

# A METHOD FOR THE DETERMINATION OF TARTRATE-LABILE, PROSTATIC ACID PHOSPHATASE IN SERUM

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L(+)-Tartrate strongly inhibits prostatic acid phosphatase (Abul-Fadl and King, 1948, 1949) while having no action on red cell phosphatase. The authors suggested the application of this phenomenon and its diagnostic value as a sensitive test in elucidating doubtful or borderline cases of prostatic cancer. Based on this principle, Fishman and Lerner (1953) described a method for the determination of serum acid phosphatase of prostatic origin. This method requires larger amounts of serum, besides involving the precipitation and separation of proteins, and is more complicated and time-consuming than the method in use here.

The method now described is the result of further work on the tartrate-labile phosphatase component and is a modification of the amino-antipyrine (A.A.P.) methods in use in this laboratory (Kind and King, 1954). For borderline cases it probably gives a truer measure of the prostatic part of the serum acid phosphatase than does the formol-stable fraction. A somewhat similar procedure has been described by Davis and Ward (1956).

## Reagents

**Citrate Buffer.**—Crystalline citric acid, 21 g., is dissolved in water, 188 ml. of *N*-NaOH added, the *pH* adjusted to 4.9 by cautious addition of *N*-NaOH or *N*-HCl, and made up to 500 ml. Preserve with a few drops of chloroform in the refrigerator.

**Substrate** (0.01 *M*-Disodium Phenyl Phosphate).—For the substrate, dissolve 2.18 g. 0.01 *M*-disodium phenyl phosphate in 1 litre of freshly boiled and cooled distilled water and preserve with a few drops of chloroform in the refrigerator.

**Stock Phenol Standard** (1 mg./ml.).—Pure crystalline phenol, 1 g., is dissolved in a litre of 0.1 *N*-HCl and preserved in the refrigerator.

**Dilute Phenol Standard** (0.01 mg./ml.).—One millilitre of stock standard diluted to 100 ml. with distilled water is preserved with a few drops of chloroform in the refrigerator.

**0.5 *N*-Sodium Hydroxide.**—This is stored in a polythene bottle and filtered when necessary.

**0.5 *M*-Sodium Bicarbonate.**—Sodium bicarbonate, 4.2 g., is dissolved in water and made up to 100 ml. The solution is stored in a polythene bottle and filtered when necessary.

**4-Amino-antipyrine** (A.A.P., 0.6%).—4-Amino-antipyrine, 0.6 g., is dissolved in water and made up to 100 ml. This solution should be stored in a brown bottle in the refrigerator and filtered before use.

**A.A.P. Reagent.**—A mixture of 0.5 *M*-NaHCO<sub>3</sub> and 0.6% 4-amino-antipyrine is made in the proportion of 1:1. The mixture is stored in the refrigerator in a brown bottle and is stable for a month.

**Potassium Ferricyanide.**—This reagent is made up as 2.4 g. per 100 ml. in water and kept in a brown bottle.

***M*-Tartrate.**—L(+)-Tartaric acid (AnalaR), 15 g., is dissolved in about 70 ml. of water, 18.5 ml. of 10 *N*-NaOH added, the *pH* adjusted to 4.9 and made up to 100 ml. Stored in the cold room (4° C.) or refrigerator with a few drops of chloroform, this solution is stable. A few crystals of Na-H-tartrate may form if the temperature goes below 4° C.: these will re-dissolve on slight warming.

It is essential that all solutions used are optically clear.

## Experimental

**Concentration of Tartrate.**—Experiments were carried out with diluted prostate extracts and seminal fluid to determine the optimum molarity of tartrate necessary completely to inhibit the prostatic component of acid phosphatase in the ranges usually encountered in blood plasma, including the higher levels found in metastatic carcinoma of the prostate. The dilutions were made with citrate buffer, as the diluted enzyme is more stable at the acid *pH*. The acid phosphatase activities of suitably diluted prostate extracts and seminal fluid were determined along with the activities in buffer-substrate mixtures containing tartrate of varying concentrations to give final molarities for tartrate ranging from 0.1 *M* to 0.0001 *M*. The buffer substrate mixtures were prepared by serial dilution of the stock molar tartrate with citrate buffer.

The results are shown graphically (Fig. 1). It is clear that for acid phosphatase activities in the ranges

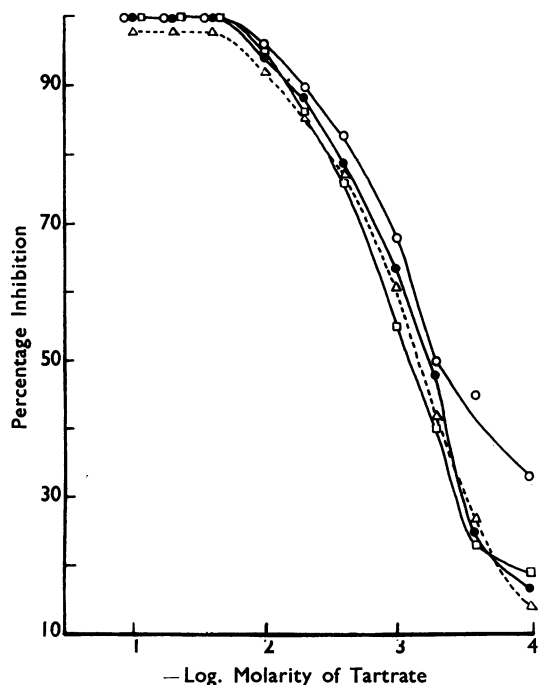


FIG. 1.—Tartrate inhibition of prostatic acid phosphatase. ○—○—○ diluted prostate extract (18 K.-A. units); ●—●—● diluted prostate extract (49 K.-A. units); □—□—□ diluted seminal fluid (131 K.-A. units); Δ—Δ—Δ serum from a patient with metastatic carcinoma of prostate (30 K.-A. units total, 29.2 units prostatic).

usually encountered in prostatic cases a final concentration of tartrate of about 0.025 M in the substrate-buffer-enzyme mixture is the optimum to give complete inhibition of the prostatic acid phosphatase, under the conditions of the test. Increasing the concentration of the tartrate beyond this serves no purpose (see also under Discussion). This was confirmed by a similar experiment on a serum of high prostatic acid phosphatase activity from a case of carcinoma of the prostate with metastases. The inhibition curve for serum follows the same pattern as the curves obtained for diluted prostate extract or seminal fluid. There is no further inhibition for molarities of tartrate greater than 0.025 M.

Recovery experiments in which diluted prostate extract or seminal fluid was added to several specimens of serum also confirmed the above finding, i.e., that a final concentration of about 0.025 M-tartrate was the optimum for complete inhibition.

**Stability of Acid Phosphatase.**—Under many conditions prostatic acid phosphatase is a very unstable enzyme. An aqueous prostatic extract loses its activity very rapidly if left out at room temperature. The same thing is true of the total, as of the prostatic, acid phosphatase activity of serum. A serum from a case of prostatic carcinoma with metastases, which had total and prostatic acid phosphatase activities of 62 and

60 units respectively while fresh, gave total and prostatic activities of only 1 and 0 respectively after standing at room temperature (ca. 20° C.) for four days. This almost complete destruction of enzyme can occur sometimes in as little as 24 hours, when serum is left at room temperature. A specimen which had an activity of 76 units showed an activity of only 4 units when left at room temperature for just over 24 hours. In another case a serum which had an extremely high activity of 956 units came down to 180 units in 48 hours. Daniel (1954) found that the pH of serum changes from 7.4 immediately after separation to 7.5 in 2 min., 7.8 in 5 min., 8.0 in 15 min., 8.4 or more in 24 hours, when left at 24° C. Serum in contact with its clot remained at pH 7.4 for a considerable time, and under these conditions the acid phosphatase was stable at temperatures below 30° C. for periods up to 24 hours.

The formol-stable as well as the tartrate-labile acid phosphatase activity of serum left in the refrigerator immediately after separation appears to be practically the same as that of serum allowed to stand with its clot at room temperature for 24 hours (Table I). The total acid phosphatase increases when the serum is allowed to stand with its clot, due to leakage of enzyme from the red cells even in the absence of any visible haemolysis.

It was found that, in general, the activity is retained for a sufficiently long time to complete the analysis, provided the serum is stored immediately after separation in the refrigerator and there has been no contamination. On the other hand, every minute the separated serum is left at room temperature the pH rises and the stability of the enzyme decreases. Sera which are freshly separated and immediately used for enzyme determination, and then left in the refrigerator, usually give slightly lower values on the following day probably due to the time the specimen is left out during the analysis.

TABLE I  
STABILITY OF ACID PHOSPHATASE (AT 0° C. AND -15° C. SEPARATED FROM CLOT, AND AT ROOM TEMPERATURE IN CONTACT WITH CLOT, KING-ARMSTRONG ACID PHOSPHATASE UNITS)

Serum No.		Total	Formol-stable	Tartrate-labile	
1	Fresh	1.5	1.3	0.3	
	24 hr. {	0° C.	1.4	1.2	0.4
		+20° C.	2.1	1.3	0.4
2	Fresh	2.1	1.6	0.3	
	24 hr. {	0° C.	1.9	1.7	0.3
		+20° C.	2.5	1.7	0.3
3	Fresh	1.6	1.3	0.3	
	24 hr. {	0° C.	1.6	1.2	0.3
		+20° C.	2.0	1.4	0.2
4	Fresh	2.3	1.8	0.3	
	24 hr. {	0° C.	2.1	1.8	0.1
		+20° C.	2.4	1.7	0.3
5	24 hr. {	0° C.	2.0	2.0	1.0
		+20° C.	2.8	2.4	1.1
	Fresh	—	96	—	
6	5 days {	0° C.	104	96	98
		-15° C.	102	100	96
		0° C.	—	104	—
14 ,, {	0° C.	—	108	—	
	-15° C.	—	—	—	

**Choice of Diluent in Case of High-activity Sera.**—With high-activity sera, dilution with saline, normal serum, or very low activity serum gives practically the same value, provided the determination is made immediately after dilution. Some typical results (Table II) also show that there is enzyme inactivation during incubation at 37° C. It is, however, preferable to dilute the serum with the citrate buffer (pH 4.9), as the enzyme is more stable at room temperature at this pH, and inactivation caused by short delays after making the dilution is thereby reduced. The alternative method of using undiluted high-activity sera for shorter incubation periods is also useful, e.g., the result of a 10 min. hydrolysis multiplied by 6 is taken to be equal to hydrolysis for one hour, i.e., units.

TABLE II  
ACID PHOSPHATASE OF DILUTED HIGH-ACTIVITY SERUM  
(UNITS PER 100 ML. OF UNDILUTED SERUM)

Period of Incubation	Diluent	
	Saline	Serum
0.5 hour	648	644
1.0 "	632	600
1.5 "	588	560

**Short Cuts in Original A.A.P. Method.** — The original A.A.P. method (Kind and King, 1954) requires the further addition of three reagents for the development of colour after the enzyme reaction is stopped by the addition of NaOH. Two of these can be mixed for use as a single reagent, viz., the A.A.P. and the bicarbonate, and this gives the same results as the original procedure.

**Method for Total and Tartrate-labile Acid Phosphatase**

Serum is separated within a few hours of clotting. For best results the estimation should be done immediately. If this is not possible, the serum should be put in the refrigerator immediately after separation and the estimation carried out (preferably) within 24 hours.

Three tubes are prepared (A=total, B=tartrate, C=control) each containing 1 ml. of buffer and 1 ml. of substrate. To tube B is added a drop (0.05 ml.) of m-tartrate (final concentration 0.023 M). The tubes are placed in a water-bath at 37° C., and after about 3 min. 0.1 ml. of serum is accurately measured into tubes A and B at timed intervals (say 30 sec.), mixed well, and the incubation is allowed to proceed for exactly one hour. (Shorter incubation periods are used for high activity sera.) The hydrolysis is stopped by the addition of 1 ml. of 0.5 N-NaOH to A, B, and C, and mixed well. Now 0.1 ml. of serum is added to the control tube C, and 2 ml. of A.A.P. reagent is added to each tube, followed by 1 ml. of ferricyanide. The contents of the tubes are mixed well after each addition.

**Standard.**—This is made up of 1.1 ml. buffer + 1 ml. of dilute standard phenol solution + 1 ml. 0.5 N-NaOH + 2 ml. A.A.P. reagent + 1 ml. ferricyanide.

**Blank.**—The blank is the same as the standard, except for water in place of the phenol solution.

The colours develop immediately and are stable for at least one hour if kept in dim light. They are read in a photoelectric colorimeter, with a green light filter (Ilford 624, 510 mμ).

**Calculation.**—This is as follows :

Total acid phosphatase (in King-Armstrong units per 100 ml.):

$$\frac{A - C}{\text{Standard} - \text{blank}} \times 0.01 \times \frac{100}{0.1}$$

$$= \frac{A - C}{\text{Standard} - \text{blank}} \times 10$$

Tartrate-labile acid phosphatase (in King-Armstrong units per 100 ml.):

$$\frac{A - B}{\text{Standard} - \text{blank}} \times 10$$

**Notes.** — For the estimation of tartrate-labile phosphatase alone, the control C is not necessary, but the simultaneous determination of the total acid phosphatase is valuable, and hence the inclusion of the control tube C.

Controls C<sup>1</sup>, similar to C but with the addition of a drop of m-tartrate, gave the same reading as C and hence it has not been necessary to include a control for tartrate.

Instead of the addition of a drop of m-tartrate, 1 ml. of substrate and 1 ml. of a buffer prepared by appropriately diluting m-tartrate with citrate buffer to give a final molarity for tartrate of 0.025 could be used for tube B. The former, however, is preferable as it makes the method simpler and more convenient. The dilution caused by the addition of the drop in B is, for practical purposes, almost negligible and does not affect the results beyond the limits of experimental error.

As nearly equal values for A and B are usual in normals and most clinical cases, since the normal tartrate-labile fraction is very low, it is generally preferable to run A and B in duplicate and to repeat doubtful or border-line cases.

Formol-stable acid phosphatase may be included by the addition of a further tube D which is identical with A except for the addition of 0.05 ml. of neutralized 20% formaldehyde, adjusted to pH 4.9.

Formol-stable acid phosphatase =  $\frac{D - C}{\text{Standard} - \text{blank}} \times 10$ .

**Results**

The total and tartrate-labile acid phosphatases were determined by the above method in a total of 65 normals (43 males and 22 females), the blood being provided from blood donors arranged through the Blood Transfusion Services. Formol-stable acid phosphatase was also determined in 26

TABLE III  
TOTAL AND TARTRATE-LABILE SERUM ACID  
PHOSPHATASE OF NORMAL PERSONS

	No.	Total Acid Phosphatase			Tartrate-labile Acid Phosphatase		
		Mean	S.D.	Range	Mean	S.D.	Range
Males	43	1.80	0.61	1.0-4.0	0.33	0.19	0.0-0.8
Females	22	2.04	0.51	1.3-3.1	0.35	0.14	0.0-0.6
Totals	65	1.88	0.59	1.0-4.0	0.335	0.18	0.0-0.8

King-Armstrong acid phosphatase units.

TABLE IV  
TOTAL, FORMOL-STABLE, AND TARTRATE-LABILE SERUM  
ACID PHOSPHATASE OF NORMAL PERSONS

No.	Total Acid Phosphatase			Formol-stable Acid Phosphatase			Tartrate-labile Acid Phosphatase		
	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.	Range
26	1.71	0.41	1.0-2.5	1.00	0.31	0.5-1.8	0.34	0.23	0.0-0.8

of these bloods. The mean values, S.D., and range of values are set out in Tables III and IV.

In most prostatic cases the formol-stable acid phosphatase has given a reliable indication as to the diagnosis, i.e., as to whether there is malignancy or not (compare Wray, 1956; King, 1957), but in a few cases the results have been equivocal and in these the tartrate-labile result has agreed with the final diagnosis. Other conditions, such as carcinoma of the breast, in both female and male, have often given results above the normal by both total and formol-stable procedures, but have nearly always yielded figures within the normal range for the tartrate-labile acid phosphatase. A further statement will be given in a future paper; a few representative results are given here (Table V).

TABLE V  
NON-PROSTATIC CASES OF ELEVATED TOTAL AND  
FORMOL-STABLE ACID PHOSPHATASE WITH NORMAL  
TARTRATE-LABILE PHOSPHATASE

Case, Age, and Sex	Units of Acid Phosphatase per 100 ml. Serum		
	Total	Formol-stable	Tartrate-labile
Carcinoma of breast:			
H. 52 M .. ..	{ 15.2	{ 12.3	{ 0
O. 72 F .. ..	{ 10.4	{ 6.6	{ 1.2*
R. 64 F .. ..	{ 10.3	{ 7.4	{ 0.1
F. 44 F .. ..	{ 7.2	{ 4.4	{ 0.1
L. 58 F .. ..	{ 12.4	{ 10.4	{ 0.4
	{ 6.0	{ 5.0	{ 0
Carcinoma of bronchus:			
M. 52 F .. ..	6.2	4.9	0
Jaundice:			
H. 19 M .. ..	5.4	4.6	0.2
B. 28 F .. ..	5.2	4.4	0.8*

\* Above normal.

## Discussion

When modifications of the King-Armstrong procedure (1934) using Folin-Ciocalteu reagent were tried for estimating the tartrate-labile component, it was found necessary to include an additional control tube C<sup>1</sup> for tartrate. This control generally gave slightly higher readings than C, and the tartrate-labile component had to be calculated on the basis (A-C)-(B-C<sup>1</sup>). This was emphasized when increasing concentrations of tartrate from 0.012 M upwards were tried, when it was found that the difference between C and C<sup>1</sup> also increased. This means that the tartrate by itself produces a certain amount of colour with the Folin-Ciocalteu reagent. The Fishman and Lerner method (1953) does not appear to take this factor into account. The final tartrate concentration in the Fishman and Lerner method is 0.019 M, but this has been increased to get optimum concentrations for tartrate. On the whole it was found that the use of Folin-Ciocalteu reagent, with the precipitation and separation of proteins which follow, makes the method more tedious and time consuming.

With the A.A.P. reagent, however, the method has been made much easier and simpler. The procedure has also been shortened by mixing the A.A.P. and bicarbonate before addition.

The p-nitrophenyl phosphate method (Ohmori, 1937; King and Delory, 1939; Bessey, Lowry and Brock, 1946) has also been tried to see how far it is advantageous. It was found that though the rate of hydrolysis with p-nitrophenyl phosphate is slightly faster at the acid pH, yet this is not appreciable enough to cut down the incubation time. The method requires no further addition of reagent beyond that of NaOH to stop the enzyme reaction, but the yellow colour produced is an unsuitable colour for ordinary photoelectric colorimetry. A quartz spectrophotometer ("unicam" SP 500) had to be used for the readings, and much of the time saved on the addition of reagents was spent in manipulating this instrument.

It was found that with the formaldehyde method the colour produced with A.A.P. is not so stable as it is with the total or tartrate-labile acid phosphatase. The colour increases slightly with time in the presence of formaldehyde and attains a somewhat different shade of rose-pink than those without formaldehyde at the end of about half an hour. The error, however, was negligible if a control tube was run with formaldehyde and the readings taken within 30 min.

The formol-stable fraction, however, is not such a sensitive index for prostatic acid phosphatase as

the tartrate-labile component. Several cases have been encountered, especially in carcinomas other than of the prostate, where the formol-stable fraction was raised in the presence of a normal tartrate-labile component (Table V). We are also assessing the value of the tartrate-labile component as determined by the above method in elucidating doubtful or border-line cases of prostatic carcinoma, especially those in which a normal formol-stable acid phosphatase is obtained.

Our experience on the stability of serum acid phosphatase is in agreement with the findings of Daniel (1954). We do not, however, favour the idea of allowing the serum to stand with the clot, as this increases the total acid phosphatase due to leakage of the enzyme from the red cells (Table I). Davison (1953) found that a serum from a case of metastatic carcinoma of the prostate retained its high activity for periods up to 112 days in the deep-freeze ( $-15^{\circ}\text{C}$ ). We find that serum acid phosphatase is equally stable at  $0^{\circ}\text{C}$ . for periods up to 14 days, provided the serum is put in the refrigerator immediately after separation (Table I).

As regards the normal range of tartrate-labile or prostatic acid phosphatase (Table III), in a total of 65 apparently healthy adult subjects (43 males and 22 females) only one male gave a value as high as 0.8 units, two males 0.7 units, and one male and two females 0.6 units. All the rest gave values below 0.6 units. We would fix 0.7 units as the upper limit of normal for prostatic acid phosphatase. This is two standard deviations (0.18) from the mean (0.335), and is 0.1 unit higher than that proposed by Fishman *et al.* (1956) and by Davis and Ward (1956), and is in accordance with the value proposed by Nobles *et al.* (1957). The distribution for prostatic acid phosphatase was negatively skewed. We would consider prostatic conditions which give values in excess of 0.7 units as suspicious.

Of the total acid phosphatase values, there were only two above 3 units (one male 4 units and one female 3.1 units) and of the 26 cases where formol-stable acid phosphatase was also done (Table IV), none above 1.8 units. There had been a trace of haemolysis in some of the specimens, and this may account for the slightly higher mean for the total

acid phosphatase for females. We do not think that there is any significant difference in the mean values for males and females whether for total acid phosphatase or for the tartrate-labile component. As the mean values for tartrate-labile acid phosphatase are practically the same for both males and females it is probable that this "prostatic" component in normal persons arises from sites other than the prostate. Haemolysis does not affect the tartrate-labile acid phosphatase, as the red cell enzyme is stable to tartrate.

### Summary

A simple method for the determination of tartrate-labile acid phosphatase using phenyl phosphate and an amino-antipyrine reagent is described. It is shown that the optimum concentration of tartrate necessary is about 0.025 M in the buffer-substrate-enzyme mixture for the complete inhibition of the prostatic component in blood serum. It is also shown that serum acid phosphatase is unstable at room temperatures but stable at  $0^{\circ}\text{C}$ .

The total and tartrate-labile acid phosphatases have been determined in 65 normal persons, and the formol-stable acid phosphatase in 26 of them. An upper limit of 0.7 King-Armstrong units is proposed for the tartrate-labile acid phosphatase in normal subjects.

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