SUMMARY

1. Muscle extracts of twenty different species of fish have been examined electrophoretically at 0.05*M*, pH 7.5.

2. Each species has a characteristic electrophoretic diagram, and considerable differences in mobility and composition exist between species.

3. The diagrams may, however, be still divided into definite mobility groups of approximately consistent quantitative size.

4. The diagrams of related species appear to be as dissimilar as those of unrelated species.

5. The effect of age and of species variants on the diagrams has been examined.

6. Elasmobranch muscle appears to contain proteins with unusually high isoelectric points.

7. The question of the identity of components of different diagrams has been discussed. Whatever the answer, it is certain that components which are present in some species are completely absent in others.

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Comparative Observations on the Chemical Properties and Biological Activities of L-Ascorbic and D-isoAscorbic Acids

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In a previous publication the course of oxidation of L-ascorbic acid under various conditions and in the presence of different accompanying substances was described (Leibowitz & Guggenheim, 1938*a*). It was shown that the stability of the vitamin in solution depends specifically on the nature of the accompanying substances and on the pH. It has also been shown that L-ascorbic acid exerts a detoxifying action on potassium cyanide, phenol and tetanus toxin (Leibowitz & Guggenheim, 1938*b*; Kligler, Guggenheim & Warburg, 1938). Some evidence, derived from titrimetric and cryoscopic data, indicated that the detoxifying effect results from the reversible formation of a chemical compound between the vitamin and potassium

cyanide or phenol. Other authors have reported that ascorbic acid diminishes the toxicity of various other substances such as arsphenamines, lead, sulphonamides, benzene, trinitrotoluene, and anaesthetics (Bicknell & Prescott, 1948). It has been tentatively suggested that the antiscorbutic function of L-ascorbic acid may be at least partially due to its ability to detoxify naturally produced toxic substances (Leibowitz & Guggenheim, 1938*b*).

Numerous clinical and experimental observations have indicated that ascorbic acid plays a role in certain forms of anaemia (Vilter, 1947; Dyke, Della Vida & Delikat, 1942; May, Nelson, Lowe & Salmon, 1950). Studies by Nichol & Welch (1950) and Welch, Nichol, Anker & Boehne (1951) have

shown that ascorbic acid promotes the formation of citrovorum factor from folic acid.

A series of chemical analogues of L-ascorbic acid has been prepared synthetically (Smith, 1946). One of these compounds, *iso*ascorbic acid (D-arbo-ascorbic acid) shows great chemical similarity to vitamin C. This compound, however, has only one-twentieth of the antiscorbutic activity of L-ascorbic acid (Dalmer & Moll, 1933; Demole, 1934). These facts afforded the opportunity of testing our hypothesis concerning the relationship between the antiscorbutic activity of vitamin C and the above-mentioned properties. The two compounds were compared as to: (1) their ability to form complexes with potassium cyanide or phenol; (2) their detoxifying effect on potassium cyanide, phenol and tetanus toxin; and (3) their ability to increase urinary excretion of citrovorum factor after administration of folic acid.

MATERIALS AND METHODS

The ability of ascorbic acid and of *iso*ascorbic acid to detoxify potassium cyanide was tested with *Escherichia coli*. The bacteria were grown in nutrient broth, and the amount of growth obtained after 24 hr. was measured turbidimetrically in a Klett-Summerson photoelectric colorimeter. Tetanus toxin was prepared in the following way: *Clostridium tetani* was grown in Tarozi broth at 37°. After 10 days the broth was filtered and centrifuged, and the toxin-containing supernatant was tested on young mice weighing 15–25 g. Appropriately diluted broth was injected intramuscularly allowing 0.1 ml./10 g. of body weight. The mice were observed for 6 days and the mortality from tetanus noted.

The conversion of folic acid into citrovorum factor was studied with six adult rats weighing 200–300 g. They received a basal diet consisting of corn starch 73, casein 18, olive oil 5, and salt mixture 4%, which was supplemented with 0.3 mg. riboflavin, 0.2 mg. thiamine, 0.1 mg. pyridoxine, 1.6 mg. calcium pantothenate, and 100 mg. choline chloride per 100 g. ration. Each rat received 100 i.u. vitamin A and 4 i.u. vitamin D twice weekly. Citrovorum factor was determined with *Leuconostoc citrovorum* (ATCC 8081) according to Sauberlich & Baumann (1948). After incubation for 3 days at 37° the acid produced was titrated with 0.02N-NaOH. Synthetic citrovorum factor (leucovorin)* in the form of the hydrated barium salt was used as standard.

RESULTS

Polarimetric studies

Experiments on the change in the rotation values of ascorbic acid and *iso*ascorbic acid induced by potassium cyanide will be described elsewhere. These experiments require special treatment

because of the complications arising from the powerful effect of pH on the optical activities of both acids. Data typical of many experiments with phenol are presented in Table 1. Small but significant changes are induced in the optical rotations of both ascorbic acid and *iso*ascorbic acid, the

Table 1. *Effect of phenol on the optical rotation of ascorbic and isoascorbic acids in aqueous solutions*

[Ascorbic acid] (M)	[<i>iso</i> Ascorbic acid] (M)	[Phenol] (M)	Incubation at 30° (hr.)	α_D (1 dm.) (°)
0.1	—	—	0	+0.39
0.1	—	—	6	+0.38
0.1	—	0.1	0	+0.36
0.1	—	0.1	6	+0.36
0.1	—	0.1	24	+0.34
0.4	—	—	0	+1.79
0.4	—	—	6	+1.77
0.4	—	—	24	+1.78
0.4	—	0.1	0	+1.74
0.4	—	0.4	0	+1.70
0.4	—	0.4	6	+1.71
0.4	—	0.4	24	+1.70
—	0.1	—	0	-0.28
—	0.1	—	6	-0.26
—	0.1	0.1	0	-0.30
—	0.1	0.1	6	-0.30
—	0.1	0.1	24	-0.31
—	0.4	—	0	-1.09
—	0.4	0.4	0	-1.15
—	0.4	0.4	6	-1.18
—	0.4	0.4	24	-1.17

dextrorotation of the former being slightly decreased and the laevorotation of the latter slightly increased. The order of magnitude of the effects is almost the same in both cases. The effects are immediate but are somewhat intensified by incubation at 30°.

Cryoscopic studies

The freezing-point depressions resulting from the mixing of equal amounts of equimolecular aqueous solutions of ascorbic acid or *iso*ascorbic acid with potassium cyanide or phenol were measured. The freezing-point depressions of the mixtures were measured immediately after mixing. The half-values of the sums of the depressions of the components were taken as 'calculated' values (Table 2).

The experimental values of the freezing-point depressions of mixtures of each of the acids with potassium cyanide or phenol were appreciably less than those calculated from separate depressions of the components, indicating a decrease in molecular concentration, due to partial combination of the acids with the reagents to form condensation or addition products. The effect was more pronounced with potassium cyanide than with phenol. No further change in the depression values was induced by incubation of the mixtures at room temperature or at 30° for 24 hr.

* Leucovorin was supplied by the Lederle Laboratories Division, American Cyanamid Company, by the courtesy of Dr T. H. Jukes.

Table 2. *Effect of potassium cyanide and phenol on cryoscopic determinations in solutions of ascorbic and isoascorbic acids*

[Ascorbic acid] (M)	[isoAscorbic acid] (M)	[KCN] (M)	[Phenol] (M)	Δ_{Found} (°)	$\Delta_{\text{Calculated}}$ (°)
0.1	—	—	—	0.203	—
—	0.1	—	—	0.210	—
—	—	0.1	—	0.354	—
0.05	—	0.05	—	0.257	0.278
—	0.05	0.05	—	0.262	0.282
0.2	—	—	—	0.402	—
—	0.2	—	—	0.412	—
—	—	—	0.2	0.389	—
0.1	—	—	0.1	0.385	0.396
—	0.1	—	0.1	0.393	0.401

Table 3. *Effect of ascorbic and isoascorbic acids on the potassium cyanide inhibition of Esch. coli*

(The figures indicate galvanometer readings obtained after 24 hr. incubation of bacteria with solutions of KCN, ascorbic acid and isoascorbic acid. Readings adjusted to 200 for culture without substances added. Average of three sets of experiments.)

[KCN] (M)	[Ascorbic acid] (M)	[isoAscorbic acid] (M)	Substances		
			Freshly dissolved	Dissolved and mixed together 24 hr. before the onset of incubation	Dissolved and kept separately for 24 hr. before the onset of incubation
—	—	—	200	200	200
—	0.01	—	258	254	255
—	—	0.01	243	212	222
0.02	—	—	0	25	25
0.01	—	—	54	81	81
0.005	—	—	71	102	102
0.02	0.01	—	0	141	54
0.01	0.01	—	51	176	118
0.005	0.01	—	77	192	113
0.02	—	0.01	10	91	23
0.01	—	0.01	33	139	103
0.005	—	0.01	93	155	125

Detoxication of potassium cyanide

Three experiments were performed. In the first experiment neutralized solutions of potassium cyanide, ascorbic acid and isoascorbic acid were added to samples of nutrient broth in amounts to give the final concentration indicated in Table 3. Sufficient water was added to bring the volume to 5 ml.; then the test tubes were inoculated with *Esch. coli*. In the second experiment similar mixtures of potassium cyanide, ascorbic acid and isoascorbic acid solutions were incubated at room temperature for 24 hr. before being added to the nutrient broth. In the third experiment which served as control for the second, the solutions of potassium cyanide, ascorbic acid and isoascorbic acid were kept separately at room temperature for 24 hr. and separately added to the nutrient broth immediately before inoculation. The amount of growth obtained after 24 hr. incubation is shown in Table 3.

It may be observed that neither ascorbic acid nor isoascorbic acid was able to affect the potassium

cyanide inhibition of growth of *Esch. coli* when added separately immediately prior to incubation. If, however, the ascorbic acid or isoascorbic acid were pre-incubated with potassium cyanide for 24 hr., the mixtures were far less toxic for the bacteria than the same concentrations of potassium cyanide alone. These findings confirm previous results (Leibowitz & Guggenheim, 1938b) obtained with ascorbic acid. It is noteworthy that no marked difference was noted between the detoxifying effect of ascorbic acid and isoascorbic acid.

Detoxication of phenol

Solutions of phenol (50 mg./ml.) were incubated with equimolar quantities of ascorbic acid or isoascorbic acid at 40° for 4 hr. The toxicity of these solutions was tested on young rats weighing 50–100 g.; the dose injected subcutaneously contained 50 mg. phenol per 100 g. of body weight. Three groups, each comprising twenty-two animals were used: the first group received phenol-ascorbic acid, the second phenol-isoascorbic acid, the third phenol alone. In the first group six rats died, in the

second twelve, whereas in the third group all twenty-two animals succumbed. The difference in mortality between the first or the second group and the third group proved to be statistically significant, whereas the difference between the first and the second group was insignificant. Ascorbic acid and *iso*ascorbic acid are, therefore, equally active in detoxifying phenol.

Detoxication of tetanus toxin

Equal volumes of the toxic broth and of solutions of ascorbic acid or *iso*ascorbic acid (1 mg./ml.) were incubated at 37° for 24 hr. In the first experiment the broth was diluted 1:100 and injected into ten mice. All these mice died during the next 6 days. Control groups given the toxin-ascorbic acid or the toxin-*iso*ascorbic acid mixture suffered no loss during the same time. In the second experiment two groups of thirty mice were injected with a solution diluted 1:8 of the toxic broth which had been incubated with ascorbic acid or *iso*ascorbic acid. The mortality during the following 6 days amounted to twenty and twenty-one mice, respectively. Thus no difference was noted in the ability of ascorbic acid and *iso*ascorbic acid to detoxify tetanus toxin.

Conversion of folic acid to citrovorum factor

Rats were maintained for 6 days on the normal diet. During the following 6 days the rats received 1 mg. folic acid daily intraperitoneally. During the third and fourth 6-day periods the rats received daily injections of folic acid and 17.6 mg. of ascorbic acid or *iso*ascorbic acid. Urine was collected and analysed for citrovorum factor during the last 3 days of each period. Table 4 shows the average daily excretion of citrovorum factor during these four periods.

Table 4. *Urinary excretion of citrovorum factor by rats*

(Average of six rats; means and standard errors.)

Substances injected	Citrovorum factor in urine (μ g./day/rat)
None	2.9 $\pm$ 0.32
Folic acid	13.5 $\pm$ 5.38
Folic acid + ascorbic acid	142.0 $\pm$ 31.25
Folic acid + <i>iso</i> ascorbic acid	153.0 $\pm$ 14.58

It follows from Table 4 that both ascorbic acid and *iso*ascorbic acid increase urinary excretion of citrovorum factor. *iso*Ascorbic acid was found to be as active in this respect as ascorbic acid.

DISCUSSION

The similarity which exists between the chemical properties of ascorbic acid and *iso*ascorbic acid

extends to their biological activities as well. The two compounds have almost the same ability to combine with potassium cyanide or phenol as measured polarimetrically or cryoscopically. Moreover, both ascorbic acid and *iso*ascorbic acid prevent the toxic effects of potassium cyanide, phenol and tetanus toxin on *Esch. coli*, rats, and mice, respectively. Ascorbic acid and *iso*ascorbic acid also show practically the same effect on the conversion of folic acid into citrovorum factor in rats. Identical actions of ascorbic acid and its analogues on other biological systems have already been described. L- and D-Ascorbic acids are equally effective in increasing the height of contraction of the isolated frog heart (Kramer, Linstead & Todd, 1943) and in reducing the toxicity of neoarsphenamine (McChesney, Barlow & Klinck, 1944). Ascorbic acid and *iso*ascorbic acid exert the same effect in promoting the oxidation of *p*-hydroxyphenylpyruvic acid (Knox & LeMay-Knox, 1951) and of tyrosine by liver homogenates (Sealock, Goodland, Summerwell & Brierly, 1952). *iso*Ascorbic acid can replace ascorbic acid as growth factor for *Trichomonas* (Cailleau, 1939) but not for trypanosomes (Lwoff, 1951).

The role played by ascorbic acid in the metabolic activation of folic acid has been studied by Welch and co-workers (Nichol & Welch, 1950; Welch *et al.* 1951). They found that the addition of either ascorbic acid or its analogue glucoascorbic acid to liver slices *in vitro* markedly increases the conversion of folic acid to citrovorum factor, and that both substances increase the excretion of citrovorum factor when administered together with folic acid. On the other hand, such potent reducing agents as cysteine and glutathione had little or no influence.

Our experiments show that *iso*ascorbic acid shares with ascorbic acid the ability to detoxify potassium cyanide and phenol and to stimulate the conversion of folic acid to citrovorum factor in the rats. Since the antiscorbutic activity of *iso*ascorbic acid is negligible, it seems impossible to assume that the antiscorbutic activity of ascorbic acid is due to the above-mentioned chemical and biological properties.

SUMMARY

1. Ascorbic and *iso*ascorbic acids (D-arabo-ascorbic acid) show similar abilities to combine with potassium cyanide and phenol in aqueous solutions.

2. Ascorbic and *iso*ascorbic acids are equally effective in detoxifying potassium cyanide, phenol and tetanus toxin, and in the conversion of folic acid into citrovorum factor.

3. The antiscorbutic activity of ascorbic acid cannot be explained on the basis of the detoxifying activity of this substance.

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The Effect of L-Ascorbic Acid on the *in vitro* Activity of Polyphenoloxidase from Potato

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In recent years many studies of the action of polyphenoloxidase on various substrates, *in vitro*, have been carried out in the presence of L-ascorbic acid (Asimov & Dawson, 1950; Miller, Mallette, Roth & Dawson, 1944; Robinson & Nelson, 1944; Sreerangachar, 1943). In these studies the L-ascorbic acid has been assigned the role of a reducing agent for any *o*-quinones produced during the oxidation of the substrates. It has been generally assumed (Asimov & Dawson, 1950; Miller & Dawson, 1941; Kendal, 1949; Sreerangachar, 1943) that the ascorbic acid has no other effect on the system, although many observers have reported that the rate of oxidation was sometimes significantly different in the presence of ascorbic acid than in its absence (Kendal, 1949; Roberts & Wood, 1950; Krueger, 1950).

Krueger (1950) made a study of the effect of ascorbic acid on the rate of oxidation of tyrosine by mushroom polyphenoloxidase and concluded that initially the ascorbic acid had a 'pro-oxidant' effect on the system since the initial oxygen uptake was substantially increased and the induction period was decreased (cf. Kendal, 1949), but later in the reaction it had an antioxidant effect since the rate

of production of L- β -3:4-dihydroxyphenylalanine (dopa) was decreased below that for the ascorbic acid-free system.

The investigations of Kertesz (1951, 1952) have shown that the observed oxidation rates of polyphenoloxidase systems in the presence of ascorbic acid are markedly dependent on the concentration of cupric ion, and since Krueger apparently did not use copper-free water for his experiments, it seemed worth while to re-investigate this system.

Our experiments, using a colorimetric procedure to determine enzyme activity, show that ascorbic acid has an inhibitory action on potato polyphenoloxidase which is not elicited in the presence of ionic copper. However, when the enzyme was treated with ascorbic acid in the absence of oxygen and the ascorbic acid subsequently removed by dialysis, it was irreversibly changed so that reactivation with cupric salts was not possible. Oxidation products of ascorbic acid, including dehydro-ascorbic acid, were found to have little effect on the enzyme.

It is suggested that the ascorbic acid affects specific structures of the enzyme (cf. Kertesz, 1951, 1952) in which copper is involved.