

# THE DETERMINATION OF SERUM ACID AND ALKALINE PHOSPHATASE ACTIVITY WITH 4-AMINOANTIPYRINE (A.A.P.)

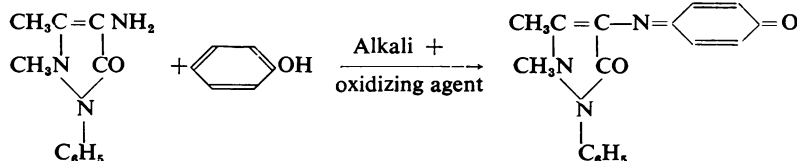
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Phenolic compounds give red or purple colours with 4-aminoantipyrine (A.A.P.) in the presence of alkaline oxidizing agents (Emerson, 1943). The amino group of the A.A.P. condenses with the phenol to give a substance which is oxidized to a coloured quinone.



Gottlieb and Marsh (1946), using alkaline ferricyanide as the oxidant, devised a method for the estimation of certain phenolic fungicides with A.A.P. The procedure was modified by Grifols (1951) for the determination of phenol in the measurement of alkaline phosphatase activity in body fluids. This author made a careful study of the factors which control the development of the colour and his results showed good correlation with those obtained by the method of King (1951). Advantages claimed for the method were that the reagent did not react with proteins, thus obviating their precipitation during the estimation, and also the colour development was comparatively rapid. The use of A.A.P. as a reagent for phenol appeared to be of potential value especially if its use could be extended to the estimation of serum acid phosphatase activity. Considerable modification of the original Grifols procedure was found to be necessary in order to measure both alkaline and acid phosphatase activity in blood. The use of calibration curves, the stability of the colour, and the effect of the presence of serum on the colour development were also investigated. Results obtained with the proposed methods in a number of normal and pathological sera were compared with those obtained with King's methods.

## Experimental

**Reagents.**—4-Aminoantipyrine hydrochloride (Hopkin & Williams, Ltd.) was used without further purification. Potassium ferricyanide was of AR standard. The sodium carbonate/bicarbonate buffer, citric acid/sodium citrate buffer, and diphenyl phosphate substrate solution were prepared according to the directions of King (1951).

Standard phenol solutions were prepared by diluting a solution containing 1 g. of pure crystalline phenol in 1 litre of water. The phenol content of this latter solution was estimated with N/10 bromine (British Pharmacopoeia, 1953).

**The Estimation of Acid Phosphatase Activity.**—In acid solutions, oxidizing agents convert A.A.P. to the deeply coloured "antipyrine red" (Emerson, 1938) so that A.A.P. and ferricyanide cannot be used to estimate phenol produced after incubation of diphenyl phosphate substrate and serum in an acid buffer at pH 4.9. In the original method of Grifols for alkaline phosphatase the pH of the final mixture after the addition of M-10 carbonate was 10.2. The corresponding mixture of serum and substrate after incubation with a citric acid buffer in the acid phosphatase method was at pH 4.9, and 1 ml. of 22.5% w/v aqueous Na<sub>2</sub>CO<sub>3</sub> was found to be necessary to produce a final pH of 10.2. Table I shows the results obtained from a series of phenol solutions of concentrations ranging from 10 to 100 µg./ml. which were estimated by the following two procedures; the concentrations of A.A.P. and ferricyanide employed were those used by Grifols (1951).

Method A	Method B
2 ml. phenol solution	2 ml. phenol solution
2 " alkaline buffer	2 " acid buffer
1 " M-10 sodium carbonate	1 " 22.5% sodium carbonate
1 " 0.06% A.A.P.	1 " 0.06% A.A.P.
1 " 0.24% potassium ferricyanide	1 " 0.24% potassium ferricyanide

TABLE I  
COMPARISON OF OPTICAL DENSITIES USING METHODS  
A AND B

Phenol ( $\mu\text{g./ml.}$ )	Method A	Method B
10	0.090	0.060
15	0.135	0.095
20	0.180	0.125
25	0.220	0.160
30	0.265	0.185
40	0.330	0.230
50	0.390	0.245
75	0.520	0.370
100	0.660	0.455

The mixtures were allowed to stand at room temperature for 15 minutes and the optical densities measured in  $\frac{1}{2}$  cm. cells in the Spekker absorptiometer using Ilford spectrum green filters No. 604.

The results show that, although the pH of the mixtures after the addition of the carbonate solutions were identical, the optical densities in method B were approximately 30% lower than those using method A. It therefore appeared that the citrate present in the acid buffer interfered in some way with the production of the colour. A similar result was observed when acid buffers containing acetate or phthalate were used. Gottlieb and Marsh (1946) reported that attempts to utilize alkaline borate and glycine buffer mixtures in their work on the estimation of phenolic fungicides were not successful because the colour development was decreased.

On the empirical reasoning that the buffer ions in some way reacted with the reagents, it was decided to increase the concentrations of A.A.P. and ferricyanide in methods A and B described above. Table II summarizes this series of experiments, a 40  $\mu\text{g./ml.}$  phenol solution being used throughout.

TABLE II  
EFFECT OF INCREASING REAGENT CONCENTRATIONS

A. A. P. (g./100 ml.)	Ferricyanide (g./100 ml.)	Optical Density	
		Method A	Method B
0.06	0.24	0.320	0.230
0.12	0.48	0.460	0.235
0.24	0.96	0.580	0.244
0.50	1.0	0.631	0.383
1.0	2.0	0.647	0.497
1.0	4.0	0.650	0.559
1.5	4.0	0.625	0.563
1.5	6.0	0.627	0.560
2.0	2.0	0.490	0.354
2.0	6.0	Turbid solutions	

The results show that there was some improvement in the optical densities obtained with increasing amounts of reagents. However, the agreement with the results obtained with the two methods was still unsatisfactory. One point which emerged from the work was that the original Grifols concentrations did not give full colour development and were inadequate for amounts of phenol corresponding to phosphatase values above 100 units. If excess phenol were present it acted as a reducing agent converting the red

quinone to a colourless leuco compound. Fig. 1 illustrates this effect using increasing amounts of phenol and 0.06% A.A.P. and 0.24% ferricyanide.

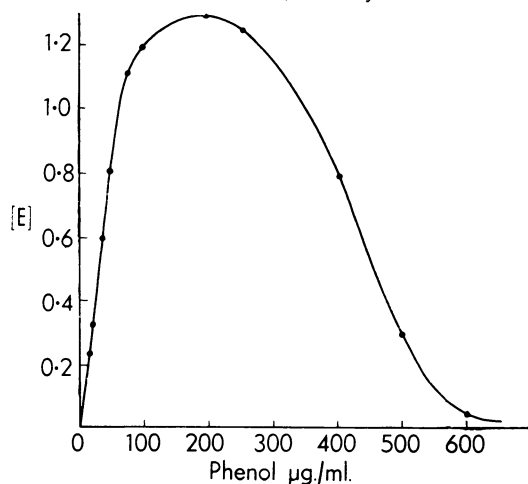


Fig. 1.—The effect of increasing the amount of phenol using 0.06% A.A.P. and 0.24% ferricyanide.

The only difference between the substances used in the alkaline and acid buffers and their subsequent treatment apart from the citric acid and citrate in the acid buffer was sodium bicarbonate. It was therefore decided to investigate the use of various carbonate/bicarbonate mixtures instead of 22.5% carbonate in method B. 4-Aminoantipyrine, 1.5 g./100 ml., and 4 g./100 ml. ferricyanide were used because the results in Table II indicated that these were the optimum concentrations of the reagents. Table III summarizes the results of these experiments using a 40  $\mu\text{g./ml.}$  phenol solution.

The results indicated that both the concentrations and ratio of the sodium carbonate and sodium bicarbonate in the mixture were important factors. Although the addition of 1 ml. of a solution containing 7.5 g./100 ml. of sodium carbonate and sodium bicarbonate gave optimum results, the addition of 2 ml. of the 4 g./100 ml. concentrations was preferred because the former mixture deposited crystals on standing at room temperature.

TABLE III  
EFFECT OF USING SODIUM CARBONATE/SODIUM BICAR-  
BONATE MIXTURES IN METHOD B\*

Composition of Mixture (g. 100 ml.)		Optical Density	
$\text{Na}_2\text{CO}_3$	$\text{NaHCO}_3$	Method A	Method B*
22.5	0	0.625	0.563
10	2.5	0.625	0.575
5	2.5	0.630	0.580
5	5	0.625	0.600
7.5	7.5	0.620	0.620
4†	4	0.545	0.550

\* The concentrations of A.A.P. and ferricyanide were 1.5% and 4.0% respectively.

† 2 ml. of this mixture added in Method B and 2 ml. of M-10 sodium carbonate added in Method A.

**Development and Stability of Final Colour.—**

Fig. 2 illustrates the increase in colour intensity with time of a phenol solution estimated by both acid and alkaline procedures. The colour with the alkaline method is fully developed in five minutes whereas with the acid method 15 minutes is necessary. The final colour is stable for at least two hours, but has diminished to approximately 90% of its maximum intensity in six hours.

**Final Method.**—The suggested procedures for acid and alkaline phosphatase estimations in serum are as follows:

Alkaline Phosphatase	Acid Phosphatase
Carbonate bicarbonate buffer, 2.0 ml.	Citrate buffer, 2.0 ml.
Diphenyl phosphate solution, 2.0 ml.	Diphenyl phosphate solution, 2.0 ml.
Serum, 0.2 ml.	Serum, 0.2 ml.
Incubate at 37° C. for 15 min., then add	Incubate at 37° C. for 60 min., then add
M-10 carbonate, 2.0 ml.	4% carbonate solution
1.5% A.A.P., 1.0 ml.	4% bicarbonate } 2.0 ml.
4% ferricyanide, 1.0 ml.	1.5% A.A.P., 1.0 ml.
	4% ferricyanide, 1.0 ml.

The alkaline phosphatase estimations are allowed to stand at room temperature for five minutes, and the acid tubes for 15 minutes (cf. Fig. 2). The optical densities are measured in a Hilger Spekker absorptiometer using Ilford spectrum green filters No. 604. The optical densities are read against control solutions prepared as above except that the serum is added after the incubation.

**Calibration Curves.**—Calibration curves as shown in Fig. 3 may be constructed by substituting 2 ml. of phenol solution varying in concentration from 5 to 100 µg./ml. for the substrate solution. In the present method one phosphatase unit is equivalent to that of King and Armstrong, i.e., 2 µg. of phenol liberated

from the substrate under the various incubation conditions; in the standard solutions used for the preparation of the calibration curves 1 µg./ml. of phenol equals 1 phosphatase unit. Each solution used for the calibration curves had 0.2 ml. serum added because the presence of serum decreases the optical density by 5 to 10% as shown in Table IV.

TABLE IV  
EFFECT OF SERUM ON COLOUR DEVELOPMENT

Phenol Solution* (µg./ml.)	Optical Densities			
	Water	Amount of Serum Added		
		0.1 ml.	0.2 ml.	0.4 ml.
10	0.148	0.140	0.135	0.130
15	0.220	0.220	0.210	0.204
20	0.298	0.289	0.291	0.273
30	0.438	0.442	0.420	0.408
40	0.592	0.565	0.558	0.534
50	0.731	0.690	0.683	0.670

\* The final volume of each solution was identical, the serum having been substituted for an equal volume of water.

**Comparison of Results with those Obtained by King's Methods.**—The results obtained with both the above methods and the methods of King (1951) for acid and alkaline phosphatase activities in a number of blood specimens are shown in Tables V and VI.

**Conclusion**

While the original method of Grifols was found to be adequate for the determination of alkaline phosphatase activity it could not be applied directly to the measurement of serum acid phosphatase activity because the anions present in the acid buffer solution interfered with the colour production. The procedure was modified by the addition of a bicarbonate/carbonate solution after the incubation and the use of much greater concentrations of A.A.P. and ferricyanide so that this interference due to the buffer anions was overcome. It has been possible to devise methods for the estimation of both types of phosphatase activity in serum using the same calibration curves. The presence of serum tends to decrease the colour intensity, and it is recommended that the calibration curves should be constructed with 0.2 ml. of serum in each estimation. This effect of serum was not studied by Grifols, who merely stated that A.A.P.

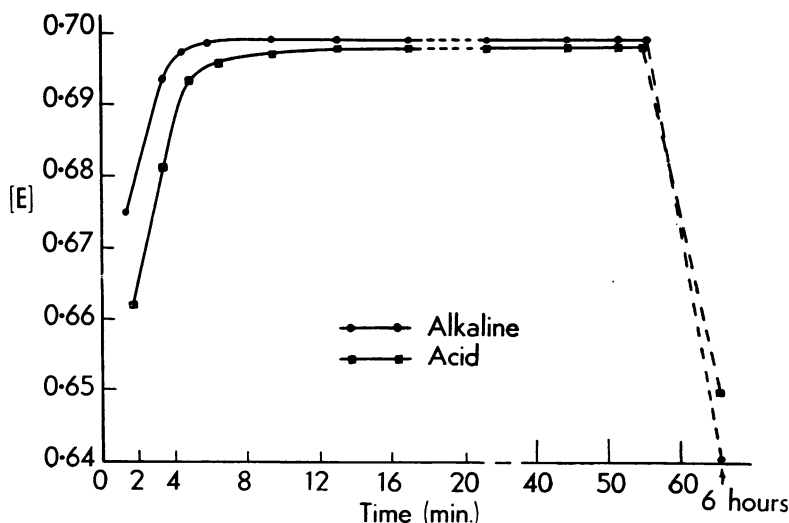


FIG. 2.—Rate of development and fading of final colour.

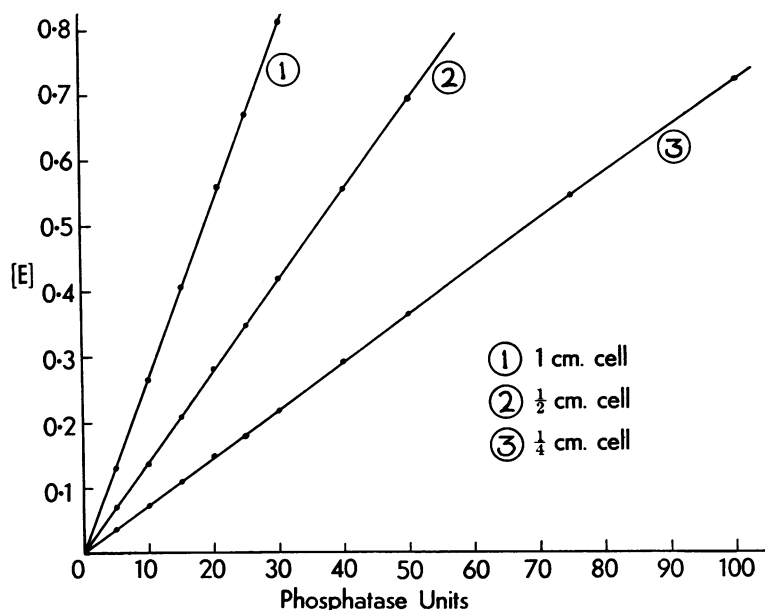


FIG. 3.—Calibration curves for both acid and alkaline phosphatase estimations using various sizes of optical cells.

did not react with plasma proteins.

The results obtained with the present methods in a number of sera from normal persons and patients suffering from jaundice and carcinoma of the prostate have been compared with the results obtained by the method of King (1951). A good agreement between the results was observed as is shown in Tables V and VI. In our opinion the present methods have several advantages over

those of King, notably in the speed and simplicity of manipulation. Serum proteins do not require precipitation, thus eliminating centrifugation and removal of a supernatant solution, and the final colour development does not involve an incubation. An estimation of serum alkaline phosphatase activity can be made in about 25 minutes, and the final colour is stable for several hours at room temperature.

### Summary

The use of 4-aminoantipyrine (A.A.P.) for the measurement of phenol in the final stage of the estimation of serum phosphatase activity has been studied.

Methods have been devised for the determination of both serum acid and alkaline phosphatase activity using the same calibration curves.

Results with these methods have been compared with those obtained using the methods of King.

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TABLE V  
COMPARISON OF METHODS FOR ESTIMATING SERUM ALKALINE PHOSPHATASE ACTIVITY

Case No.	Diagnosis	Phosphatase Activity (King-Armstrong Units)	
		King	Present Method
1	Normal adults	6	7
2		10	9
3		7	7
4		7	8
5	Normal child	20	19
6	Infective hepatitis	11	13
7		19	21
8		29	28
9		40	40
10	Obstructive jaundice	54	52
11		98	102
12		128	124
13	Carcinoma of pancreas—post-operative specimen	33	33
14	Paget's disease	30	30

TABLE VI  
COMPARISON OF METHODS FOR ESTIMATING SERUM ACID PHOSPHATASE ACTIVITY

Case No.	Diagnosis	Phosphatase Activity (King-Armstrong Units)	
		King	Present Method
1	Normal subjects	2	1
2		2	3
3		3	3
4		2	2
5	Carcinoma of prostate with metastases	26	27
6		70	68
7		42	42
8		17	18