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Invalidation of Plasma Ascorbic Acid Values by Use of Potassium Cyanide.

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Farmer and Abt[1] proposed a method for the determination of reduced ascorbic acid in blood plasma deproteinized by tungstic acid, which was titrated with 2,6-dichlorophenolindophenol. The method required 5 cc. of blood, "which is centrifuged immediately, the plasma removed, and a tungstic acid filtrate prepared." This quotation is given to emphasize the fact that the authors were aware of the rapid destruction of ascorbic acid in blood.[2] When the micro-method[3] requiring but 0.5 cc. of blood was developed, we recommended the substitution of metaphosphoric acid for tungstic acid as a deproteinizing agent. The dependence of plasma level upon the vitamin C intake was shown. Abt, Farmer and Epstein[4] applied these methods to the establishment of plasma values for normal individuals of various age groups as well as the variations encountered in clinical material.

In attempting to apply the macro-method, modified to the extent of substituting 10% for 5% metaphosphoric acid as a deproteinizing agent, Pijoan, Townsend and Wilson[5] report varying and inconsistent results in presumably normal individuals. They apparently overlooked the statement of Farmer and Abt as to the necessity of immediate analysis of the freshly drawn blood, and report losses in blood plasma permitted to stand for periods of 2 to 3 hours, at 26°C. and at 0° to 5°C.

In order to prevent this destruction of plasma ascorbic acid, Pijoan and Klemperer[6] and Pijoan and Eddy[7] recommended the

1 Farmer, C. J., and Abt, A. F., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1625.

2 Contrary to the statement of Schultz, M. P.: Discussion of paper of Rinehart, J. F., Greenberg, L. D., Baker, F., and Choy, F., *J. Am. Med. Assn.*, 1937, **109**, 1394.

3 Farmer, C. J., and Abt, A. F., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 146.

4 Abt, A. F., Farmer, C. J., and Epstein, I. M., *J. Pediat.*, 1936, **8**, 1.

5 Pijoan, M., Townsend, S. R., and Wilson, A., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 224.

6 Pijoan, M., and Klemperer, F., *J. Clin. Inv.*, 1937, **16**, 443.

7 Pijoan, M., and Eddy, *J. Lab. Clin. Med.*, 1937, **22**, 1227.

addition of potassium cyanide (with potassium oxalate) to the tubes used for blood collection. This is based on the observation of Euler, Myrbock, and Larsson[8] that the oxygen uptake of ascorbic acid solutions, as measured in the Barcroft-Warburg apparatus, gave "badly reproducible values" unless potassium cyanide (1 ml. 0.005 N KCN per 10 mg. ascorbic acid) was added. Euler, *et al.*, believed that the potassium cyanide precipitated traces of heavy metals present in the system, which were responsible for the erratic values. The catalytic effect of copper was confirmed by Barron, De Meio, and Klemperer,[9] and its catalysis of oxidation of ascorbic acid added to human blood serum shown in an article by Barron, Barron, and Klemperer.[10] According to one observation of the latter authors, using whole blood of the dog, the presence of red blood cells exerts no influence on the rate of oxidation.

We, therefore, welcomed the suggestion of Pijoan and associates that potassium cyanide could be used to prevent the rapid destruction of ascorbic acid in whole blood and plasma. After some experience with the method as given by Pijoan and Eddy[7] (10 mg. KCN for 10 cc. blood) and its proportionate use in the smaller samples as required for our micromethod, we found that the use of potassium cyanide gave enhanced ascorbic acid values; in fact, potassium cyanide in aqueous solutions of metaphosphoric acid decolorizes 2,6-dichlorophenolindophenol. This was overlooked by Pijoan and associates, otherwise metaphosphoric acid-potassium cyanide blanks would have been recommended as a routine titration procedure.

The recently proposed method of Mindlin and Butler,[11] employing a photoelectric colorimeter, depends upon the increased light transmission through a 2,6-dichlorophenolindophenol solution due to partial reduction by ascorbic acid. Since a photoelectric colorimeter is not available, we are not in a position to evaluate in this simple manner the reduction of the dye upon addition of cyanide, and therefore must depend upon visual perception of the endpoint as obtained by titration. In the collection of blood, Mindlin and Butler add one drop of 5% KCN (approx. 2.5 to 3 mg.) to tubes

8 Euler, H. v., Myrbock, K., and Larsson, H., *Z. f. Physiolog. Chem.*, 1933, **217**, 1.

9 Barron, E. S. G., DeMeio, R. H., and Klemperer, F., *J. Biol. Chem.*, 1935-36, **112**, 625.

10 Barron, E. S., Barron, A. G., and Klemperer, F., *J. Biol. Chem.*, 1937, **116**, 563.

11 Mindlin, R. S., and Butler, A. M., *J. Biol. Chem.*, 1938, **122**, 673.

receiving 4 to 5 cc. of blood. It is obvious that a suitable blank should be obtained, taking this factor into account.

By a study of the reaction velocity between metaphosphoric acid plasma filtrates and 2,6-dichlorophenolindophenol buffered to pH 4.1, Minton and Butler find that no significant error in evaluating ascorbic acid is introduced by reductions caused by glutathione or cysteine within one-half minute. In the usual titration procedure, the unbuffered dye is added to the metaphosphoric acid plasma filtrate, which has a reaction about pH 2, thereby eliminating interference from -SH compounds.

Experiment I. The decolorization (blue to pink) of 2,6-dichlorophenolindophenol by potassium cyanide in 2.5% metaphosphoric acid solution.

In these experiments, the dye solution had an equivalent of 0.02 mg. ascorbic acid per cc. For titration, 0.2 cc. of a 2.5% metaphosphoric acid solution containing potassium cyanide in amounts ranging from 0.25 to 5.0 mg. per cc. was used. Titrations were made using the authors' micropipette. The data are presented in Table I.

It will be seen that the decolorization is not a straight line function of the amount of cyanide in the solution being titrated. The effect is relatively greater in lower concentrations of KCN.

TABLE I.
The decolorization of 2,6-Dichlorophenolindophenol by KCN.

Period of Standing (Room Temp.)	KCN-HPO_3 Solution mg./cc.	Titre† cc. Dye for 0.2 cc. Solution cc.	Blank 0.2 cc. 2.5% HPO_3 cc.	Dye Decolorized by KCN cc.
Immediate	5	.066	.005	.061
½ hr.	5	.065	.004	.061
1 ”	5	.064	.004	.060
2 ”	5	.065	.004	.061
Immediate	1	.023	.005	.018
½ hr.	1	.021	.004	.017
1 ”	1	.022	.005	.017
2 ”	1	.021	.004	.017
Immediate	.25*	.011	.004	.007
½ hr.	.25	.011	.004	.007
1 ”	.25	.010	.004	.006
2 ”	.25	.011	.004	.007

*The amount of KCN in 2 cc. Plasma-HPO_3 filtrate used by Pijoan and associates is 0.4 mg. In the authors' micromethod, the amount would be 0.05 mg. in 0.2 cc. filtrate.

†The end point persists for 20 seconds. Fading occurs on longer standing, as in blood.

Experiment II. The stability of ascorbic acid in 2.5% metaphosphoric acid solution.

In this experiment, approximately 1 mg. of ascorbic acid* was dissolved in 100 cc. of 2.5% HPO_3 solution. To 50 cc. we added 12.5 mg. KCN. The data (Table II) are expressed as cc. of dye (1 cc. ≙ 0.02 mg. ascorbic acid) required to titrate 0.2 cc. portions (by micropipette) after various periods of standing. The 50 cc. flasks containing the samples were lightly corked.

TABLE II.
Stability of Ascorbic Acid.

	Without KCN			With KCN (0.25 mg./cc.)		
Time of Standing (Room Temp.)	Titre	Blank 0.2 cc. 2.5% HPO_3	Dye Reduced by Ascorbic Acid (alone)	Titre	Blank 0.2 cc. 2.5% HPO_3	Dye Reduced by Ascorbic Acid (+KCN)
Immediate	.033	.004	.029	.040	.004	.036
15 min.	.032	.004	.028	.038	.004	.034
30 "	.032	.004	.028	.038	.004	.034
60 "	.030	.004	.026	.037	.004	.033
90 "	.031	.004	.027	.037	.004	.033
180 "	.030	.004	.026	.037	.004	.033

The data indicate no advantage in preservation of ascorbic acid in 2.5% metaphosphoric acid by KCN, while the enhancement of the titration is clearly shown.

Experiment III. The enhancement of apparent plasma ascorbic acid values by the use of KCN.

Twenty cc. of blood were taken from the vein of an individual. Of this, 10 cc. were placed in a tube containing 10 mg. KCN and 15 mg. potassium oxalate. The remaining 10 cc. were placed in a tube containing 15 mg. potassium oxalate alone. Five cc. of each blood were centrifuged, the plasma pipetted off, and placed in respective tubes. One cc. of each plasma was removed and deproteinized according to the method of Pijoan and Eddy,[7] then 2 cc. of the resulting plasma-HPO_3 filtrate titrated immediately (A in Table III). After 30 minutes, a second 1 cc. sample of each plasma was deproteinized and titrated in the same way (B in Table III).

The unused 5 cc. portions of each whole blood sample were allowed to stand in the laboratory for 30 minutes. They were then centrifuged, deproteinized, and titrated as above (C in Table III).

The data are given in Table III.

* We wish to express our appreciation to Merck and Co., Inc., Rahway, N. J., for a generous supply of ascorbic acid (Cebione).

TABLE III.
Ascorbic Acid Values (mg.%) from Individual Blood as Effected in KCN.

	No KCN mg.%	With KCN mg.%
A. Plasma separated immediately from blood	.616	.836
B. Plasma 30 minutes after separation from blood*	.528	.700
C. Plasma removed after blood had stood 30 min.*	.572	.616

*Room temperature.

From the figures on plasma separated immediately from blood when drawn, it is seen that the use of cyanide gives an enhancement of 0.220 mg. %, which is approximately 25% of the value of ascorbic acid present. These data are typical of 5 similar experiments.

Experiment IV. The enhancement of apparent ascorbic acid values in plasma as determined by the micromethod of Farmer and Abt, from blood containing potassium cyanide.

Blood from the same individual was collected in 2 tubes, one containing potassium oxalate, the second, oxalate with KCN in the amount of 1 mg. per cc. blood. As in the previous experiment with the macromethod, a portion of each blood was centrifuged imme-

TABLE IV.
Influence of KCN on Plasma Ascorbic Acid Values Determined by Micromethod of Farmer and Abt. (Figures are expressed as mg.%.)

Time of Standing (Room Temp.)	Plasma Separated and Deproteinized Immediately No KCN	Plasma Separated Immediately. Deproteinized at Time Interval Shown		Plasma Separated from Whole Blood at Time Interval Shown, then Immediately Deproteinized	
		No KCN	With KCN	No KCN	With KCN
Blood A					
Immediate	1.20	1.20	1.40	1.20	1.40
½ hr.	1.20	1.12	1.36	1.16	1.32
1 ”	1.16	1.00	1.16	1.08	1.16
2 ”	1.08	1.00	1.08	1.04	0.96
6 ”	0.76	0.60	0.60	0.68	0.56
Blood B					
Immediate	.64	.64	.80	.64	.80
¼ hr.	.64	.64	.80	.60	.76
½ ”	.64	.52	.72	.56	.68
1 ”	.64	.44	.68	.60	.60
2½ ”	.60	.44	.64	.52	.60
Blood C					
Immediate	.36	.36	.64	.36	.64
½ hr.	.32	.28	.56	.32	.48
1 ”	.28	.20	.52	.28	.40
2 ”	.28	.16	.44	.20	.40
3 ”	.24	.16	.44	.20	.36
5 ”	.24	.12	.36	.16	.32

diately, then deproteinized in the usual way (0.1 cc. plasma + 0.1 cc. water + 0.2 cc. 5% HPO_3) and 0.2 cc. deproteinized plasma titrated with 2,6-dichlorophenolindophenol (1 $\simeq$ 0.02 mg. ascorbic acid) using our micropipette. A portion of each plasma was held for later titration at hourly intervals.

The remainder of each blood sample was allowed to stand at laboratory temperature and a portion of each centrifuged and deproteinized at intervals as shown in Table IV. We present data on 3 bloods of ascorbic acid contents frequently observed.

The above data are typical of similar studies conducted upon 17 different bloods.

Whole blood to which KCN is added becomes hemolysed after a short period of standing.

Bloods differ one from another considerably in the loss of reductive power upon standing.

Conclusion. For dependable ascorbic acid values, blood should be centrifuged, the plasma deproteinized, and the plasma-HPO_3 filtrate titrated in immediate sequence after the blood is drawn. Whole blood which stands in a closed small phial, with a minimum air space, may be depended upon to give results of clinical value for ½ hour. The higher values obtained with bloods to which KCN has been added represent an enhancement due to the action of KCN upon the 2,6-dichlorophenolindophenol, and in no wise a more accurate determination of their ascorbic acid content. This is particularly true with blood of low ascorbic acid value. KCN does not prevent the loss of ascorbic acid from blood.

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Vitamin B_1 Metabolism in Man. Excretion of B_1 in Urine and Feces.

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Goodhart and Jolliffe[1] have emphasized the need for a practical chemical method for studying vitamin B_1 metabolism. The fer-

[1] Goodhart, Robert, and Jolliffe, Norman, *J. Am. Med. Assn.*, 1938, **110**, 414.