FAILURE TO FIND BOUND ASCORBIC ACID IN PLASMA*

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(Received for publication, April 17, 1950)

The fact that in experimental scurvy the concentration of ascorbic acid in the cellular elements of blood decreases more slowly than does the concentration in plasma (2–4) has led to the hypothesis that ascorbic acid might be bound1 to cellular protein (5, 6). There is, however, little direct evidence to support such a hypothesis. Saha, Majumdar, and Guha (7, 8) demonstrated the presence of bound ascorbic acid (ascorbigen) in trichloroacetic acid filtrates of laked whole blood. Ghosh and Guha (9) identified a similar substance in extracts of cabbage, which would not dialyze through parchment or cellophane membranes. If these two substances are similar, then ascorbic acid is bound to a relatively small nondialyzable molecule. Guha et al. did not, however, indicate whether bound ascorbic acid was located in the cellular elements, in the plasma, or in both.

Investigators disagree on the question of whether or not bound ascorbic acid occurs in plasma. Use of such physical methods as ultrafiltration (10), ultracentrifugation (11), refractometry (12), and electrophoresis (13) has led to conflicting results. Consequently, we undertook a systematic study of the physicochemical behavior of ascorbic acid in plasma, utilizing several of the physical methods previously tried in an effort to explore further this problem.

Methods

Diffusion—A small bag made of either collodion or Visking2 sausage skin, containing 6 ml. of the test solvent, was suspended in an Evelyn

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1 Throughout this paper the term "bound ascorbic acid" will be used in referring to this hypothetical compound.

2 The Visking Corporation of Chicago, Illinois, donated a generous supply of this seamless dialyzing tubing.
colorimeter tube which contained 6 ml. of test solvent plus ascorbic acid. One colorimeter tube containing a similar solution but without the suspended bag served as a heat control in each experiment. Ascorbic acid was determined in each of the three solutions both at the beginning and at the end of the diffusion by the method of Roe and Kuether (14). All the tests were carried out in a water bath at 37°. The rate of diffusion of ascorbic acid was studied in a variety of test solvents for intervals of time up to 2 hours.

In order to compare the results of various experiments conducted over a wide range of ascorbic acid concentrations, a measure of the rate of diffusion was adopted which was independent of concentration; viz., the ratio of the diffusion gradient at time t to the initial gradient ($\Delta t/\Delta_0$). This ratio was expressed as a percentage.

Ultrafiltration—Ultrafiltration was accomplished by an adaptation of the centrifugal method described by Clegg (15). During centrifugation, which lasted 3 to 5 hours, the temperature was usually maintained by a refrigeration apparatus at about 5°. In a few experiments the temperature was not controlled. In all experiments 1 ml. of 6 per cent metaphosphoric acid was placed in each centrifuge tube to insure the stability of the ascorbic acid in the ultrafiltrate. 5 ml. aliquots of the various test solutions were added to each of six or eight cellophane tubes and centrifuged. Ultrafiltrates containing protein, as shown by a precipitate in the metaphosphoric acid solution, were discarded. The protein-free ultrafiltrates and the test solutions remaining in the cellophane tubes were pooled. The volumes were measured as accurately as possible in a 25 ml. graduate. Aliquots of the pooled solutions were analyzed for ascorbic acid by the phenylhydrazine method (14). Appropriate controls were included and similarly analyzed.

Spectrophotometry—Absorption Spectrum of Ascorbic Acid—The absorption spectra of ascorbic acid in equimolar KCN, plasma diluted 1:100 with distilled water, a solution of the amino acids mixture, 10 per cent (Merck), diluted 1:100 with distilled water, and 0.55 M NaCl were measured between 220 and 320 mp. Ascorbic acid in equimolar KCN was then added to the diluted plasma, the diluted amino acids mixture, and NaCl solution, respectively, and the absorption spectra were remeasured. A 1.14 X 10^{-4} M solution of ascorbic acid in equimolar KCN was used throughout.

Method of Continuous Variations—The principle of the method of continuous variations has been described by Vosburgh and Cooper (16). In general this method consists of measuring at a fixed wave-length the optical densities of equimolar solutions of two substances mixed in vary-

* A Beckman spectrophotometer (model DU) was used.
ing molar ratios from 0 to 1. Since in the case of plasma the molarity is unknown and in the case of the amino acids solution only approximately known, the following procedure was adopted. The optical densities of various dilutions of plasma and the amino acid mixture, several ascorbic acid concentrations (in equimolar KCN), and ascorbic acid in equimolar KCN added to diluted plasma or amino acid mixture were measured at 250, 265, and 280 mμ (Table III).

Because of the absorption characteristic of plasma and amino acids in the ultraviolet region, it was impossible to measure accurately the absorption spectrum of ascorbic acid in solutions of these substances more concentrated than 1:100.

_Determination of Ascorbigen._—The method of acid hydrolysis described by Guha _et al._ (7–9, 17–19) was used with the following modifications. Ascorbic oxidase was omitted. The trichloroacetic acid centrifugates were analyzed both by the method of Roe and Kuether (14) and by an indophenol method (20). Plasma and serum were substituted for whole blood.

**Results**

_Diffusion._—The results of experiments on the rate of diffusion of ascorbic acid in plasma and Ringer-Locke's solution are shown graphically in Fig. 1. Since the rate of diffusion was similar in fresh plasma, refrigerated plasma, and reconstituted plasma (Cutter) and in Ringer-Locke's solution, 6 per cent metaphosphoric acid solution, and distilled water, the data from experiments with these two series of solutions have been grouped together as "plasma" and "Ringer-Locke's solution." In all, thirty-five tests were carried out with plasma and twenty-two with Ringer-Locke's solution. In twenty-one experiments with plasma in which diffusion was allowed to continue for 30 minutes, the standard deviation was ±5.2 for \( \Delta q_{30}/\Delta q \). In eleven comparable experiments with Ringer-Locke's solution the standard deviation was ±3.1.

The question may be raised as to whether ascorbic acid naturally occurring in plasma diffuses at a different rate than crystalline ascorbic acid in aqueous solution added to plasma. Our results failed to reveal any difference.

Once the rate of diffusion of ascorbic acid in plasma and in Ringer-Locke's solution had been measured experimentally, it was possible to apply Fick's law of diffusion to test whether or not ascorbic acid was diffusing freely under the conditions of these experiments. Theoretical

4 Dr. Wolfe of the Michael Reese Hospital donated a generous supply of Kahn-positive citrated plasma.
Δt/Δ0 values, calculated\(^5\) on the basis of the experimental data, are shown in Fig. 1 for comparison with the experimentally determined ratios. Three significant facts are apparent: Free diffusion of ascorbic acid occurs in either medium for about 30 minutes, the systems fail to come to equilibrium, and ascorbic acid diffuses more slowly in plasma than in Ringer-Locke's solution.

The latter finding, at first, seems to confirm the hypothesis that ascorbic acid is bound to a non-diffusible constituent of plasma. An alternative hypothesis, however, can be suggested; viz., that intermolecular frictional forces retard the rate of diffusion. Such a hypothesis is in accord with the observation of others (22) that the rate of diffusion of an ion can be accelerated or retarded by collision with other electrolytes or non-electrolytes present in the same solution. In experiments designed to explore this hypothesis, the collodion sack was replaced by one of Visking's sausage skin. The test solvents included plasma and various solutions of electro-

\(^5\) The details of these calculations have been reported elsewhere by Sargent and Golden (21).
lytes and non-electrolytes. All the experiments were conducted for 30 minutes. The results are summarized in Table I.

With the change of membrane the difference in the rate of diffusion of ascorbic acid in plasma and in Ringer-Locke's solution diminished; yet by the "t" test (23) it remained highly significant. Experiments with solutions of various derivatives of protein (Table I) revealed that, as the structure of the material was simplified from plasma to amigen and from amigen to amino acids, the rate of diffusion was essentially unchanged. 0.55 \text{M} \text{NaCl} also retarded the rate of diffusion of ascorbic acid. In all these various media the diffusion of ascorbic acid was significantly slower than in Ringer-Locke's solution. Therefore, the results support the hypothesis that a non-specific intermolecular frictional force can account for the slower rate of diffusion of ascorbic acid in plasma.

### Table I

\( \Delta z \) for Ascorbic Acid Diffusion through Visking Membrane in Various Environments at 37°

<table>
<thead>
<tr>
<th>Environment</th>
<th>( \Delta z/\Delta o )</th>
<th>Range</th>
<th>( \sigma )</th>
<th>( t )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>61 (6)*</td>
<td>60-63</td>
<td>±1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.1% amigen in Ringer-Locke's solution</td>
<td>65 (4)</td>
<td>61-72</td>
<td>±4.1</td>
<td>1.90</td>
<td>0.09</td>
</tr>
<tr>
<td>9.1% amigen in distilled water</td>
<td>56 (4)</td>
<td>50-64</td>
<td>±5.2</td>
<td>2.04</td>
<td>0.08</td>
</tr>
<tr>
<td>10% amino acid mixture (0.39 M)</td>
<td>62 (5)</td>
<td>58-67</td>
<td>±3.2</td>
<td>0.64</td>
<td>0.50</td>
</tr>
<tr>
<td>0.55 M NaCl†</td>
<td>56 (8)</td>
<td>46-64</td>
<td>±5.8</td>
<td>1.93</td>
<td>0.080</td>
</tr>
<tr>
<td>Ringer-Locke's solution‡</td>
<td>46 (6)</td>
<td>44-49</td>
<td>±2.0</td>
<td>14.2</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

* The figures in parentheses indicate the number of experiments.
† NaCl versus Ringer-Locke's solution; \( t = 3.78; P <0.005. 
‡ Includes tests with distilled water.

#### Ultrafiltration (Table II)—The ascorbic acid concentration in the ultrafiltrate, collected during two control experiments, did not differ appreciably from that in the original solution, in an aliquot maintained in an ice box, and in the solution from the cellophane bag. In the six experiments with plasma, there was with one exception little difference between the original ascorbic acid concentration and that of the ice box control. A rather large loss of ascorbic acid occurred in the plasma in the cellophane tubes. Appropriate controls demonstrated that the loss could not be attributed to a destructive action of the cellophane on ascorbic acid but rather to the fact that during centrifugation difficulty was experienced in maintaining the temperature at 5°. The influence of temperature on these experiments is clearly shown in the test carried out without thermal control. In spite of this situation, statistical analysis of the data indicated that
there was no significant difference between the ascorbic acid concentration in the ultrafiltrate and that in the final plasma contained in the cellophane tubes.

**Spectrophotometric Studies; Absorption Spectrum**—The absorption spectrum of ascorbic acid was measured in the presence of solutions of some of the substances which slowed its rate of diffusion. Two series of experiments were made over the range, 220 to 310 μ. The spectrophotometer was calibrated before each series. In the first series log ε reached a maximum⁶ (4.11) at 265 μ when ascorbic acid in equimolar KCN was used. In the presence of plasma, amino acids, and NaCl, log ε was also maximal at 265 μ, being respectively 4.13, 4.13, and 4.11. In the second

<table>
<thead>
<tr>
<th>Environment</th>
<th>Original ascorbic acid concentration (mg. per 100 ml.)</th>
<th>Ascorbic acid concentration in ice box control (mg. per 100 ml.)</th>
<th>Final ascorbic acid concentration (mg. per 100 ml.)</th>
<th>Ascorbic acid concentration of ultrafiltration (mg. per 100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water*</td>
<td>1.75</td>
<td>1.75</td>
<td>1.80</td>
<td>1.84</td>
</tr>
<tr>
<td>6% metaphosphoric acid*</td>
<td>1.18</td>
<td>1.05</td>
<td>1.02</td>
<td>1.00</td>
</tr>
<tr>
<td>Plasma (Cutter)</td>
<td>3.35</td>
<td>2.85</td>
<td>2.70</td>
<td>2.33</td>
</tr>
<tr>
<td>Fresh pooled plasma</td>
<td>2.50</td>
<td>2.20</td>
<td>1.90</td>
<td>2.05</td>
</tr>
<tr>
<td>“ “</td>
<td>1.85</td>
<td>1.85</td>
<td>1.49</td>
<td>0.90</td>
</tr>
<tr>
<td>“ “</td>
<td>1.60</td>
<td>1.55</td>
<td>1.18</td>
<td>0.98</td>
</tr>
<tr>
<td>“ “</td>
<td>1.45</td>
<td>1.45</td>
<td>1.35</td>
<td>1.29</td>
</tr>
<tr>
<td>“ “</td>
<td>1.25</td>
<td>1.30</td>
<td>0.37</td>
<td>0.58</td>
</tr>
<tr>
<td>Mean†</td>
<td></td>
<td></td>
<td>1.49</td>
<td>1.36</td>
</tr>
</tbody>
</table>

* No thermal control during centrifugation.
† t = 0.32; P = 0.76.

series log ε was maximal at about 270 μ, being 4.16 for ascorbic acid⁶ in equimolar KCN and 4.15 for ascorbic acid in diluted plasma and in dilute solution of amino acids. In both these series, log ε, calculated on the basis of no reaction, agreed remarkably well with those of the standard ascorbic acid solution.

**Method of Continuous Variations**—A typical experiment with plasma and an analysis of all the results are given in Table III. One may conclude from these data that the method of continuous variations did not indicate

⁶ Rosenberg (24) states that, for ascorbic acid in equimolar KCN, log ε is 3.93 at 263 μ.
any significant alteration in the ascorbic acid molecule when mixed with the various concentrations of plasma and amino acids used in this study.

Acid Hydrolysis—In three experiments, no increase could be detected in the concentration of ascorbic acid in heated trichloroacetic acid cen-

<table>
<thead>
<tr>
<th>Composition of mixture</th>
<th>Plasma, 265 mp</th>
<th>Per cent measurement with Y &gt; ±3σ†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma or amino acids</td>
<td>Ascorbic acid</td>
<td>Measured optical density</td>
</tr>
<tr>
<td>1:20</td>
<td>0.00</td>
<td>2.15</td>
</tr>
<tr>
<td>1:30</td>
<td>0.25</td>
<td>1.54</td>
</tr>
<tr>
<td>1:40</td>
<td>0.50</td>
<td>1.42</td>
</tr>
<tr>
<td>1:50</td>
<td>0.75</td>
<td>1.45</td>
</tr>
<tr>
<td>1:60</td>
<td>1.00</td>
<td>1.49</td>
</tr>
<tr>
<td>1:70</td>
<td>1.25</td>
<td>1.59</td>
</tr>
<tr>
<td>1:80</td>
<td>1.50</td>
<td>1.70</td>
</tr>
<tr>
<td>1:90</td>
<td>1.75</td>
<td>1.88</td>
</tr>
<tr>
<td>0</td>
<td>2.00</td>
<td>1.60</td>
</tr>
</tbody>
</table>

Mean* ............................................................... 19

* Nine experiments were performed with the seven mixtures. In only twelve instances, or 19 per cent of 63 measurements, did Y exceed 3σ.
† The experimental error of the method was ±0.02. In order for a difference, Y, to be considered significant, it must be greater than 3 times σ; i.e., 0.02 × 3 or 0.06. At this level the probability is 0.0027 that Y is due to chance alone (23).

<table>
<thead>
<tr>
<th>Change in Ascorbic Acid Concentration in Fresh Plasma after Treatment according to Method of Guha; Analyses by Indophenol and Hydrazine Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifugate, unboiled</td>
</tr>
<tr>
<td>Indophenol</td>
</tr>
<tr>
<td>mg. per 100 ml.</td>
</tr>
<tr>
<td>1.09</td>
</tr>
<tr>
<td>2.10</td>
</tr>
<tr>
<td>1.20</td>
</tr>
</tbody>
</table>

trifugates (Table IV). Agreement between concentrations as determined by the two analytical methods was excellent. The conclusion formed is that the plasma tested contained no ascorbigen (as defined by Guha et al.). Matusis (25) reached a similar conclusion.
DISCUSSION

The results of these studies rule out the presence of Guha's ascorbigen in plasma. They offer no support for the claim of Ruskin and Jonnard (12) that serum proteins will bind 0.5 to 0.8 mg. per ml. of ascorbic acid. The studies of other investigators (e.g., 26, 27) on binding of organic ions by proteins have indicated that one manifestation of binding was a change in the absorption spectrum from that characteristic of the ion in pure solution. This fact, together with our negative findings, indicates that plasma proteins probably do not bind ascorbic acid. Schubert (13), however, claims to have shown the existence of a "loosely bound ascorbic acid" by electrophoresis, although his ultrafiltration experiments were negative. In a series of poorly controlled studies he showed that both ascorbic acid and albumin acted on anions and concluded that ascorbic acid was thus bound to albumin. His facts do not support his conclusions.

SUMMARY

A physicochemical and chemical study was made of the behavior of ascorbic acid in plasma, amino acids, and special control solutions.

The techniques employed in the study were diffusion, ultrafiltration, spectrophotometry, and acid hydrolysis.

The results of experiments by these various techniques concurred. No bound non-diffusible ascorbic acid could be demonstrated in plasma.

We are especially grateful to Dr. R. E. Johnson, Dr. D. MacFadyen, and Dr. R. R. Scallock for their encouragement and advice during the progress of these studies and for their assistance in preparing this report. To Dr. William H. Forbes, under whose direction the problem was first attacked, the senior author is indebted for a working hypothesis for the investigation.

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