissue. The present method is not only simple and fast, but it also has a small control and is essentially specific for prostatic acid phosphatase. It is based on the estimation of alpha-naphthol liberated in the enzymatic hydrolysis of alpha-naphthyl phosphate, by coupling with tetrazotized orthodianisidine and measuring the resulting azo dye colorimetrically. This substrate has been used in the histochemical localization of acid phosphatase,[10] for identifying semen on clothing,[11] and for estimating urinary acid phosphatase.[1]

REAGENTS

*Buffer-substrate.** The tablet contains 0.67 mg. of sodium alpha-naphthyl acid phosphate in a mixture of citrates designed to yield a pH of 5.2 in the reaction mixture.

*Color developer.** The tablet contains 0.4 mg. of tetrazotized orthodianisidine in a stabilized form.

Physiologic saline solution. NaCl, 0.85 per cent.

Standard alpha-naphthol in serum.† Reconstitute lyophilized vial with 1.0 ml. of distilled water. Alternatively, the standard may be prepared as follows: dissolve exactly 94.5 mg. of pure alpha-naphthol in 10 ml. of ethanol in a 100-ml. volumetric flask and dilute to 100 ml. with water. Dilute this standard 1:10 with normal serum. The diluted standard contains 9.45 mg. of alpha-naphthol per 100 ml.

PROCEDURE

Place 1 tablet of buffer-substrate in a suitable container calibrated at 5.0 ml. It was observed that 15- by 150-mm. test tubes calibrated at 5.0 ml. are very satisfactory. Dissolve the tablet in 0.5 ml. of water and warm to 37 C. in a water-bath. Add exactly 0.2 ml. of serum, mix by lateral shaking, and incubate at 37 C. for exactly 30 min. Remove the tube from the water-bath and cool it in a beaker of water at 15 to 20 C. At the same time add 1 tablet of color developer and dissolve it by crushing with a glass rod. (To add the serum, it is convenient to use pipets calibrated to contain 0.2 ml. The pipet, that is left in the tube, can be used to crush the tablet.) Wash off the glass rod and dilute to the 5.0-ml. mark with water. Transfer the solution to a cuvet and read the optical density at 530 mμ, using water as a blank, exactly 3 min. after the addition of the tablet of color developer. When several serums are assayed, it is convenient to start

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* These reagents are available for quantitative and semiquantitative use as Phosphatabs, Acid, from Warner-Chilcott Laboratories, Morris Plains, New Jersey.

† Available as a service from Warner-Chilcott Laboratories, Morris Plains, New Jersey.

the samples at intervals of 2 min. Determine a serum blank by diluting 0.2 ml. of serum to 5 ml. with physiologic saline solution and reading the optical density at 530 mμ.

Determine the optical density of the standard exactly as for the unknown serums. No incubation is required for the standard.

CALCULATIONS

The unit of acid phosphatase is defined as the amount of enzyme that will liberate 1 mg. of alpha-naphthol per hr. (This unit is equivalent to approximately 0.18 Bodansky units.)

In the incubated samples it is necessary to correct for the color that is not due to alpha-naphthol liberated by acid phosphatase. This may be done by running a control in which the tablet of color developer is added immediately after the serum; the resulting color of the diluted mixture is read exactly 3 min. later. A simpler procedure for color correction, however, was devised. The control color originates from 3 sources: (1) the serum, (2) the reagents, and (3) a reaction between the proteins in serum and the diazonium salt. The contribution of the first source is deducted by subtracting the serum blank from the total color. The contribution of the other 2 sources has been determined by measuring the difference between the optical densities of the total control and the serum blank. In 40 serums (including lipemic, hemolyzed, and icteric samples), this color was found to be equivalent to 1.1 ± 0.2 units (approximately 0.2 Bodansky units) with extreme values of 0.6 and 1.6 units. Because of the small variability of this value, satisfactory results are obtained by subtracting only the serum blank from the incubated sample optical density, and then subtracting 1.1 units from the calculated activity.

The standard contains 9.45 mg. of alpha-naphthol per 100 ml. Inasmuch as the time of incubation is 30 min., this quantity of alpha-naphthol corresponds to 18.9 units, and the total color, minus the serum blank, is equivalent to 18.9 + 1.1, or 20.0 units. The activity of acid phosphatase of the unknown serum is calculated as follows:

$$\left(\frac{\text{Optical density of unknown} - \text{blank}}{\text{Optical density of standard} - \text{blank}}\right) 20 - 1.1 = \text{units per 100 ml.}$$

RESULTS

Limits of assay. Serums of up to 40 to 50 units can be assayed by the above procedure. Inasmuch as the rate of reaction is constant, the range of the assay can be increased by a factor of 2 or 3 by decreasing the incubation to 15 or 10 min. An incubation time of less than 10 min. is not practical. After the development of color, the samples may be diluted 2- or 3-fold by adding an additional 5 or 10 ml. of water. If they must be diluted more than 3-fold to be read, it is recommended that the assay be repeated with the sample of serum diluted, inasmuch as the azo dye may precipitate if the concentration is very high. Variations in the concentration of protein from 4 to 8 per cent do not affect the color produced, but, inasmuch as protein is necessary to keep the azo dye in solution, highly active serums can not be diluted with water or saline. Phosphatase-free serum is the ideal diluent. Serum left at 37 C. for a few hours, or overnight at room temperature, is essentially free of acid phosphatase.[12]

Correlation with other assays. Thirty-one serums from patients with prostatic involvement were assayed by 4 methods: (1) the present method, (2) the modification of Shinowara, Jones, and Reinhart[6] of the procedure of Bodansky,[4] (3) the adaptation of Gutman and Gutman[5] of the assay of King and Armstrong,[8] and (4) the method of Bessey and his associates[3] as modified by Hudson and his associates.[7] In some instances 2 or more serums were pooled to yield samples large enough for all assays. The results of the analyses are listed in Table 1 in increasing degree of activity as determined by the present method. It is apparent that all the methods were in substantial agreement except for the modified method of Bessey-Lowry. It has been demonstrated[2] that *p*-nitrophenyl phosphate, the substrate for this method, is hydrolyzed

TABLE 1

A COMPARISON OF ASSAYS FOR ACID PHOSPHATASE IN SERUM

Serum Number	Present Method	Bodansky	King-Armstrong	Bessey-Lowry
	units/100 ml.	*units/100 ml.*	*units/100 ml.*	*units/l.*
1	1.9	0.1	2.4	0.6
2	2.8	0	2.3	0.8
3	4.6	0.4	4.6	1.3
4	5.0	0	5.9	2.1
5	5.1	0.6	3.8	0.8
6	5.8	1.1	4.7	0.7
7	6.3	0.7	5.1	0.9
8	6.5	0.8	5.4	0.9
9	6.6	1.1	5.2	0.7
10	6.9	0.6	7.8	2.3
11	8.1	1.1	5.2	0.8
12	8.9	1.1	5.3	0.8
13	9.4	1.3	6.3	1.1
14	11.0	1.6	6.4	0.9
15	12.0	2.4	7.5	1.2
16	12.1	2.1	8.4	1.4
17	12.4	0.9	14.4	3.9
18	12.5	2.1	8.1	1.4
19	13.9	2.5	9.1	1.3
20	22.6	4.4	13.0	1.8
21	22.6	4.9	14.0	1.6
22	28.8	4.3	15.3	2.4
23	29.5	4.8	16.3	2.6
24	30.0	5.8	16.7	2.1
25	30.4	5.9	16.7	2.0
26	41.0	8.1	24.4	2.8
27	41.2	7.4	22.1	2.6
28	47.9	9.4	27.1	3.0
29	72.4	14.4	45.8	5.1
30	75.6	19.3	70.3	6.2
31	162.6	30.3	73.3	10.0

more readily by erythrocytic than by prostatic acid phosphatase. The lack of agreement between the Bessey-Lowry and the other methods suggests that the hydrolysis of *p*-nitrophenyl phosphate was primarily due to acid phosphatase not of prostatic origin.

The relation between the various units was determined by plotting the data obtained by one assay against those of another. One Bodansky unit was observed to be equal to approximately 5½ of our units. To convert our units to those of King and Armstrong, it is necessary to divide our units by 2 and add 2. It was observed that serum contains an average of 2 King-Armstrong units of apparent activity of acid phosphatase, not of prostatic origin, that the Bodansky and the present method do not measure.

Normal range of assay. The average activity of prostatic acid phosphatase in the serum of 56 apparently normal, healthy men was 2.0 ± 0.7 units per 100 ml., with extremes of 0.9 and 5.5. The average of 33 apparently normal women was 1.5 ± 0.5 units per 100 ml., with extremes of 0.5 and 2.6. If the normal range is defined as the average ± 2 standard deviations, it would be 0.6 to 3.4 units per 100 ml. Comparison of the values of the present method with the accepted upper limit of the normal range of the assays of Bodansky and King and Armstrong would suggest an upper limit of normal of approximately 5 units per 100 ml. Observed values of more than 5 units should, therefore, be looked upon with suspicion.

DISCUSSION

Seligman and co-workers described an assay[9] for phosphatases in serum using beta-naphthyl phosphate as the substrate. These authors used this isomer because the azo dye formed with beta-naphthol was more readily extractable with ethyl acetate than the azo dye formed with alpha-naphthol. We have observed that with their method a significant amount of azo dye remains bound to the precipitated protein. In the present method, the conditions are such that the azo dye forms a solution that is optically clear, and the tedious steps of protein precipitation and ethyl acetate extraction are avoided.

The present method was found to be 40 to 100 times as specific for prostatic acid phosphatase as all methods studied, except for the modification of Shinowara, Jones, and Reinhart of the method of Bodansky.[2] The procedure of Bodansky, however, involves many time-consuming manipulations and the use of unstable reagents. In addition, the relatively large control considerably increases the error in the determination.

SUMMARY

A new method for the determination of prostatic acid phosphatase in serum is pre-

sented. The method is based on the coupling of alpha-naphthol liberated from alpha-naphthyl phosphate with tetrazotized orthodianisidine. The method is simple and rapid, and the reagents are available in convenient, stable form. The method is specific for prostatic acid phosphatase.

SUMMARIO IN INTERLINGUA

Es presentate un nove methodo pro le determination de phosphatase acide prostatic in sero. Le methodo es basate super le accopulamento de alpha-naphthol (liberate ab phosphato de alpha-naphthyl) con orthodianisidina tetrazotisate. Le methodo es simple e rapide, e le reagentes requirite es disponibile in forma stabile e convenibile. Le methodo es specific pro phosphatase de acido prostatic.

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