## A REACTION OF ASCORBIC ACID WITH α-AMINO ACIDS

DURING the course of an investigation of reactions between ascorbic acid and amines,[1] we observed that α-amino acids reacted with ascorbic acid in the presence of hydrogen peroxide and heat to yield a reddish-colored solution. Ammonia water (U.S.P.) may be substituted for the amino acids. The following α-amino acids were studied and found to be suitable as reagents: asparagine, glycine, leucine, isoleucine, tyrosine, cystine, cysteine, valine, phenylalanine, aspartic acid and dl-alanine. The latter was finally adopted as a standard reagent.

This reaction appears to be specific for ascorbic acid, since it is not given by other reducing substances, ketones, phenols or carbohydrates such as sorbitol, glucose, fructose, mannose, xylose, trehalose, arabinose, starches, d-glucurone, and therefore, it is not based either upon the reducing property of[2] or upon the carbohydrate structure[3] of ascorbic acid.

When this reaction is carried out in aqueous media the sensitivity is limited to solutions containing 50 mg per cent. of ascorbic acid or more, whereas, if the reaction is conducted in glacial acetic acid the sensitivity is increased to 2.5 mg per cent., but the color fades rather rapidly at room temperature.

The employment of dilute copper sulfate solutions instead of $H_2O_2$ increases the sensitivity of the test in glacial acetic acid media still further. This does not hold for aqueous solutions.

*Experimental:* The following reagents were used for the detection of ascorbic acid: (a) 5 per cent. dl-alanine dissolved in distilled water; (b) superoxol (Merck) in various dilutions depending upon the approximate amount of ascorbic acid reacting—from 30 per cent. to 0.3 per cent. To each cc of ascorbic acid solution (for spectrophotometric estimation—5 cc) were added 1 cc of 5 per cent. dl-alanine and 0.05 cc of $H_2O_2$ of various strengths. The test-tubes containing the final solution were placed in boiling water for 2 minutes and were allowed to cool for 15 minutes, and the final colors estimated either visually or spectrophotometrically. (For the test in glacial acetic acid the ascorbic acid was made up in various concentrations in that medium, the alanine reagent in a 1.0 per cent. glacial acetic acid and heated in a water bath for 2 minutes. 0.05 cc of an 0.5 per cent. copper sulfate was used to accelerate the reaction. It was found that the addition of alcohol following heating stabilizes the color.)

For spectrophotometrical determination a total volume of 10 cc were used (5 cc dl-alanine, 0.25 $H_2O_2$ and varying amounts of ascorbic acid made to volume with distilled water).

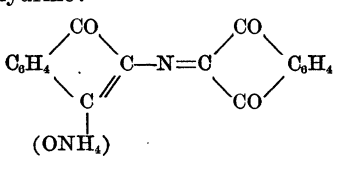
When small amounts of ascorbic acid were used (6 mg/cc or less) the $H_2O_2$ used was 0.075 per cent. by volume; between 6 and 12 mg/cc ascorbic acid, the final per cent. of $H_2O_2$ was 0.025, whereas for amounts in excess of 12 mg ascorbic acid, a final per cent. of 0.0075 may be used. With smaller amounts of ascorbic acid and low percentage of $H_2O_2$ the development of color was slow (may take 15 minutes after boiling); whereas, with larger amounts of ascorbic acid and high percentage of $H_2O_2$ the color develops during the 2 minutes of boiling. It was found that an excess of hydrogen peroxide will destroy colors produced by small amounts of ascorbic acid and that larger concentrations of hydrogen peroxide were necessary for full development of color using larger amounts of ascorbic acid. The following are the concentrations of peroxide found to be most suitable for maximum color development, using 5 per cent. solution of alanine throughout: between 1–6 mg ascorbic acid per cc—3.0 per cent. $H_2O_2$; between 6–12 mg ascorbic acid per cc—3.0 per cent., and beyond 12 mg ascorbic acid per cc—10 per cent. of $H_2O_2$ (0.025 cc $H_2O_2$ per cc in all cases).

The color in the test exhibited a maximum absorption between 505–510 mμ in aqueous solution. Upon prolonged standing the color shifts to the yellow end of the spectrum exhibiting a maximum absorption of about 400 mμ. Colorimetric estimation can also be made using these yellow colors.

The reaction is a colorimetric one, that is, within certain ranges the intensity of the red color varies as the concentration of ascorbic acid. Within narrow ranges the test follows Beer's Law; in other words, the K value within these ranges is fairly constant.

*Theory:* It is well known that the $-N=N-$ group is chromophoric and that the $-NH_2$ or $NH_3$ groups are necessary for color development in the ninhydrine and murexide reactions. In both cases the reaction involves two molecules of the reagent connected by $-N=H-$ chromophoric grouping. Only compounds containing an α-amino to a carboxyl group give the ninhydrine test. The constitution of the coloring matters resulting from the interaction of $-NH_2$ with ninhydrine, murexide and the possible reaction with the ketone structural formula of dehydroascorbic acid are as follows:

(1) Ninhydrine:

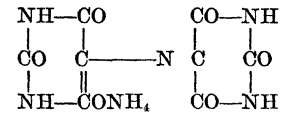



[1] Mr. Louis S. Schwartz, of the Van Patten Pharmaceutical Company, suggested an experimental study of ascorbic acid – NH compounds. We thank him and his firm for the suggestion and for support in carrying out this project.

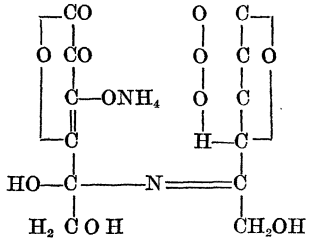
[2] O. A. Bessey and C. G. King, *Jour. Biol. Chem.*, 103: 687, 1933.

[3] J. H. Roe, *Jour. Biol. Chem.*, 116: 609, 1936.

(2) Murexide (Slimmer and Stieglitz formula):



(3) Ascorbic acid (proposed formula):



In support of the theory, it may be argued that in both the ninhydrine and the present test only compounds containing amino group α to a carboxyl are reacting and that we found that in the murexide test the customary reagent, $NH_3$ water, may be substituted by α-amino compounds (dl-alanine). Also, if dehydroascorbic acid is tested directly with ammonia water and heat, a red color develops without the aid of an oxidizing agent. The facts that the test is more delicate if performed in glacial acetic acid (non-aqueous) media and that copper may be substituted for hydrogen peroxide in the test, indicate that ascorbic acid must be first converted to dehydroascorbic acid to react with $-NH_2$ to yield a colored compound.

It may be stated that the suggestion of Harding and MacLean[4] that 0.2 cc of pyridine to be used for increasing the sensitivity of the ninhydrine test is not applicable to the ascorbic acid reaction because pyridine, along with some other compounds, yields a yellow color in the above procedure which obscures the faint red colors developed.

*Conclusions:* A new reaction and preliminary colorimetric test of distinctive specificity has been developed by using dl-alanine and $H_2O_2$ as reagents for the detection of ascorbic acid. It is probable that the color develops with dehydroascorbic acid in a manner similar to the ninhydrine or murexide tests. While the new test may not be fully applicable at the present time to determine ascorbic acid in animal and human materials, it may be used to test the identity of ascorbic acid in official (*e.g.,* U.S.P.) preparations.

We are greatly indebted to Dr. J. H. Roe for checking the reaction and for his encouragement to continue this research.

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[4] V. J. Harding and R. M. MacLean, *Jour. Biol. Chem.,* 20: 217, 1915.

## IN VITRO GROWTH AND MULTIPLICATION OF THE MALARIA PARASITE, PLASMODIUM KNOWLESI[1, 2]

WHETHER one is considering the biochemical, biological, immunological or chemotherapeutic aspects of the malaria problem, questions arise which could be more readily answered by experimentation on parasites grown *in vitro* than on those sheltered by the host. This well-recognized fact has led many previous investigators to attempt the *in vitro* cultivation of the malaria parasite. Undoubtedly, the most successful and best-documented work to date on the erythrocytic form has been that of Trager,[3, 4] who clearly demonstrated that the bird malaria parasite, *P. lophurae,* would survive *in vitro* up to 16 days at body temperature. Never did Trager observe an increase *in vitro* in the total number of parasites comparable to that seen in the host. The parasite population in his experiments usually remained constant for the first few days and then declined. He concluded that, though development must be continuing in such preparations, the death rate, particularly after the first few days, far exceeds the birth rate.

We wish to report here a brief summary of the results obtained during the past year on the *in vitro* cultivation of the erythrocytic form of the malaria parasite, *P. knowlesi,* in which we have regularly observed growth and good multiplication. *P. knowlesi* was chosen for our work because this parasite has a 24-hour cycle, produces a heavy infection of red cells in the monkey, *Macaca mulatta,* and will also produce infection in man. Moreover, since our chief interest lay in making biochemical and metabolic studies on the parasite, we preferred to deal with a host possessing a non-nucleated red blood cell.

Two types of techniques have been developed. One, which we have termed the rocker-dilution, consists of the dilution of 1 part of whole blood with 3 parts of a nutrient fluid whose composition is given in Table 1. This mixture, usually 6.0 ml in total volume though 50 ml have been used, is placed in a tube or flask equipped with gas inlet and outlet tubes. The container is then placed on a rocking machine which just keeps the red cells in gentle motion. A slow flow of 5 per cent. $CO_2$: 95 per cent. air is passed into the vessel without being allowed to bubble through the liquid. The whole procedure is carried out under sterile conditions and the cultivation performed at

[1] From the Department of Biological Chemistry, Harvard Medical School, and the Department of Comparative Pathology and Tropical Medicine, Harvard School of Public Health and Harvard Medical School, Boston.

[2] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the President and Fellows of Harvard College.

[3] W. Trager, *Jour. Exp. Med.,* 74: 441, 1941.

[4] W. Trager, *Jour. Exp. Med.,* 77: 411, 1943.