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## PROTECTIVE ACTION OF OXALIC ACID IN RELATION TO ASCORBIC ACID AGAINST ITS OXIDATION BY THE ASCORBIC ACID OXIDASE \*

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In a previous paper (FONSECA RIBEIRO & CARDOSO, 1950) in which we demonstrated the anti-oxidative action of the oxalic acid in relation to adrenalin, we also suggested the possibility that the addition of oxalic acid to solutions of iron or copper acted by making the ionization of the salts of these metals difficult. For this reason there was presented a hypothesis that oxidative processes, catalyzed by the ions of iron or copper, should be inhibited or delayed by oxalic acid. In fact, we verified in the above mentioned paper that the addition of oxalic acid to the solution of ascorbic acid makes them more stable. In consequence, it should be reasonable to admit that oxalic acid acting by the formation of a weak dissociable combination with copper or iron present in the oxidative system could also act in those processes, due to enzymes having an active group formed by copper or iron. To verify this hypothesis experimentally we decided to study the action of oxalic acid in the oxidative process due to the ascorbic acid oxidase for this enzyme belongs to those having copper in their active group. A supporting reason for the choice of this material for our study was the possibility of finding an explanation to the well known fact of the existence in animal or vegetable materials of substances, not yet identified, which protect ascorbic acid against the oxidation by the ascorbic acid oxidase (BARRON, BARRON & KLEMPERER, 1936 and PIMENTA, 1941).

Indeed, the experiments we undertook confirmed the suggested hypothesis of the existence of a definite protective action of oxalic acid in relation to ascorbic

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acid in its oxidation by the ascorbic acid oxidase. We will now present those experimental results as well as those related to the behaviour of oxalic acid in the oxidation of ascorbic acid by the sole presence of the copper ion and its comparison with the similar action exerted by thiourea in the oxidation of ascorbic acid by copper, which was demonstrated by HUELIN & MYEE STEPHENS (1948).

#### EXPERIMENTAL

The solutions of ascorbic acid we used were always prepared at the time of the experiment and titrated by Tillmans reagent.

We used, for the oxidative action, the ascorbic acid oxidase, prepared from cucumbers (*Cucumis sativus*) following the technic of FUJITA & SAKAMOTO (1938) slightly modified by LESER (1941). The enzyme was previously titrated by the method of TAUBER & KLEINER (1935). The action of the ascorbic acid oxidase on the ascorbic acid in all the experiments was standardized to the time of 15 minutes and to the temperature of 40°C, in a water bath, thus fulfilling the most favorable conditions for enzymatic action.

We found it convenient to work with the quantity which, at the already mentioned time and temperature, promoted the oxidation of about 50% of the initial ascorbic acid. The quantity of one tenth of a ml of the stock solution was the indicated one for quantities of ascorbic acid near 0.2 mg per ml.

The enzymatic action was always inhibited, after the desired time, by the addition to each test tube of 2 ml of a 10 per cent solution of metaphosphoric acid.

To stabilize the pH during the experiments we always used buffers of disodium phosphate and citric acid according to MCILVAINE (*in* CLARK, 1928). Buffers' efficiency was potentiometrically controlled.

#### INFLUENCE OF pH

In preliminary tests we verified that the protective action of oxalic acid was influenced by the pH of the medium. We decided then to determine what variation in the intensity of this action would result from varying the pH value. For this, using the same amounts of ascorbic acid and oxalic acid we varied the pH from 3.5 to 6.0.

We obtained the following results.

TABLE 1

INFLUENCE OF pH ON THE PROTECTIVE ACTION OF OXALIC ACID  
IN RELATION TO ASCORBIC ACID, IN ITS OXIDATION BY THE ASCORBIC  
ACID OXIDASE

Ascorbic acid (initial) 1 ml = 0.204 mg (in each tube)

Ascorbic acid oxidase 0.1 ml of the stock solution (in each tube)

Temperature: 40° C — Time: 15' — After this time  $\text{HPO}_3$  was added to  
each tube — 2 ml of a 10% (w/v) solution

| Tube n.º | Oxalic acid | Phosphoric-citric<br>buffer (5 ml) pH | Ascorbic acid<br>(residual) mg |
|----------|-------------|---------------------------------------|--------------------------------|
| 1        | 1 mg        | 3.5                                   | 0.206                          |
| 2        | —           | 3.5                                   | 0.195                          |
| 3        | 1 mg        | 4.0                                   | 0.202                          |
| 4        | —           | 4.0                                   | 0.174                          |
| 5        | 1 mg        | 4.5                                   | 0.188                          |
| 6        | —           | 4.5                                   | 0.102                          |
| 7        | 1 mg        | 5.0                                   | 0.055                          |
| 8        | —           | 5.0                                   | 0.007                          |
| 9        | 1 mg        | 5.5                                   | 0.007                          |
| 10       | —           | 5.5                                   | Nil                            |
| 11       | 1 mg        | 6.0                                   | Nil                            |
| 12       | —           | 6.0                                   | Nil                            |

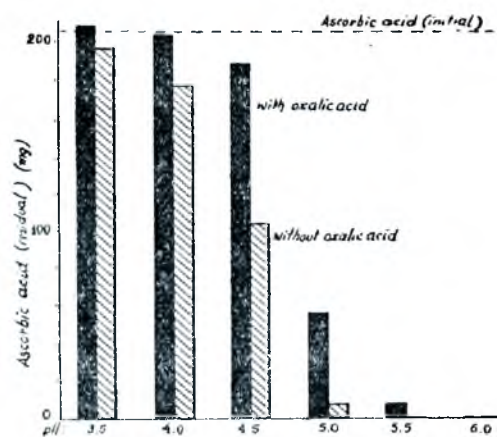


FIG 1 - INFLUENCE OF pH

As can be seen by these results the protective action of oxalic acid was more evident at pH 4.5 and at this same pH ascorbic acid oxidase alone acted

satisfactorily. For that reason we began to use pH 4.5 systematically in the following experiments.

Having established this preliminary we proceeded to make the quantitative study of the protective action of oxalic acid; for this purpose we made a series of experiments of which the following one (Table 2, represented by Fig. 2) is quite representative.

TABLE 2

PROTECTIVE ACTION OF OXALIC ACID IN RELATION TO ASCORBIC ACID IN THE PRESENCE OF ASCORBIC ACID OXIDASE. VARIATION IN RELATION TO THE AMOUNTS OF OXALIC ACID

Ascorbic acid (initial) 1 ml = 0.2 mg (in each tube)

Buffer pH 4.5-5 ml (in each tube)

Temperature: 40°C — Time: 15' — After this time  $\text{HPO}_4$  was added to each tube — 2 ml of a 10% (w/v) solution

| Tube n.º | Oxalic acid mg | Ascorbic acid oxidase | Ascorbic acid (residual) mg |
|----------|----------------|-----------------------|-----------------------------|
| 1        | —              | —                     | 0.195                       |
| 2        | —              | 0.1                   | 0.103                       |
| 3        | 0.01           | 0.1                   | 0.107                       |
| 4        | 0.01           | 0.1                   | 0.108                       |
| 5        | 0.02           | 0.1                   | 0.110                       |
| 6        | 0.02           | 0.1                   | 0.109                       |
| 7        | 0.04           | 0.1                   | 0.119                       |
| 8        | 0.04           | 0.1                   | 0.119                       |
| 9        | 0.06           | 0.1                   | 0.128                       |
| 10       | 0.06           | 0.1                   | 0.128                       |
| 11       | 0.08           | 0.1                   | 0.135                       |
| 12       | 0.08           | 0.1                   | 0.135                       |
| 13       | 0.10           | 0.1                   | 0.138                       |
| 14       | 0.10           | 0.1                   | 0.138                       |
| 15       | 0.15           | 0.1                   | 0.148                       |
| 16       | 0.15           | 0.1                   | 0.148                       |
| 17       | 0.20           | 0.1                   | 0.152                       |
| 18       | 0.20           | 0.1                   | 0.152                       |
| 19       | 0.40           | 0.1                   | 0.169                       |
| 20       | 0.40           | 0.1                   | 0.168                       |
| 21       | 0.60           | 0.1                   | 0.175                       |
| 22       | 0.60           | 0.1                   | 0.175                       |
| 23       | 0.80           | 0.1                   | 0.180                       |
| 24       | 0.80           | 0.1                   | 0.181                       |
| 25       | 1.00           | 0.1                   | 0.184                       |
| 26       | 1.00           | 0.1                   | 0.186                       |
| 27       | 1.50           | 0.1                   | 0.188                       |
| 28       | 1.50           | 0.1                   | 0.189                       |
| 29       | 2.00           | 0.1                   | 0.193                       |
| 30       | 2.00           | 0.1                   | 0.194                       |

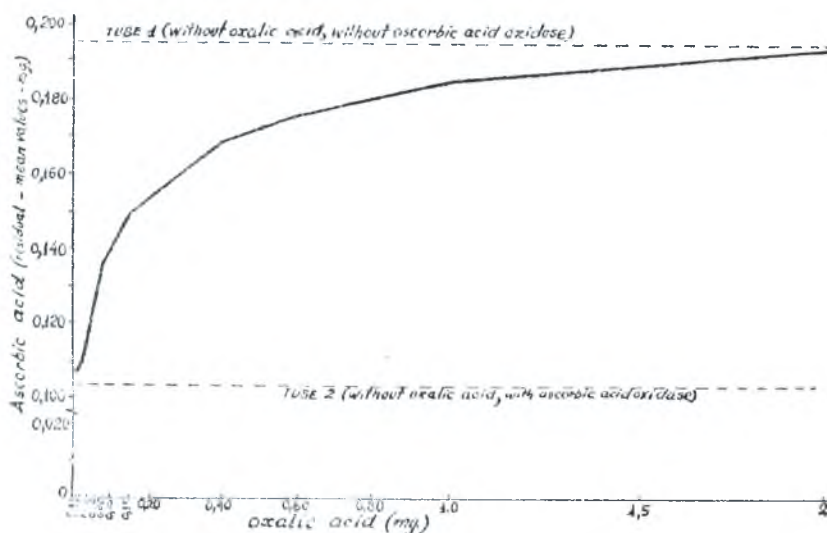


Fig. 2 - VARIATION OF THE PROTECTIVE ACTION OF OXALIC ACID IN RELATION TO ITS AMOUNT.

Trying to adapt a curve to the obtained data we started from the consideration that the curve should have a superior asymptote  $K = 1950$ . After a series of interpolatory attempts we obtained as a most adaptable curve a modified exponential (Gompertz' type) having as its equation:

$$y = 1950 - 715.47 \times 0.98142^x$$

The development of the interpolated curve presents, initially, ordinate values greater than the observed ones; at the intermediary phase it presents lesser values, giving afterwards a very good adaptation. The mean of the differences, taken as absolute values, represents 3.63% of the mean of the values of  $y$ .

Having demonstrated the protective action of the oxalic acid in relation to ascorbic acid against its oxidation by the ascorbic acid oxidase which is an enzyme containing copper as its action group, it seemed to us that the comparative study of the action of the cupric ion as oxalate and that of another salt would be timely. For this purpose we selected copper sulphate.

The following experiments (Table 3) shows the results which were obtained (they are also represented by Fig. 3).

TABLE 3

COMPARISON BETWEEN THE OXIDATIVE ACTION OF  $\text{CuSO}_4$  AND THAT OF  $\text{Cu}(\text{COO})_2$  IN RELATION TO ASCORBIC ACID

Ascorbic acid (initial) 1 ml = 0.234 mg (in each tube)

Buffer pH 4.5 — 1 ml (in each tube)

Temperature: 40°C — Time: 15' — After this time  $\text{HPO}_3$  was added to each tube — 2 ml of a 10% (w/v) solution

| Tube n.º | $\text{CuSO}_4$<br>(Cu "in" $\mu\text{g}$ ) | $\text{Cu}(\text{COO})_2$<br>(Cu "in" $\mu\text{g}$ ) | Ascorbic acid<br>(residual) mg |
|----------|---|---|--------------------------------|
| 1        | —   | —   | 0.220                          |
| 2        | 1   | —   | 0.161                          |
| 3        | —   | 1   | 0.217                          |
| 4        | 2   | —   | 0.138                          |
| 5        | —   | 2   | 0.208                          |
| 6        | 3   | —   | 0.129                          |
| 7        | —   | 3   | 0.208                          |
| 8        | 4   | —   | 0.121                          |
| 9        | —   | 4   | 0.204                          |
| 10       | 5   | —   | 0.116                          |
| 11       | —   | 5   | 0.205                          |
| 12       | 6   | —   | 0.115                          |
| 13       | —   | 6   | 0.202                          |
| 14       | 7   | —   | 0.111                          |
| 15       | —   | 7   | 0.202                          |
| 16       | 8   | —   | 0.108                          |
| 17       | —   | 8   | 0.195                          |
| 18       | 9   | —   | 0.108                          |
| 19       | —   | 9   | 0.198                          |
| 20       | 10  | —   | 0.107                          |
| 21       | —   | 10  | 0.196                          |

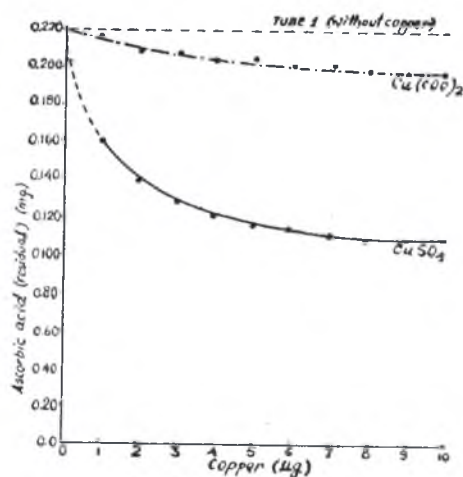


FIG. 3 - COMPARISON BETWEEN THE OXIDATIVE ACTION OF  $\text{CuSO}_4$  AND THAT OF  $\text{Cu}(\text{COO})_2$  IN RELATION TO ASCORBIC ACID

In this way we confirmed the results obtained by HUELIN & MYEE STEPHENS (1948), who, in pure solutions, observed a good protection with oxalic acid in relation to ascorbic acid in its copper catalyzed oxidation. These authors also verified that thiourea presented a prominent protective action against the copper catalyzed ascorbic acid oxidation, in pure solutions. With the purpose of verifying whether thiourea possessed the same protective power against the oxidation by the ascorbic acid oxidase and to compare its action with that of oxalic acid we proceeded to the experiment reported below.

TABLE 4

COMPARATIVE STUDY OF THE ACTION OF OXALIC ACID AND OF THIOUREA IN THE OXIDATION OF ASCORBIC ACID BY COPPER AND BY THE ASCORBIC ACID OXIDASE

Ascorbic acid (initial) 1 ml = 0.206 mg (in each tube)

Buffer pH 4.5 — 5 ml (in each tube)

Temperature: 40°C — Time: 15' — After this time HPO<sub>2</sub> was added to each tube — 2 ml of a 10% (w/v) solution

| Tube n.º | CuSO <sub>4</sub><br>(Cu in µg) | Oxalic acid<br>mg | Thiourea<br>mg | Ascorbic<br>acid<br>oxidase | Ascorbic acid<br>(residual)<br>mg |
|----------|---------------------------------|-------------------|----------------|-----------------------------|-----------------------------------|
| 1        | —                               | —                 | —              | —                           | 0.197                             |
| 2        | 10                              | —                 | —              | —                           | 0.118                             |
| 3        | 10                              | 1.0               | —              | —                           | 0.165                             |
| 4        | 10                              | —                 | 1.0            | —                           | 0.140                             |
| 5        | —                               | —                 | —              | 0.1                         | 0.106                             |
| 6        | —                               | 0.5               | —              | 0.1                         | 0.167                             |
| 7        | —                               | 1.0               | —              | 0.1                         | 0.174                             |
| 8        | —                               | 2.0               | —              | 0.1                         | 0.183                             |
| 9        | —                               | —                 | 0.5            | 0.1                         | 0.100                             |
| 10       | —                               | —                 | 1.0            | 0.1                         | 0.097                             |
| 11       | —                               | —                 | 2.0            | 0.1                         | 0.108                             |

By the above given results we can verify that in the oxidation of ascorbic acid by copper, thiourea in spite of its definite protective action was less efficient than an equal quantity of oxalic acid under the conditions of our experiments. HUELIN & MYEE STEPHENS (1948), working in different conditions, found that the protective action of thiourea was more efficient than that of oxalic acid.

On the other hand we can see that thiourea showed no protective action in relation to the oxidation by the ascorbic acid oxidase.

## DISCUSSION

There can be no doubt that oxalic acid in certain conditions of pH interferes with the activity of ascorbic acid oxidase on its specific substratum, ascorbic acid; such an interference corresponds to a decrease of the activity that the enzyme should have shown under the conditions of the experiment.

It can also be verified that the catalytic action of copper in the oxidation of ascorbic acid is partially inhibited by oxalic acid as well as by others substances such as thiourea as was verified by HUELIN & MYEL STEPHENS (1948) and xanthine as observed by GIRI & KRISHNAMURTHY (1941). Nevertheless thiourea proved to be completely inactive against ascorbic acid oxidase as well as xanthine which also proved inactive as was pointed out by the research of FONSECA RIBEIRO & BONOLDI (1943).

It is therefore evident that we cannot admit the same mechanism of action for thiourea and xanthine on one hand, and for oxalic acid on the other. The latter acts perhaps by its capacity of reacting with copper to form a weak dissociable combination even in the case in which the metal constitutes the action group of an enzyme like ascorbic acid oxidase; of course we should admit here the possibility of a complex through which the enzyme would lack the cupric ion indispensable to its action.

The evident action of oxalic acid in the protection of ascorbic acid in the presence of ascorbic acid oxidase enables us to suggest its possible protective role in many animal or vegetable substrata in which the existence of substances inhibiting ascorbic acid oxidase was demonstrated long ago. Really the existence of oxalic acid in many animal or vegetable tissues may be suggested but the fact that its importance has been considered as secondary until now discouraged its systematic detection except when its presence revealed itself by relatively great quantities. In spite of this point of view, it has been observed (FONSECA RIBEIRO & CARDOSO, 1950) that oxalic acid protects adrenalin, tyrosin, and other substances against the copper catalyzed oxidation besides protecting ascorbic acid against the specific action of ascorbic acid oxidase. These observations and the possibility of other copper or iron containing enzymes being influenced in their activity by oxalic acid seem to be such as to confer on it a more significant role in the interpretation of vital phenomena, in the future.

## SUMMARY

1. Oxalic acid has a definite protective action on ascorbic acid in its oxidation by ascorbic acid oxidase, an enzyme whose active group contains copper; this action is best revealed at pH 4.5.



2. Oxalic acid exerts also its protective action on ascorbic acid oxidation by copper; the comparison between the oxidative effect of copper oxalate and that of copper sulphate shows that the latter is much more intense.

3. The protective action of thiourea exerts itself in the oxidation by copper though less intensively than that of oxalic acid but it does not reveal itself in the oxidation by ascorbic acid oxidase.

4. The protective action of oxalic acid in the oxidation of ascorbic acid by the ascorbic acid oxidase suggests a new field of investigation in the branch of knowledge of those substances, not identified up to the present, which, in natural substrata, protect the ascorbic acid against such an oxidation.

## REFERENCES

- BARRON, E. S. G. — BARRON, A. G. & KLEMPERER, F. — 1936 — *J. Biol. Chem.*, **116**: 563-73
- CLARK, W. M. — 1928 — The determination of hydrogen ions. 3rd ed. Baltimore, Williams and Wilkins Company
- FONSECA RIBEIRO, D. DA & BONOLDI, V. — 1943 — *Rev. Fac. Med. Vet.*, S. Paulo, **2**(3): 87-97
- FONSECA RIBEIRO, D. DA & CARDOSO, F. A. — 1950 — *Rev. Fac. Med. Vet.*, S. Paulo, **4**(2) 235-40
- FUJITA, A. & SAKAMOTO, T. — 1938 — *Biochem. Ztschr.*, **297**:10-23
- GIRI, K. V. & KRISHNAMURTHY, P. V. — 1941 — *Nature*, London, **147**:59
- HUELIN, F. E. & MYEE STEPHENS, I. — 1948 — *Aust. J. Sci. Res.*, **1**:50-7
- LESER, W. S. P. — 1941 — Da importância na higiene alimentar em nossa população, do suprimento de vitamina C pela "Musa chinensis", Sweet (banana nanica), e "Musa paradisiaca", L. sub-espécie "sapientum" Schum., variedade maçã (banana maçã). Tese. São Paulo
- PIMENTA, N. — 1941 — Da proteção do ácido ascórbico observada em dosagens pelo método enzimático. Experimentação na manga ("Mangifera indica", L.). Tese. São Paulo
- TAUBER, H. & KLEINER, I. S. — 1935 — *Proc. Soc. Exp. Biol. & Med.*, **32**:577-8

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