CCVII. SOME OBSERVATIONS ON THE REDUCTION OF IRON BY TISSUE EXTRACTS AND BY ASCORBIC ACID, WITH A NOTE ON THE STABILIZATION OF ASCORBIC ACID SOLUTIONS.

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THE investigations of a large number of workers have made possible a general conception of the metabolism of iron. The retention of iron in the body, blood formation and other aspects of iron metabolism are conditioned by the reduction of food iron in the alimentary tract and its absorption from the duodenum in the ferrous state [McGowan, 1930; Lintzel, 1933]. The absorbed iron is believed to be oxidized in the blood and transported, presumably as a ferric compound of globulin [Starkenstein & Harvalik, 1933], to the liver where it is either utilized in haemoglobin synthesis or stored largely in the reduced state. The presence of ascorbic acid, in relatively large concentration in the intestinal mucosa [Hopkins, 1934; Zilva, 1935] and in the liver, invites speculation as to the role which this substance may play in the absorption of iron from the alimentary tract and in the reduction of blood iron in the liver.

The secondary anaemia of human and guinea-pig scurvy is well known. How far the anaemia is the result of a derangement of iron metabolism due to the lack of a specific reducing substance (ascorbic acid) is not definitely known. Stacy & Chew [1932] believe the fundamental cause to be retarded erythropoiesis. The oral administration of iron was shown to be ineffective in alleviating the anaemia of human scurvy [Mettier *et al.* 1930]. In a series of experiments with guinea-pigs in which scurvy was produced by feeding the diet of Harris *et al.* [1932], we observed an anaemia of similar severity to that found by Stacy & Chew [1932]. During the recovery which followed the injection of 3 mg. of ascorbic acid daily the haemoglobin and red-cell count returned to the normal level. When next the ascorbic acid was withheld and each guinea-pig received instead a daily intraperitoneal injection of 3.2 mg. of Fe⁺⁺ or Fe⁺⁺⁺ in the form of Mohr's salt or ferric chloride the animals again lost weight, all became anaemic and died of scurvy within 12 days. When 6 guinea-pigs were fed the same diet and received daily injections of 3.2 mg. Fe⁺⁺ as Mohr's salt, severe scurvy marked by inanition, rapid loss in weight and the development of a pronounced secondary anaemia developed in all the animals. We believe with Stacy and Chew that the hemorrhages which occurred in any of these animals could not be responsible for the degree of anaemia which ensued.¹

The failure to maintain normal haematopoiesis in animals suffering from advanced scurvy by the parenteral administration of iron is not surprising. The animals received a diet which, owing to the inclusion of egg-yolk, had a high iron content (0.021 % Fe). Presumably the iron stored in the liver should have been

¹ In view of the negative findings in these experiments it was considered that no useful purpose would be served by giving the detailed results.

sufficient to permit the haemoglobin content of the blood to be maintained at the normal level during the short period of extreme inanition. It may be concluded that the anaemia of scurvy is not due to a diminished absorption of iron. Eichholtz & Unrath [1935] have found that feeding mice with ascorbic acid along with iron did not increase the amount of so-called "catalytically active" iron available to the organism.

If a derangement in iron metabolism is responsible for the anaemia of scurvy it would appear to be localized in the mechanism which brings about the reduction of iron in the liver. The results of the experiments just described would not exclude this possibility if, as Starkenstein & Harvalik [1933] believe, injected ferrous iron is rapidly oxidized by the blood. In fact direct evidence in this regard is not easily obtained. The experiments *in vitro* now to be described are mainly concerned with certain aspects of the reduction of iron salts by tissue extracts and by ascorbic acid and constitute an attempt to obtain indirect evidence linking ascorbic acid in the chain of processes involved in the metabolism of iron.

1. THE REDUCTION OF FERRIC ION BY TISSUE EXTRACTS.

The technique and the accuracy of the method employed to determine the amount of iron reduced under the conditions of succeeding experiments is illustrated below in a brief summary of the results of an experiment which show, as anticipated, that the reaction between ascorbic acid and ferric ion involves a two-electron change.

Composition of the test solution:

8 ml. of phthalate or acetate buffer.

1 ml. of a ferric chloride solution containing 0.50 mg. Fe.

1 ml. of approximately 0.001 *M* ascorbic acid solution.

4.4 mg. B.D.H. ascorbic acid plus 3.0 ml. 0.001 *M* solution of sodium diethyldithiocarbamate and diluted to 25 ml. with ordinary distilled water.

This solution was found, by iodine titration, to contain 0.173 mg. ascorbic acid per ml. A note at the end of this paper explains the use of the carbamate reagent.

The final *pH* of the mixture was determined by the quinhydrone electrode. Solutions of *pH* 2.2 to 5.5 were employed.

Titanium titration:

Using the micro-technique of McFarlane [1936] repeated titrations of 2.0 ml. aliquots of the above mixtures gave values varying from 0.190 to 0.199 ml. of titanous chloride solution. The total iron (0.10 mg. Fe⁺⁺⁺) in a 2 ml. aliquot required 0.249 ml. of the titanous chloride solution for its quantitative reduction.

Therefore 0.190 ml. TiCl₃ solution is equivalent to 0.0763 mg. Fe and 0.199 ml. TiCl₃ solution is equivalent to 0.080 mg. Fe.

1 ml. 0.001 *M* ascorbic acid solution at *pH* 2.2–5.5 reduced 0.120–0.102 mg. Fe.

Calculated on the basis that one molecule of ascorbic acid reduces two atoms of iron.

1 ml. 0.001 *M* ascorbic acid solution should reduce 0.112 mg. Fe.

At physiological hydrogen ion concentrations ascorbic acid, according to Szent-Györgyi [1934] forms a stable complex with ferrous iron. A simple calculation, using the values for the "inorganic" iron content of tissues as given by Tompsett [1935] or McFarlane [1934] shows that the amount of ascorbic acid usually considered to be contained in these tissues is more than sufficient to hold the iron in the reduced state. Measurement of the degree to which trichloroacetic

acid extracts of tissues reduce 2:6-dichlorophenolindophenol forms the basis of the well known procedure of Birch *et al.* [1933] for the estimation of ascorbic acid. It becomes of interest to determine the degree to which tissue extracts will reduce ferric ion relatively to their reducing intensity as measured by the dye titration. These measurements have been made and the results are presented in Table I.

The general procedure employed to obtain these data was to triturate a weighed quantity of the tissue with 5 ml. of a 20% solution of trichloroacetic acid and a small amount of quartz sand.¹ The suspension was centrifuged and the precipitate washed with 20 ml. of 1% trichloroacetic acid solution. The combined centrifugates were made up to a definite volume. An aliquot was transferred to a small Erlenmeyer flask and to it was added an equal volume of phthalate-HCl buffer of pH 4.5 and a measured volume, usually 2 ml., of a standard ferric chloride solution containing 0.05 mg. Fe⁺⁺⁺ per ml. The pH of the mixture was approximately 2.5. The amount of iron reduced was determined and its equivalent as ascorbic acid calculated by the procedure already described.

According to Fugita & Iwataki [1935] the estimation of ascorbic acid is more accurately carried out using metaphosphoric acid instead of trichloroacetic acid extracts. Simultaneous determinations have also been made using their procedure.

Table I. *The ascorbic acid content of various tissues as calculated from the amount of iron reduced by trichloroacetic acid extracts and from the amount of dye reduced by trichloroacetic acid or metaphosphoric acid extracts.*

Tissue	Trichloroacetic acid extract		Metaphosphoric acid extract	% of indophenol value estimated by	
	Iron reduction	Dye titration	Dye titration	(a) Iron reduction	(b) Colorimetric†
1. Rat livers	0.21*	0.29	0.27	72	67
	0.22	0.28	—	75	—
2. Rat spleens	0.30	0.50	—	60	100
	0.31	0.48	—	64	88
3. Rat kidneys	0.14	0.34	0.22	41	46
	0.13	0.35	—	40	62
4. Chick livers	0.13	0.38	0.35	—	—
	0.11	0.37	0.37	—	—
5. Ox adrenals:					
A. Fresh	0.59	1.19	1.21	53	—
	0.78	1.24	1.30	63	—
	0.58	1.67	—	34	30§
	0.49	1.62	—	30	—
B. Frozen, 4 months	0.44	0.96	0.96	46	—
	0.42	0.96	—	44	—
6. Orange juice	0.45†	0.47	—	96	—

* mg. per g. of tissue—average value of duplicate analysis.

† mg. per ml.

‡ Taken from the paper of Fugita *et al.* [1935, Table VIII].

§ Guinea-pig adrenals.

It will be observed (Table I) that the amount of ascorbic acid in these animal tissues as calculated from the amount of iron reduced is always lower than that

¹ The quartz sand was freed from iron by extraction with hot concentrated HCl.

obtained by the dye titration. This is particularly so in the case of the kidney and the adrenal. In general the results by iron reduction are in good agreement with those which were obtained by Fugita *et al.* [1935] using a colorimetric procedure involving the reduction of sodium tungstate in alkaline solution. According to Van Eekelen [1935] this colorimetric method gives low results when applied to the adrenal gland owing to the interfering action of adrenaline. Difficulties attending the estimation of ascorbic acid in kidney extracts are mentioned by Hopkins & Slater [1935] who found that the results are dependent upon the rate at which the dye titration is carried out. It still remains to be determined whether any of the values given in Table I represent the true ascorbic acid content of the particular tissue.

Ascorbic acid estimations in orange juice by iron reduction and by dye titration have consistently given results which were practically identical.

Millikan [1935] has shown that the rate of reduction of 2:6-dichlorophenol-indophenol by cysteine is very much slower than that obtained with ascorbic acid. We have observed a similar difference in the rates at which ascorbic acid and glutathione reduce ferric ion. When 1 ml. of a 0.001 *M* solution of ascorbic acid is added to each one of a series of solutions ranging in *pH* from 3.1 to 5.0 and composed of 3.0 ml. phthalate-HCl or NaOH buffer, 0.5 ml. ferric chloride solution containing 0.05 mg. Fe^{+++} and 0.5 ml. of 0.001 *M* $\alpha\alpha'$ -dipyridyl solution, the pink colour of ferrous dipyridyl develops immediately and in a few minutes reaches an intensity indicating quantitative reduction. When the experiment is repeated using 1 ml. of a 0.001 *M* solution of crystalline reduced glutathione instead of ascorbic acid no evidence of reduction is obtained in the first hour. A trace of colour is observed after 3 hours which develops slowly to reach maximum intensity in about 24 hours. It would thus appear that sulphydryl compounds exert little, if any, influence upon the reduction of iron by tissue extracts under the conditions of these experiments.

2. THE REDUCTION OF IRON SALTS BY ASCORBIC ACID.

Assuming that ascorbic acid plays some role in the reduction of the iron in tissues, the question now arises as to the nature of the iron compound(s) reduced at the hydrogen ion concentration of the tissue. Obviously some complex of iron and not ferric ion is involved. When ferric chloride is added to a series of Sørensen's phosphate mixtures (*pH* 4.7–8.0) the iron is precipitated at approximately *pH* > 6.0 and the precipitated iron, we have found, is not reduced by ascorbic acid. We have already shown [McFarlane, 1934] that about 60 % of the non-haematin iron in liver tissue is precipitated in the trichloroacetic acid fraction and is reducible by sodium hydrosulphite. Further, when a solution of ferric chloride is added to liver pulp the added iron is completely precipitated by 50 % alcohol. The iron extracted from tissues by trichloroacetic acid is all in the ferrous form and presumably is present in the tissues as a soluble ferrous complex possibly as ferrous ascorbate. Considering these facts and the observations of Starkenstein and Harvalik, already referred to, it would appear probable that an iron-protein complex is involved.

We have taken leicithovitellin as an example of such an iron-protein compound. To a solution of leicithovitellin, prepared according to McFarlane [1932], and buffered at *pH* 7.4 were added $\alpha\alpha'$ -dipyridyl and ascorbic acid. A pink colour developed slowly and reached an intensity indicating quantitative reduction of the iron in a few hours. Reduced glutathione under the same conditions also reduced the iron of leicithovitellin but at an even slower rate.

It is now well known that hydroxy-organic acids combine with iron to form soluble unionized compounds. The influence of pH on the reduction of ferric lactate by ascorbic acid was next investigated.

At $pH < 4.1$ one mole of ascorbic acid reduced 2 atoms Fe.

At $pH 4.6-5.2$ one mole of ascorbic acid reduced 1 atom Fe.

At $pH > 5.6$ ascorbic acid failed to reduce any iron.

According to Smythe [1931] lactic acid behaves as a dibasic acid when titrated in the presence of ferric chloride, the pK_a for the alcoholic hydroxyl group being about 3.8. With ferric glutamate solutions we found that:

At $pH < 4.9$ one mole of ascorbic acid reduced 2 atoms Fe.

At $pH 5.1-5.9$ one mole of ascorbic acid reduced 1 atom Fe.

At $pH > 6.1$ no reduction took place.

Smythe & Schmidt [1930] have observed that glutamic acid will retain some iron in solution at $pH 5.0$ but that it is all precipitated at $pH 6.0$.

The inference from these findings is that some form of iron-protein combination is a conditioning factor in so far as the reduction of tissue iron by ascorbic acid is concerned.

3. A NOTE IN REGARD TO THE INHIBITION OF THE CATALYSIS OF THE OXIDATION OF ASCORBIC ACID BY HEAVY METALS.

Mawson [1935] has shown that whilst the action of copper and of ferrous or ferric iron in catalysing the aerobic oxidation of ascorbic acid is retarded by glutathione, cysteine, cystine and H_2S the same protective action of tissue extracts is not quantitatively accounted for on the basis of their content of sulphhydryl compounds. Recently Hunter [1935] has isolated taurine in considerable amounts from the adrenal gland. Taurine, we have found, exerts no influence upon the copper catalysis of ascorbic acid oxidation. At the same time we have made some general observations in regard to the stabilizing action of several sulphur-containing compounds.

We first found that a concentration of copper between 1×10^{-4} and 5×10^{-5} mg. just effects the complete oxidation, in 1 hour at 38° , of 0.18 mg. of ascorbic acid in a total volume of 5 ml. of phosphate solution,¹ $pH 6.6$. The effects of several

Table II. *Showing the influence of various sulphur compounds on the catalysis of ascorbic acid oxidation by copper.*

Composition of the test solutions:

1.0 ml. 0.001 M ascorbic acid solution (standardized by iodine titration).

1.0 ml. copper sulphate solution (2×10^{-4} mg. Cu per ml.).

1.0 ml. 0.0001 M solution of the sulphur compound.

2.0 ml. phosphate buffer $pH 6.6$.

Sulphur compound	% oxidation after incubation at 38° for 1 hour
Control—no sulphur compound	100
Taurine	100
Glutathione	85
Cysteine hydrochloride	33
Cystine	27
Sodium diethyldithiocarbamate	zero

¹ Sørensen's phosphate mixture. The primary and secondary phosphates employed were three times recrystallized from glass-distilled water. All filtrations were made using Jena sintered filters.

compounds in inhibiting this oxidation were next investigated with the results shown in Table II. The general procedure used to estimate the reduced ascorbic acid remaining in the test solution was to add an excess of a solution of 2:6-dibromophenolindophenol, which had been standardized against a standard ascorbic acid solution and against titanous chloride, and to back-titrate the excess of the dye with titanous chloride.

It is well known that at extreme dilutions sodium diethyldithiocarbamate forms an orange-yellow coloured undissociated complex with copper. Compared with the other sulphhydryl compounds the action of glutathione was slight. Taurine or glycine even in 0.01 *M* solution has no effect. Even in the presence of the carbamate reagent ascorbic acid solutions in ordinary distilled water become increasingly unstable at *pH* > 7.5.

Further experiments (Table III) have shown that the oxidation of the ascorbic acid in orange juice left standing in the laboratory for 9 hours is almost completely inhibited by adding sodium diethyldithiocarbamate and $\alpha\alpha'$ -dipyridyl but is not affected by adding either reagent singly. In fact the addition of $\alpha\alpha'$ -dipyridyl alone appeared to result in an acceleration of the oxidation. Glutathione has practically the same effect as the combination of dipyridyl and carbamate reagent.

Table III. *Showing the effect of $\alpha\alpha'$ -dipyridyl, sodium diethyldithiocarbamate or glutathione on the rate of oxidation of ascorbic acid in orange juice.*

Test solutions	Ascorbic acid (mg./ml. orange juice)		% oxidation
	Zero hour	After 9 hours	
1 ml. orange juice + 2 ml. H_2O *	0.46	0.18	61
1 ml. orange juice + 1 ml. 0.001 <i>M</i> dipyridyl + 1 ml. H_2O	0.41	0.09	78
1 ml. orange juice + 2 ml. 0.001 <i>M</i> dipyridyl + 1 ml. H_2O	0.39	0.06	85
1 ml. orange juice + 1 ml. 0.001 <i>M</i> carbamate + 1 ml. H_2O	0.44	0.16	64
1 ml. orange juice + 1 ml. 0.001 <i>M</i> carbamate + 1 ml. 0.0001 <i>M</i> dipyridyl	0.46	0.42	9
1 ml. orange juice + 2 ml. 0.02 <i>M</i> glutathione	0.46	0.40	13

* Three times redistilled from glass and also used as the solvent for the other reagents employed.

The observation of Mawson in regard to the factors influencing the stability of ascorbic acid in fruit juice is therefore confirmed and extended. The applications of these reagents in facilitating the ascorbic acid analysis of plant juices and in the preparation of stable aqueous solutions of ascorbic acid are obvious.

SUMMARY.

The results of some experiments *in vitro* on the reduction at different *pH* values of ionic iron, iron in combination with lactic acid, glutamic acid or protein by ascorbic acid indicate that the reduction of tissue iron *in vivo* by ascorbic acid must involve some form of iron-protein complex. The relative capacities of extracts of several tissues to reduce Fe^{+++} and 2:6-dichlorophenolindophenol have been quantitatively measured.

The catalysis of ascorbic acid oxidation by copper is inhibited by the following substances, in order of decreasing activity, sodium diethyldithiocarbamate, cystine, cysteine and glutathione but not by taurine or glycine. The aerobic oxidation of ascorbic acid in orange juice is inhibited by adding $\alpha\alpha'$ -

dipyridyl and sodium diethyldithiocarbamate together. It is not affected by adding the carbamate reagent alone but may actually be accelerated by the single addition of dipyridyl.

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