THE OXIDATION OF ASCORBIC ACID AND ITS REDUCTION IN VITRO AND IN VIVO*

By HENRY BORSOOK, HORACE W. DAVENPORT,† CECIL E. P. JEFFREYS, and ROBERT C. WARNER†

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena)

(Received for publication, June 8, 1936)

The outstanding chemical property of ascorbic acid (vitamin C) is that it is a reducing agent. The suggestion is obvious that its physiological function may be associated with this property, and, if it is oxidized reversibly, with its behavior in an oxidation-reduction system. It is desirable therefore to know the oxidation-reduction potential of ascorbic acid.

A number of attempts were made to measure this potential (2–6) both before and after it was established that ascorbic acid (earlier called hexuronic acid) and vitamin C were identical. In all except one of these studies it was reported that ascorbic acid does not yield thermodynamically reversible potentials. More or less rapid negative potential drifts were observed and the final steady value was independent of the initial concentration of the oxidized form. The one claim that a reversible potential was obtained (3) was based upon inadequate evidence; and later studies have shown that the order of magnitude of the potential given was widely inaccurate.

The difficulty here is 2-fold. Both the reduced and the reversibly oxidized forms of ascorbic acid react slowly with the electrode, and, what is more important, the reversibly oxidized

* Nearly all of the work described in this paper, with the exception of the glutathione experiments, was presented at the meeting of the American Chemical Society at San Francisco, August, 1935. Its submission for publication was delayed for the sake of presenting a more complete treatment of the reducing mechanism of oxidized ascorbic acid in the tissues. A preliminary account of some of the glutathione experiments has appeared (1).

† California Fruit Growers Exchange Fellow.

form, now commonly called dehydroascorbic acid, undergoes an irreversible change in aqueous solution above pH 4 at ordinary temperatures. When these two factors were taken into account, it was possible to obtain a fairly accurate value for the reversible potential of the first oxidation stage (7). This behavior of ascorbic acid and the value of the potential given were confirmed by Wurmser and de Loureiro (8). Somewhat later Fruton (9) reported values for this potential 100 millivolts more negative than those obtained by Wurmser and de Loureiro and by ourselves. It is shown below that the potential reported by Fruton pertains to a second oxidation stage.

This instability of dehydroascorbic acid is responsible for the variation in antiscorbutic potency of oxidized solutions of the vitamin, as well as for the complex behavior of ascorbic acid *in vitro*. The greater part of the present study deals with different aspects of this irreversible change. We have attempted on the one hand to elucidate some of the difficulties which have been encountered in determining the reversible oxidation-reduction potential of ascorbic acid, and on the other to follow, guided by the *in vitro* findings, the fate of dehydroascorbic acid *in vivo*.

The work reported here falls into four parts—physicochemical measurements, nutrition, and physiological experiments, and a study of the interaction of oxidized ascorbic acid and glutathione. In order to facilitate following the argument through a varied and extended series of experiments we shall present here a description of the main features of the irreversible change in dehydroascorbic acid. As stated, dehydroascorbic acid undergoes a spontaneous, irreversible change at ordinary temperatures in aqueous solution at hydrogen ion concentrations less than pH 4. This change is responsible for the negative potential drift observed in electrometric measurements of the oxidation-reduction potential, and for the loss in reversibility of the first oxidation stage. The product of this change is a stronger acid than dehydroascorbic acid, and is a more powerful reducing agent than ascorbic acid itself. It is distinguished from dehydroascorbic acid also in that it is not reduced by H_2S in acid solution, nor by glutathione in neutral or alkaline solution. It is not antiscorbutic; whereas dehydroascorbic acid possesses very nearly the same antiscorbutic potency as the reduced form of the vitamin (the form in which most of it is

found in nature), although the "half-life" of dehydroascorbic acid *in vitro* at the pH and temperature of the tissues is only a few minutes. The rates of appearance of all of these manifestations of the irreversible change in dehydroascorbic acid exhibit the same dependence on the hydrogen ion concentration. They are also all independent of the presence of air or oxidizing agents. The irreversible change is therefore not an oxidation. It is also independent of the oxidizing agent used to form dehydroascorbic acid. The resolution of the paradox between the high antiscorbutic potency of dehydroascorbic acid and its rapid loss in potency *in vitro* at the pH and temperature of the blood and tissues turned out to be simply that dehydroascorbic acid is rapidly reduced *in vivo* to ascorbic acid. The principal reducing agent here is glutathione. Apart from this rapid reduction we found no evidence of any greater stability of dehydroascorbic acid *per se* in blood or in the tissues than *in vitro*.

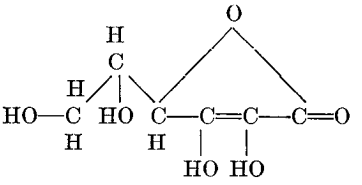
Physicochemical Measurements

An account of these measurements is presented first because these data are necessary for an appraisal of the possibilities of ascorbic acid as a reducing agent *in vivo*.

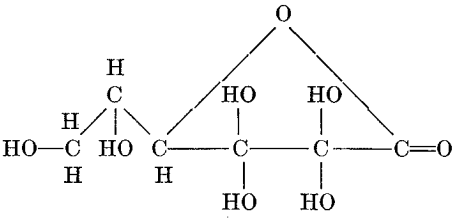
Ascorbic acid can be made to take up the equivalent of at least 3 atoms of oxygen in the course of its oxidation, in three separate steps. The oxidation-reduction potentials of these three steps were estimated, the potential of the first by electrometric and colorimetric methods, of the second and third by a colorimetric method only, and with less precision.

We also determined the orders of magnitude of the first acid dissociation constants of dehydroascorbic acid and of the product of its irreversible change.

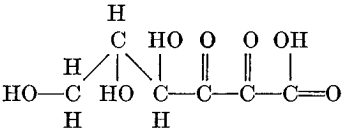
Interposed between the three oxidation stages are several irreversible non-oxidative changes. In order to make the description of the interrelation of all these reactions clearer we shall present here the formulæ for the different compounds involved and the relations which we propose exist between them. The formulæ are those given by Herbert *et al.* (10). Ascorbic acid is represented by formula (I); dehydroascorbic acid by (II). It is the hydrated oxidation product of (I). The first reversible oxidation stage is



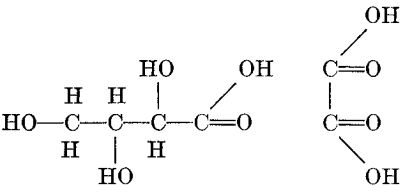
I



II



III



IV V

(I) $\rightleftharpoons$ (II). (III) is the product of the irreversible change in dehydroascorbic acid. From formula (III) it is 2,3-diketo-*l*-gulonic acid. We shall refer to it hereafter as diketogulonic acid. In the second oxidation diketogulonic acid eventually gives rise to *l*-threonic acid (IV) and oxalic acid (V). It is at present uncertain whether the reversible step in the second oxidation stage is (III) $\rightleftharpoons$ (IV) + (V), or from (III) to some intermediary compound which eventually breaks down to (IV) and (V). It would seem

that the second alternative is the more probable. In the third oxidation stage we are uncertain even regarding the compound which is oxidized. It is a rapid reaction only on the alkaline side of neutrality. Tentatively we would suggest that it is the *l*-threonic acid (IV) which is oxidized, and that the alkaline reaction is necessary for the cleavage of the intermediary which is in equilibrium with diketogulonic acid in the reversible second oxidation stage.

We wish to emphasize that neither the magnitude of the oxidation-reduction potentials and ionization constants given, nor the properties of the reactions described below, nor the application of this information to physiological questions depends in any way on structural considerations. Wherever reference is made below to diketogulonic acid, what is meant is the product of the irreversible non-oxidative change in dehydroascorbic acid.

Electrometric Measurement of Oxidation-Reduction Potential of First Oxidation Stage—These measurements were carried out at 35.5° with the vacuum technique previously described (11). The principle of the method is the measurement of the potentials acquired by noble metal electrodes when these are immersed in solutions containing known proportions of the reduced and oxidized forms of the substance investigated.

Mixtures containing varying proportions of reduced and oxidized ascorbic acid were made in citrate-phosphate buffers at the hydrogen ion concentrations given in Table I. The oxidized form was prepared by oxidation of ascorbic acid with iodine. The specimen of ascorbic acid used gave a titration value of 100 per cent on the basis that 1 molecule of ascorbic acid requires 2 equivalents of iodine. No reversibly oxidizable dye nor enzyme was added. All solutions used were kept at ice temperature and rendered nearly air-free by aeration with nitrogen. The different mixtures were prepared immediately before their transfer to the electrode vessels. These were evacuated with an oil pump for 3 minutes at room temperature, followed by 2 minutes at 35°. During the evacuation at room temperature the solutions froze. Platinum foil electrodes were used. The same results were obtained with plain or gold-plated electrodes. The electrode vessels were rocked continuously in an air bath maintained at 35.5°. Readings were taken at frequent intervals until either a steady

potential was established or a uniform drift of the potential was observed.

TABLE I

Oxidation-Reduction Potentials at 35.5° of Different Mixtures of Reduced and Oxidized Ascorbic Acid at Different Hydrogen Ion Concentrations

pH	Concentration of reduced form	Concentration of oxidized form	Reduced / Oxidized	Calculated potential difference = 0.0306 log (reduced)/(oxidized)	Observed potential difference*	E'_0 values; *i.e.*, when (reduced) ÷ (oxidized) = 1		Potential drift $-dE/dt$	Period of observation	
						Calculated	Observed		Total	Of constant $-\frac{dE}{dt}$
	moles × 10^3	*moles* × 10^3		*mv.*	*mv.*	*mv.*	*mv.*	*mv. per hr.*	*min.*	*min.*
2.04	2.43	0.05	4.82	18	17	+0.281	+0.283	0	465	75
2.04	2.43	2.12	1.20			+0.281	+0.281	0	420	75
2.68	0.74	1.38	0.54	18	18	+0.242	+0.242	0	705	75
2.68	2.96	1.38	2.14			+0.242	+0.242	0	700	95
3.30	0.72	1.41	0.51	18	17	+0.204	+0.205	0	240	80
3.30	2.87	1.41	2.03			+0.204	+0.204	0	235	75
4.01	0.73	1.40	0.52	18	19	+0.166	+0.167	1.4	265	150
4.01	2.93	1.40	2.07			+0.166	+0.166	1.4	260	165
4.65	0.75	1.38	0.55	18	19	+0.138	+0.137	1.9	370	120
4.65	3.01	1.38	2.18			+0.138	+0.136	1.6	365	120
5.31	1.91	1.82	1.05	18	17	+0.119	+0.118	3.0	580	270
5.31	1.91	0.45	4.20			+0.119	+0.120	3.0	570	240
5.75	4.55	0.56	8.18	37	38	+0.106	+0.108	5.9	440	210
5.75	1.14	2.23	0.51			+0.106	+0.109	7.2	435	240
6.43	3.81	1.52	2.50	18	−49	+0.080	+0.096	20.9	295	110
6.43	0.95	1.52	0.63			+0.080	+0.045	31.3	290	110

* The minus sign of the observed potential difference for the pair at pH 6.43 indicates that the mixture with the lower ratio of reduced to oxidized ascorbic acid was 31 millivolts more negative instead of 18 millivolts more positive than the other member of this pair.

The electrode equation was obtained by the derivation described earlier (11). For the mechanism, oxidized ascorbic acid + $2H^+$ + $2(e)$ → reduced ascorbic acid, the equation is

$$E_{obs.} = \bar{E} - \frac{RT}{F} \text{pH} - \frac{RT}{2F} \ln \frac{(\text{reduced})}{(\text{oxidized})} - \frac{RT}{2F} \ln \frac{K_r}{K_o}$$

$$- \frac{RT}{2F} \ln \frac{K_o + (H^+)}{K_r + (H^+)}$$

where (reduced) and (oxidized) indicate the concentrations of the total reduced and oxidized forms respectively, K_r and K_o the first dissociation constants of the reduced and oxidized forms considered as monovalent acids. For simplicity we have ignored the second dissociation constants. Birch and Harris (12), and Karrer *et al.* (4), found the value of pK_{r_1} to be about 4.17. By a colorimetric titration procedure described below we found the value of pK_o to be approximately 9.0. The value of n was taken as 2, which was indicated by the iodine titration and corroborated by the agreement between the observed and calculated E'_0 values in Table I.

The data in Table I may be divided into two groups: the three most acid pairs in which the potentials attained steady values, and the remaining five from pH 4.01 to 6.43 inclusive in which the potentials did not attain steady values. In the latter group, after the first rapid negative change, the potential changes slowed down to drifts which were uniform for hours; *i.e.*, $-dE/dt$ in each case was a constant. The magnitude of this constant was greater the higher the pH.

The notable feature of the first group of data is that several hours elapsed before steady potentials were attained. Once attained the differences between these values in the different mixtures were sufficiently close to the theoretical differences for the differences in the ratios of (reduced)/(oxidized) to warrant the conclusion that they are thermodynamically reversible potentials. This conclusion is supported further by the fact that the E'_o values (E'_o is the calculated potential at any specified pH when (reduced)/(oxidized) = 1) fell on the theoretical curve calculated from the electrode equation. The values in this pH region, from 2 to 3.3 inclusive, have been confirmed by Wurmser and de Loureiro who employed a similar electrometric method (8), by our later colorimetric measurements, and by Ball (13) who has worked out a rapid electrometric method (Table IV).

The second group of data in Table I does not give values of the

thermodynamically reversible potentials directly. Such values were obtained on the assumption first, that the constant negative drifts which characterize this group are the result of an irreversible change in the oxidized form (dehydroascorbic acid) described above, and second, that this irreversible change is a first order reaction. On the basis of the second assumption it follows that d log (oxidized)$/dt$ is a constant. From the electrode equation it is seen that the potential, $E_{obs.}$, is a linear function of log (oxidized). $-dE_{obs.}/dt$ will therefore be a constant. This was observed in every case. The E'_0 values given in Table I were obtained on the basis of these two assumptions by subtracting from the observed potential at any moment during the interval in which $-dE_{obs.}/dt$ was constant the product of the value of this constant and the time which had elapsed from the moment when the mixture containing the oxidized ascorbic acid was brought to 35°; *i.e.*, $(-dE_{obs.}/dt) \times t$.

The E'_0 values so obtained fell on the same theoretical E'_0-pH curve as those in the more acid group which were obtained without these assumptions or extrapolations. The theoretical E'_0-pH curve for ascorbic acid changes its slope at pH 4.2 from 60 to 30 millivolts per pH unit. The extrapolated E'_0 values fell on this curve both in the region in which the slope is changing and on the later straight line portion. This coincidence is the more striking because the extrapolation slopes are progressively steeper with increasing pH. The values given in the recent paper by Ball (Table IV) are in accord with the order of magnitude of the E'_0 values obtained by our extrapolation.

At pH 6.43 the situation became too complicated to be interpreted. The potential difference between the two mixtures was in the reverse of the theoretical direction; the extrapolation curves were very steep and different in the two mixtures. As a result the extrapolated E'_0 values were different for the two mixtures, and both were more positive than the theoretical curve at this pH. We have set these values aside for these reasons, and also because diketogulonic acid begins to behave as a reducing agent at this pH (see below) and presumably therefore also affects the electrode in the manner of a reductant. This introduces a number of disturbing effects on the electrode potential which we cannot discuss here. Their influence is small up to pH 5.75. Beyond this point they dominate the picture.

Relation between Regeneration of Ascorbic Acid by H_2S in Solutions of Dehydroascorbic Acid and Rate of Irreversible Change in Dehydroascorbic Acid—In this and in the next two sections we shall present independent evidence that the negative drift in the electrode potentials observed at hydrogen ion concentrations less than at pH 4 is a consequence, as we have assumed above, of a spontaneous irreversible change in dehydroascorbic acid.

Dehydroascorbic acid is restored practically quantitatively to ascorbic acid by H_2S in acid solution. After its conversion to diketogulonic acid this property is lost. Table II is a summary of some experiments which show that the irreversible change in dehydroascorbic acid, judged by this criterion, begins at about pH

TABLE II

Variation with pH of Rate of Irreversible Change in Dehydroascorbic Acid Measured by Yield of Ascorbic Acid Recovered after Treatment with H_2S

pH	Per cent of original reducing capacity recovered by treatment with H_2S after incubation *in vacuo* at 23° for			
	1 hr.	2 hrs.	4 hrs.	6 hrs.
3.0	100	95	98	98
4.0	98	98	100	98
4.5	95	93	90	88
6.0	86	80	73	60
7.0	61	41	30	20
8.0	21	18	15	14
9.0	16	12	11	11

4, and becomes progressively faster with increasing pH above this point. The variation with pH in the behavior toward H_2S is the same in both these respects as the electrode potential drifts. The failure of H_2S to regenerate ascorbic acid from diketogulonic acid shows that dehydroascorbic acid and diketogulonic acid are not in equilibrium, and that the change is therefore an irreversible one.

The experimental procedure was as follows: the buffer solution used (McIlvaine's series) was pipetted into the main, lower compartment of a Thunberg tube, and the plain aqueous ascorbic acid solution previously oxidized with iodine, into the overhang. After evacuation with an oil pump the contents of the tube were mixed and then allowed to stand at room temperature (20–25°) for the times indicated. At the end of the specified period the

vacuum was broken, 0.1 N hydrochloric acid was immediately added to bring the pH to 2.0, the contents of the tube were transferred to a small Erlenmeyer flask, hydrogen sulfide was bubbled through for 3 hours, after which the 2-way stop-cock was closed and the solution allowed to stand overnight under hydrogen sulfide. Next morning this was removed by a stream of nitrogen, and the solutions titrated with 2,6-dichlorophenol indophenol.

Barron and his collaborators (14) measured the rates of irreversible oxidation of ascorbic acid by oxygen (catalyzed by $CuCl_2$) at different hydrogen ion concentrations. Their figures are essentially the same as those in Table II, which represent the rates of irreversible change in dehydroascorbic acid *in vacuo* (and in the absence of oxidizing agents). It is therefore probable that the irreversibility in the "irreversible" oxidation of ascorbic acid at hydrogen ion concentrations less than at pH 4 resides in the non-oxidative change of dehydroascorbic to diketogulonic acid, and not in any special mode of oxidation of ascorbic acid prior to this change. Accordingly the rate of its "irreversible" oxidation is governed by the rate of this irreversible change.

Inability of Glutathione to Regenerate Ascorbic Acid from Products of Irreversible Change in Dehydroascorbic Acid—A possible alternative explanation for the above observations in the experiments with H_2S is that the equilibrium between dehydroascorbic acid and diketogulonic acid readjusts itself very slowly in the acid solutions in which the H_2S was used. Hence, although the equilibrium is disturbed by the conversion of the existing dehydroascorbic acid to ascorbic acid, very little more dehydroascorbic acid and thence ascorbic acid are formed from the diketogulonic acid.

This explanation is implicit in the description given by Herbert *et al.* (10) of the changes which they observed in neutral, alkaline, and dilute mineral acid solutions (anaerobic) of dehydroascorbic acid. They followed, among other changes, the mutarotation of these solutions, and found that nearly the same final rotatory power was attained in the alkaline and in the acid solutions. They designated the final mixture as an equilibrium mixture. They also referred to the slowness with which the lactone ring of dehydroascorbic acid is reconstituted in acid solution.

This alternative explanation was excluded by experiments with glutathione (these are described in more detail in a later section).

At pH 7 and more alkaline solutions, where the irreversible change in dehydroascorbic acid is very rapid, glutathione quickly reduces the latter substance to ascorbic acid, and has no such effect at this or at any other pH on the products of its irreversible change. Therefore, for all practical purposes, we may designate the change in dehydroascorbic acid as an irreversible one. The results of an experiment carried out at pH 7 are shown in Table III.

The technique used in this experiment was as follows: 3.5 cc. of a phosphate buffer at pH 7 were transferred to the lower part of

TABLE III

Inability of Glutathione (200 Mg. Per Cent) to Regenerate Ascorbic Acid from Products of Irreversible Change in Dehydroascorbic Acid (10 Mg. Per Cent), at pH 7.0, and 37.5°, in Vacuo

Incubation of oxidized ascorbic acid		Fraction of original oxidized ascorbic acid recovered in reduced state
Before mixing with glutathione	After mixing with glutathione	
min.	*min.*	*per cent*
0	30	90
5	30	70
15	30	36
30	30	14
60	30	5
90	30	2
120	30	<1
120	60	<1
120	120	<1
120	240	<1
120	1440	<1
120	2880	<1

a Thunberg tube. The solution was then frozen by immersing the tube in an alcohol-solid CO_2 bath. 1 cc. of a dehydroascorbic acid solution (formed by oxidation of ascorbic acid with I_2) was next added above the frozen cake, and then frozen in the same way. The glutathione (dry) and 0.5 cc. of buffer solution were placed in the overhang. After the tube was thoroughly evacuated the ice cake was allowed to thaw, and pumping continued for 2 minutes after it had entirely melted. The tubes were then set away in a water bath at 37.5°. The contents of the upper and lower compartments were kept separate for different lengths of

time indicated in Table III. They were then mixed and the incubation continued as indicated in Table III. At the end of the incubation the vacuum was broken, the solution immediately acidified with metaphosphoric acid to a final concentration of 2 per cent, and the ascorbic acid present estimated by titration with 2,6-dichlorophenol indophenol. Controls with dehydroascorbic acid and glutathione alone were also carried through for the maximum incubation periods.

The figures in Table III show that as the period of incubation of the oxidized ascorbic acid solution at pH 7 was prolonged prior to mixing with the glutathione, the yield of ascorbic acid subsequently obtained diminished. This experiment provides further evidence that the irreversible change in dehydroascorbic acid is not an oxidation. The reconstitution of the lactone ring of dehydroascorbic acid, if the above formulæ are correct, evidently is as difficult in neutral as in acid solution.

Glutathione does disturb the course of the reactions, and possibly an equilibrium, among the irreversible products of dehydroascorbic acid. The control solution which contained dehydroascorbic acid initially and to which no glutathione was added invariably became brownish yellow after several hours. The solution to which glutathione was added after 2 hours incubation, *i.e.* after nearly all the dehydroascorbic acid had undergone its irreversible change, remained colorless even after 48 hours. Yet neither solution gave any appreciable titration with the dye on acidification.

Ionization Constants of Dehydroascorbic Acid and of Diketogulonic Acid—Another evidence of the irreversible change in dehydroascorbic acid is the resulting increase in strength of the acid group. This affords striking visual evidence of the transformation. It can be demonstrated by the following colorimetric procedure we have employed to measure the ionization constants of dehydroascorbic acid and of diketogulonic acid.

1 cc. of an aqueous 0.01 M solution of ascorbic acid oxidized with iodine was measured into the overhang of a Thunberg tube. The main (lower) compartment contained a suitable pH indicator, and a quantity of alkali equivalent to the HI formed in the oxidation of the ascorbic acid, plus an additional quantity which was varied from 0.1 to 0.8 mole equivalent of the ascorbic acid in the overhang.

Before the contents of the upper and lower compartments were mixed the Thunberg tubes were thoroughly evacuated. After being mixed, the initial pH of the solution was estimated by comparing the color immediately after mixing with the color of the same dye in standard buffer solutions. With thymol blue and phenolphthalein the color changes in a few seconds from the alkaline to the acid color following the formation of the more strongly acid irreversible product of dehydroascorbic acid. By successive trials the color formed immediately on mixing was bracketed more and more closely within those of the series of buffer standards.

The estimated initial pH values extended over a range from 8.05 to 9.2. These yielded the value of $pK = 9.0 \pm 0.1$ for the ionizable hydrogen of dehydroascorbic acid. This hydrogen ion probably arises from an enolic group.

The first acid dissociation constant of diketogulonic acid was determined in the same manner except that the final, equilibrium colors were measured. About 24 hours were required at 25° for the attainment of this stage. The acidities of the different solutions fell within the pH ranges of brom-cresol green and brom-phenol blue, yielding a pK of approximately 3.3. The group involved here is probably a carboxyl group.

We obtained some indication here of a second ionizable hydrogen, with a pK between 7 and 8. We were unable to measure it more precisely with the above technique because all the dyes usable in the appropriate pH range—brom-thymol blue, chlorophenol red, phenol red, neutral red, and brom-cresol purple—were attacked by the oxidized ascorbic acid when more than 1 equivalent of additional base was added. As a result the color intensity or hue was changed. However, even in the changed state these dyes responded to changes in pH and so permitted a rough guess of the final pH. But it is possible that this ionizable hydrogen arises from some secondary oxidation product of diketogulonic acid (after reaction with the dye) rather than from this substance itself.

Colorimetric Observations on Three Oxidation Stages of Ascorbic Acid—When the reducing action of solutions of ascorbic acid and of dehydroascorbic acid are studied by colorimetric methods over a pH range from 2 to 9, the three oxidation stages of ascorbic acid

are clearly shown. The first, that of ascorbic acid to dehydroascorbic acid appears without the intervention of the second and third steps in the pH range from 2 to 4. The second, that of diketogulonic acid to an unstable intermediary, which in an alkaline reaction breaks down to *l*-threonic acid and oxalic acid, can be isolated in the pH range between 5.5 and 7.5 by beginning with dehydroascorbic acid. The actual reductant here is diketogulonic acid, arising in this pH range from the irreversible change in dehydroascorbic acid. The third oxidation stage occurs only in alkaline reactions. We can only guess at the substance which is oxidized. It seems that it is probably *l*-threonic acid.

The usual Thunberg vacuum technique was employed in these experiments with a series of reversible oxidation-reduction dyes ranging from *o*-cresol indophenol to methylene violet, and a series of buffers (McIlvaine's series) spaced at 0.5 pH intervals from pH 2 to 9. At each pH with each dye the reducing action of ascorbic acid and of dehydroascorbic acid was observed. The degree of reduction of the dye was estimated by comparison with known dilutions of oxidized dye. Where the reduction did not go to completion, it was taken as an equilibrium value when the degree of reduction observed remained unchanged for 24 to 48 hours, and not more than 2 to 3 days were required for the attainment of this value.

The details of the procedure were as follows: Aqueous stock solutions of the following dyes given in descending order of their oxidation-reduction potentials, *o*-cresol indophenol, thionine, methylene blue, indigotetra-, indigodi-, and indigomonosulfonate, brilliant alizarin blue, and methylene violet, were diluted with the buffer solution used to a final concentration of 0.00005 M. 2 cc. of this solution were pipetted into the lower compartment of a Thunberg vessel, 1 cc. of an aqueous solution of 0.005 M ascorbic or of dehydroascorbic acid into the overhang. Where ascorbic acid was used, HCl and KI equivalent to the HI present in the solution of dehydroascorbic acid were added. The vessels were evacuated as in the electrometric measurements, mixed, and then set away in water baths. At each pH two series of experiments were carried out, one at 37°, the other at 25°. The reduction rates were of course slower at the lower temperature, but otherwise the

results were essentially the same as those at 37°. We shall give the details only of the observations at the higher temperature.

Colorimetric Measurement of Reducing Property of Ascorbic Acid. First Oxidation Stage—Ascorbic acid rapidly completely reduced *o*-cresol indophenol and thionine over the whole pH range. With methylene blue equilibrium was attained at pH 4.5, and with indigotetrasulfonate at pH 2.5, 3.0, 3.5, and 4.0. The computed E_0' values are given in Table IV. At higher pH values reduction of these two dyes was eventually complete, or nearly so. This was the result of the intervention of the more powerful reducing

TABLE IV

Reversible Oxidation-Reduction Potentials of Ascorbic Acid. Relation between $E_0'\left(\frac{(Reduced)}{(Oxidized)} = 1\right)$ *and pH*

pH	First oxidation stage			Second oxidation stage	
	Electrometric		Colorimetric		
	35°, Borsook and Keighley (7)	30°, Ball (13)	37°, authors	35°, authors	25°, Fruton (9)
2.5	+0.252	+0.242	+0.235		
3.0	+0.223	+0.212	+0.206		
3.5	+0.193	+0.184	+0.185		
4.0	+0.166	+0.158	+0.155		
4.5	+0.145	+0.136	+0.146		
5.0	+0.127	+0.118			
5.5	+0.112	+0.102		+0.015	+0.019
6.0				−0.031	−0.021
6.5				−0.068	−0.051

action of the products of the irreversible change in the dehydroascorbic acid which arises from the oxidation of the ascorbic acid by the dyes. There was a negligible or no reduction of the dyes more negative than indigotetrasulfonate.

Colorimetric Measurement of Reducing Property of Solutions of Dehydroascorbic Acid. Second Oxidation Stage—The dehydroascorbic acid solutions did not reduce any of the dyes in 24 hours at hydrogen ion concentrations from pH 2.5 to 4.0 inclusive. At pH 4.5 between 5 and 10 per cent of *o*-cresol indophenol, thionine, methylene blue, and indigotetrasulfonate were reduced in 24 hours. Above this pH the reduction of these dyes was much more rapid.

o-Cresol indophenol was completely reduced at pH 5 in 4 hours, at pH 6.0 in 45 minutes, at pH 7 in 6 minutes, and at pH 8 in 3 minutes. The reduction of thionine and methylene blue was slower. At pH 5, 30 hours elapsed before the reduction of the thionine was complete, and 44 hours with methylene blue. At pH 6.0 the reduction times for these two dyes were 2.5 and 12 hours; at pH 7.0, 17 and 55 minutes; at pH 8.0, 4 and 14 minutes. With indigodisulfonate equilibrium degrees of reduction were attained at pH 5.5, 6.0, and 6.5. The computed E_0' values are given in Table IV. Indigomonosulfonate was only slightly reduced in 100 hours at pH 6.5, it was 75 per cent reduced in this time at pH 7.0, completely reduced in 3 hours at pH 8.0, and in 5 minutes at pH 9.0. Brilliant alizarin blue was not reduced below pH 7. At this pH the reduction was less than 10 per cent in 10 hours. It was 100 per cent in 2 hours at pH 8.0, and in 25 minutes at pH 9.0. There was no reduction of methylene violet even at pH 9.0.

The first point to be noted in these observations is the proof that dehydroascorbic acid can become a reducing agent. This property first appeared at pH 4, and then became progressively more conspicuous with increasing pH. With the most positive dye used, *o*-cresol indophenol, the reduction times with the solutions of dehydroascorbic acid were always greater than with those of ascorbic acid. Thus from pH 2.5 to 4, the two times were infinity and less than 1 minute respectively; at pH 5, 100 and < 1 minute; at pH 6, 45 and < 1 minute; at pH 7, 6 and < 1 minute; and pH 8, 3.5 and < 1 minute. These differences in reactivity of the reduced and oxidized forms make it possible to determine vitamin C by titrimetric methods with indophenols, and are some of the reasons for the empirically discovered superiority of the titration at pH 2.5 (15), to that at or near neutrality, as it was first done by Tillmans (16).

The failure of solutions of dehydroascorbic acid to reduce any of the dyes at acidities greater than at pH 4 was not solely because its conversion to diketogulonic acid is too slow here. Diketogulonic acid itself is inactive as a reducing agent below pH 4. We have carried out experiments in which the transformation of dehydroascorbic acid to diketogulonic acid was allowed to proceed to completion at pH 7, 8, and 9. The solutions were then acidified, and no reducing power was found.

The second point is that the reducing power of solutions of

dehydroascorbic acid coincides with respect to the pH at which it first appears and to its development with increasing pH, with the potential drift in the electrometric measurements, and with the loss in the property of reduction to ascorbic acid by H_2S. These three independent pieces of evidence attest in complete accord to the spontaneous change in dehydroascorbic acid. The fact that the latter substance, the oxidant of the first oxidation stage, becomes a more powerful reducing agent (by 0.1 volt at pH 5.5 for example, Table IV) than ascorbic acid, is further proof that the change is an irreversible one. This is the explanation for the inability of ascorbic acid to reduce dyes which are reduced under the same conditions by solutions which consisted initially solely of dehydroascorbic acid; *e.g.*, indigodisulfonate at pH 7. If the dye is too negative to be reduced by ascorbic acid, no dehydroascorbic acid appears, and hence no diketogulonic acid is formed, which is the actual reductant here.

We have pointed out above that there are two possible formulations for the second oxidation stage. One is diketogulonic acid to an intermediary which breaks down in alkaline reactions to *l*-threonic acid and oxalic acid; the other is diketogulonic acid directly to *l*-threonic acid and oxalic acid. Experiments, which space limitations do not permit us to discuss here, favor the first of these two alternatives.

Colorimetric Observations on Third Oxidation Stage—At pH 9.0 the dehydroascorbic acid solutions quickly completely reduced all the dyes used except methylene violet. The complete reduction of indigomonosulfonate and brilliant alizarin blue would not be expected from an extrapolation of the equilibrium values obtained with dehydroascorbic acid (transformed to diketogulonic acid) and indigodisulfonate at pH 5.5 to 6.5 inclusive.

Our interpretation of this second increase in reducing power is that it is a third oxidation stage, *i.e.* the oxidation of a product of the oxidation of diketogulonic acid, rather than the oxidation of a transformation product of diketogulonic acid, *i.e.* a different second oxidation stage.

This interpretation is supported by the following observations in the literature. Heard and Welch (17) found that at pH 7.36 and 38° 1 molecule of ascorbic acid took up 3 atoms of oxygen. Similarly Herbert *et al.* (10) observed that further oxidation of

ascorbic acid occurred after potassium permanganate equivalent to 2 atoms of oxygen had been used. Fruton (9), who studied the second oxidation stage, noted that at pH levels above 7.5 "the equilibria obtained were less reproducible and did not accord with the 0.06 slope fitting the readings in the more acid region."

In view of this uncertainty regarding the mechanism of the third oxidation stage, it is not worth while even to guess at its oxidation-reduction potential. It is sufficient to note that it is much more negative than the second oxidation stage.

In Table IV are collected the E'_0 values we have obtained for the first and second oxidation stages. Our values for the first oxidation stage are in reasonably good agreement with those recently reported by Ball who employed a different electrometric method from ours.

We have assigned the oxidation-reduction potentials of ascorbic acid reported by Fruton to the second oxidation stage, although the implication in the communication in which they appeared is that they pertain to the first oxidation stage. The initial material in Fruton's experiments was ascorbic acid. It was oxidized *in vacuo* by dyes; and E'_0 values for ascorbic acid were computed from the observed equilibrium degrees of reduction of these dyes whose oxidation-reduction potentials are known. The justification for our interpretation of his data is as follows: The pH range in which reproducible and concordant potentials were obtainable in these experiments was from 5.7 to 7.2. This is the range in which it is not possible by measuring the equilibrium state to obtain the reversible potentials of the first oxidation stage because of the transformation of dehydroascorbic acid to diketogulonic acid; and it is precisely the range of stability of the product of this second oxidation stage. The time required for the attainment of equilibrium in Fruton's experiments varied from 1.2 to 18 days (at 25°). The data in Tables II and III show that most of the dehydroascorbic acid formed in the first oxidation stage must have undergone its irreversible transformation in this time. The potential of the first oxidation stage is 100 millivolts more positive than that given by Fruton. On this point observers in three different laboratories using three different methods are now in accord. Further, both the theoretical and experimentally observed values for $-dE'_0/d\text{pH}$ for the first reversible oxidation are 30 millivolts per

pH unit in the range from pH 5.5 to 7.0 instead of 60 millivolts as found by Fruton. Finally the E'_0 values we obtained for the second oxidation stage, where the initial material was dehydroascorbic acid, are of the same order of magnitude as those given by Fruton (Table IV).

The dehydroascorbic acid used in all of the foregoing experiments was obtained by oxidation of ascorbic acid with iodine. Shwachman, Hellerman, and Cohen (18) have suggested that the oxidation of ascorbic acid by iodine may be atypical. This suspicion was aroused by their observation of greater reducing power in solutions containing ascorbic acid than our values for the potentials for the first reversible oxidation indicated. The explanation for their observations is that their experiments were carried out on the alkaline side of neutrality, where not only the first oxidation stage, but also the second and third more negative reductions occur. We have studied the properties of solutions of ascorbic acid oxidized by oxygen catalyzed by copper, charcoal, 2,6-dichlorophenol indophenol, bromine, and ferricyanide. The same reversible and irreversible changes were observed as when the oxidation was carried out with iodine.

Nutrition Experiments

Dehydroascorbic acid possesses nearly the same antiscorbutic potency as ascorbic acid (19–21). Oxidized solutions of the vitamin, particularly if these are the natural juice, lose their potency on standing. It has been assumed that this loss is the result of "irreversible oxidation." The experiments described in the foregoing section led us to question this interpretation.

Nutrition experiments were undertaken to examine this and two other questions: whether the reconversion of diketogulonic acid to dehydroascorbic, which cannot be done *in vitro* by means of H_2S in acid nor by glutathione in neutral or alkaline solution, can occur *in vivo*; and whether some mechanism exists *in vivo* which prevents or retards the transformation of dehydroascorbic acid to diketogulonic acid.

The general method and rationale of these experiments were as follows: Solutions containing initially the same amount of dehydroascorbic acid were incubated at different hydrogen ion concentrations. As a result of these differences in pH different amounts

of dehydroascorbic acid were transformed to diketogulonic acid. The same volumes of these solutions were then administered to guinea pigs whose only source of vitamin C was these solutions. The amount of dehydroascorbic acid remaining in each solution at the end of the incubation was determined by treatment with H_2S after acidification and estimation of the regenerated ascorbic acid by titration. The antiscorbutic potency of these solutions was tested by comparison with graded protective and subprotective doses of orange juice. If the antiscorbutic potency of the incubated solutions turned out to be greater than that of (the ascorbic acid equivalent of) the dehydroascorbic acid remaining at the time of administration to the animal, it would be evidence of the reconversion of diketogulonic acid to dehydroascorbic acid; if it was much less, then there is no mechanism *in vivo* preventing or retarding the transformation of dehydroascorbic acid to diketogulonic acid; if it was nearly the same, then a mechanism does exist *in vivo* which protects dehydroascorbic acid from its irreversible transformation.

The last result was the one obtained.

The standard technique described by Sherman and Smith (22) was used in these experiments. Two sets were carried out: one in which the incubation of the dehydroascorbic acid solutions until the moment of administration to the animal took place *in vacuo*, the other in air. Dehydroascorbic acid was prepared by oxidizing solutions of ascorbic acid with iodine. Aliquots were then brought to pH 4.0, 6.0, 7.0, and 9.0 by means of buffers. These were then incubated for an hour either in air or *in vacuo*. The following amounts of dehydroascorbic acid (expressed in terms of ascorbic acid) remained at the end of this time, in the solutions at pH 4.0, 6.0, 7.0, and 9.0 respectively, 1.4, 1.2, 0.75, and 0.2 mg. per cc. In the series where the incubation took place *in vacuo* the mixing with the buffer occurred after the tubes were evacuated. All these solutions were prepared freshly each day. A volume of one of these containing originally 1.5 mg. of ascorbic acid was administered daily to each animal either subcutaneously or by mouth.

The controls were the basal ration alone, and supplemented daily with one of the following: 3 cc. of orange juice, 1.5 mg. of reduced ascorbic acid, the buffer solutions, an amount of KI equal

to that given in the solutions of oxidized ascorbic acid, these with and without buffer, and with and without 3 cc. of orange juice. Where it was possible two sets of similar controls were carried, one in which the control solution was given subcutaneously, in the other by mouth.

Each experimental and control group consisted of five animals.

The method of assay was based on the scurvy score of each animal determined post mortem either after the animal had died or was killed. The following stigmata of the disease were appraised: the extent and number of hemorrhages, the beading and enlargement of the costochondral junctions, the softness of the mandibles, scapulæ, and ribs, and the looseness of the incisors.

These experiments showed that the antiscorbutic potency of the oxidized and incubated solutions of ascorbic acid was quantitatively very nearly equal to the amount of the reduced form which could be recovered from them by treatment with H_2S. The same results were obtained whether the solutions were incubated *in vacuo* or in air, and whether these solutions were administered subcutaneously or by mouth.

The fact that these solutions were slightly less antiscorbutic than the ascorbic acid equivalent of the dehydroascorbic acid which they contained was probably the result of transformation of the latter substance to diketogulonic acid and other products before it reached its site of action in the tissues.

We may conclude from these experiments that diketogulonic acid and its transformation and oxidation products possess no antiscorbutic potency. The tissues resemble H_2S and glutathione in that they are unable to convert these substances to dehydroascorbic or ascorbic acid. This point is interesting because it is possible to recover 75 per cent or more of ascorbic acid from the products of the irreversible transformation of dehydroascorbic acid by concentrating their acid solution to a syrup in the presence of HI. This procedure therefore has no physiological analogue.

In a recent communication Roe and Barnum (23) reported that dehydroascorbic acid possesses only one-quarter the antiscorbutic potency of ascorbic acid. This observation is in disagreement with those of all other workers who have investigated this point (19–21) and with our own observations. For example we found no sign of scurvy in guinea pigs receiving for 60 days 1.2 mg. of

dehydroascorbic acid as their only source of vitamin C, and only moderate scurvy after the same length of time in animals receiving 0.75 mg. of dehydroascorbic acid. 1 mg. of ascorbic acid daily is the minimum protective dose for the standard animals used in these experiments.

The explanation for this discrepancy is that Roe and Barnum neutralized their solutions of oxidized ascorbic acid before administering them. They were misled by their method of analysis, boiling with HCl containing $SnCl_2$, and determination of the resulting furfural, which indicated erroneously that dehydroascorbic acid remains unchanged in neutralized solutions for as long as 3 hours after its preparation. (See Table II.)

We attempted to analyze the blood, liver, and adrenals of the experimental animals for ascorbic and dehydroascorbic acids in order to observe whether the tissues reduce the latter to the former substance. The ascorbic acid was titrated in salicylic or metaphosphoric acid filtrates with 2,6-dichlorophenol indophenol. To our surprise we were unable to attain an end-point at all comparable to that apparently obtained by all other workers with guinea pig tissues, or by ourselves with the plasma of seven other animal species, human urine, rat liver, intestine, kidney, and adrenal, and plant tissues. There appeared to be a reducing substance in the blood, liver, and adrenals of all the guinea pigs in our experiments which did not reduce the dye as quickly as ascorbic acid, but did so quickly enough to prevent us from obtaining an end-point in which we had any confidence. The value obtained with the dye titration depended too much on the speed of titration. This interfering substance reduced iodine, and was not removed by the mercury precipitation of Emmerie and van Eekelen (24). A relatively enormous quantity was present. In the following section conclusive evidence is presented that human and rat tissues convert dehydroascorbic acid to the reduced form.

Fox and Levy (21) administered reversibly oxidized ascorbic acid to guinea pigs and found large enough amounts of the reduced form in the tissues after 60 days to make it seem certain that dehydroascorbic acid is reduced in guinea pig tissues. Apparently they did not encounter, or were not hindered by, the reducing substance which marred our titrations.

Physiological Experiments

The nutrition experiments showed that dehydroascorbic acid is protected *in vivo* from rapid transformation to the antiscorbutically impotent diketogulonic acid. We sought by some physiological experiments to locate the site of this protection of dehydroascorbic acid, and to obtain some information regarding its mechanism. The experiments consisted in following the fate of dehydroascorbic acid and of ascorbic acid when these were added to blood, plasma, minced and intact isolated tissues, and also after their ingestion.

Analytical Methods—The following analytical methods were used. Ascorbic acid in blood, tissues, and urine, was titrated with 2,6-dichlorophenol indophenol after acidification with metaphosphoric acid. According to the volume of solution to be titrated the titration was carried out with a capillary burette (25), or a semimicroburette containing 1 cc. in a length of 45 cm., or in a sugar burette. Ascorbic acid cannot be recovered quantitatively from plasma or tissue filtrates after precipitation with trichloroacetic acid, sulfosalicylic acid, or sodium tungstate and sulfuric acid. It can be recovered quantitatively from metaphosphoric acid blood and tissue filtrates (26). Accordingly metaphosphoric acid was used (in a final concentration of 2 to 4 per cent) both for purposes of acidification only—with urine or solutions of ascorbic acid and glutathione—and for protein precipitation and acidification with blood and tissues.

In urine only a fraction of the reducing material titratable with 2,6-dichlorophenol indophenol is ascorbic acid. Among the other reducing substances are polyphenols such as homogentisic acid, pigments, and chromogens (27). The pigments, and probably other reducing substances, can be removed from urine acidified with sulfosalicylic or metaphosphoric acid by shaking for a few minutes with Lloyd's reagent—100 mg. of Lloyd's reagent for 10 cc. of solution. At the same time about 10 per cent of the ascorbic acid present is removed. The total amount of dye-reducing material removed in this way varied, expressed in terms of ascorbic acid, from 0.6 to 6 mg. per 100 cc. of urine. These quantities were negligible for our comparative purposes, especially as Barrenscheen and his collaborators had shown that the excretion of this fraction was fairly constant in any one individual. The figures for urine

given below are those for urine not treated with Lloyd's reagent. The conclusions were identical with the values obtained after treatment with Lloyd's reagent.

Dehydroascorbic acid was determined by treating the metaphosphoric acid filtrates with H_2S in the usual manner: a stream of wet H_2S was passed through the solution for 3 hours, the material was left standing overnight in a stoppered flask filled with H_2S, and this treatment was followed by removal of the H_2S by a vigorous stream of wet nitrogen for 3 to 5 hours.

When whole blood (beef, cat, dog, human, pig, rat, and sheep) is precipitated with either trichloroacetic acid, sulfosalicylic acid, sodium tungstate plus sulfuric acid, or metaphosphoric acid, nearly all the ascorbic acid whether initially present, or added, is oxidized to dehydroascorbic acid. Similar precipitation of the plasma or serum leaves practically all the ascorbic acid in its reduced state. Our experience here differs from that of Kellie and Zilva (28) who reported complete recovery of added ascorbic acid in this form after precipitating whole blood or a buffer solution containing red blood corpuscles with trichloroacetic acid. Their experience is the more surprising as they used trichloroacetic acid as precipitant. Confirming Fujita and Iwatake (26) we have been unable to recover added ascorbic acid quantitatively as such even when added to serum, when trichloroacetic acid was used as precipitant. Van Eekelen and his collaborators (29) have reported findings similar to ours—when whole blood is treated with an acid protein precipitant, nearly all of the ascorbic acid present is oxidized to dehydroascorbic acid. These authors accordingly revised their earlier opinion that a considerable fraction (if not all) of the ascorbic acid in human blood is in the reversibly oxidized form. Their present conclusion, that of Kellie and Zilva (30), of Farmer and Abt (31), and ours, is that practically all (if not all) of the ascorbic acid in plasma and serum is in the reduced state. This conclusion differs, with respect to human blood, from that of Plaut and Bülow (32) who found most of the ascorbic acid in serum in the form of dehydroascorbic acid.

Stability of Ascorbic Acid and of Dehydroascorbic Acid in Blood and in Plasma—Table V is a summary of a typical group of experiments in which the stability of ascorbic acid and of dehydroascorbic acid in Ringer's solution, plasma, and whole blood was studied.

The data show the gradual disappearance of added ascorbic acid from all three solutions. The rate of this disappearance was nearly the same in Ringer's solution and in plasma, approximately half remaining after 4 hours. In whole blood, *i.e.* in plasma in contact with its corpuscles, the vitamin was much more stable.

TABLE V

Stability of Ascorbic Acid and Dehydroascorbic Acid in Ringer's Solution, Plasma (Human), and Whole Blood (Human), under 95 Per Cent Oxygen and 5 Per Cent CO_2 at 37.5°

Solution	Concentration and form of added ascorbic acid	Ascorbic acid, mg. per cent, found after						
		Form	0.75 hr.	1 hr.	1.5 hrs.	2.5 hrs.	4 hrs.	6 hrs.
	mg. per cent							
Ringer's, pH 7.4	12 Ascorbic acid	Reduced	9.0		7.6	6.9	5.9	4.6
		After H_2S	9.9		8.4	7.7	6.5	5.0
Plasma	0	Reduced	1.0		0.6	0.3	0.2	0.2
		After H_2S	1.7		0.8	0.6	0.5	0.5
	40 Dehydro-ascor-bic acid	Reduced	1.5		2.0	2.4	2.5	2.7
		After H_2S	3.7*		3.8*	4.3*	4.8*	4.5*
	19.4 Ascorbic acid	Reduced	16.3		14.1	11.9	8.8	6.6
		After H_2S	18.6		14.7	12.9	10.3	8.2
Whole blood, plasma only analyzed	0	Reduced	1.2		1.2	0.8	0.8	0.7
		After H_2S	1.5	1.9†	1.3	1.3	1.3	1.1
	20 Dehydro-ascor-bic acid	Reduced	1.5		2.7	3.6	4.9	6.3
		After H_2S	3.0*	9.7†	4.5*	4.3*	5.9*	6.5*
	12 Ascorbic acid	Reduced	21.0		19.3	18.2	17.1	15.4
		After H_2S	20.1	9.4†	18.9	19.6	19.0	14.6

* In these solutions the rate of reduction of the dye was definitely slower than in the others, or in plain aqueous acid solutions of ascorbic acid. The end-points, once attained, were quite stable. It is questionable whether these figures may be taken as ascorbic acid.

† Figures for whole blood precipitated as such. Filtrate treated with H_2S.

Nearly three-quarters was recovered in the reduced form after 4 hours. (Nearly all of the ascorbic acid added to blood remains in the plasma; hence the figure of 20.1 mg. per cent when the amount added gave a concentration in whole blood of only 12 mg. per cent.) We have frequently found greater stability in whole blood over a period of 2 hours than is recorded here. In both

human and rat blood we have recovered 95 to 97 per cent of the ascorbic acid added in the reduced form.

The preexisting ascorbic acid in plasma and in whole blood exhibited the same difference in stability as the added ascorbic acid. The figures show also that only a small fraction of the ascorbic acid (whether preexisting or added) which had disappeared was found in the reversible oxidized form. There is, therefore, no effective mechanism in the blood for protecting dehydroascorbic acid.

These observations on the greater stability of ascorbic acid in whole blood than in plasma are in accord with those of Kellie and Zilva (28) on the protective influence of red blood corpuscles added to buffer solutions. De Caro and Giani (33) record a lower stability of ascorbic acid in defibrinated guinea pig blood than in serum. Their result is probably an artifact of their analytical procedure—precipitation of the whole blood and serum with trichloroacetic acid.

When dehydroascorbic acid was added to the blood, Table V shows that after $\frac{3}{4}$ hour only 4 per cent was recovered as ascorbic acid by subsequent treatment with H_2S. This finding and those after longer incubation periods show that dehydroascorbic acid is not reduced at any significant rate in blood or plasma; and also, in accord with the conclusion drawn from the fate of preexisting or added ascorbic acid, that there is no effective mechanism in blood for the protection of dehydroascorbic acid. This substance is transformed to diketogulonic acid as rapidly in blood as in a buffer solution at this pH (Table III).

We have drawn these conclusions although, as Table V shows, we observed a slow increase in reducing material after the addition of dehydroascorbic acid to plasma and to whole blood. We doubt whether this material is ascorbic acid. The filtrates in which it was contained reduced the dye more slowly than solutions of ascorbic acid. We found no evidence of a mechanism in blood capable of reducing either preexisting or added dehydroascorbic acid. The low recovery of ascorbic acid after treatment with H_2S from plasma and blood to which dehydroascorbic acid had been added also argues against this reducing material being ascorbic acid.

Plaut and Bülow (32) came to the conclusion from *in vitro* experi-

ment with serum, and *in vitro* and injection experiments with cerebrospinal fluid that dehydroascorbic acid is not reduced in either of these fluids *in vitro* or *in vivo*.

Roe and Barnum (23) deduced from evidence similar to that in Table V the existence of an enzyme in the blood, which is capable of reducing dehydroascorbic acid. The interpretation of their data is uncertain because the results are expressed as titration figures. They added 100 mg. per cent of dehydroascorbic acid and incubated the blood or plasma for 3 hours at 38°. When their titration figures are converted to mg. per cent on the assumption that their figures for plasma represent the normal ascorbic acid content, the fraction of the added dehydroascorbic acid which they recovered as ascorbic acid (without H_2S treatment) was less than in our experiments. Their data and those in Table V are therefore in accord in that the hypothetical mechanism in the blood for the reduction of dehydroascorbic acid, if it exists at all, reacts too slowly to save most of the dehydroascorbic acid from irreversible transformation.

The question whether or not the reducing material which slowly appears after the addition of dehydroascorbic acid to blood is ascorbic acid must be left until some such method as ultraviolet spectroscopy is applied to the problem.

Distribution of Ascorbic Acid between Blood and Corpuscles—Table VI shows that in seven animal species the bulk of added ascorbic acid does not enter the corpuscles. No allowance was made in the figures given for oxidation of some of the added ascorbic acid, which certainly occurred. Only in the cases of the pig and sheep were there any large differences between the concentrations found in the plasma and those calculated on the basis of complete impermeability of the corpuscle, and no oxidation. But even in these two specimens the amounts found in the plasma were much larger than if there had been equipartition between plasma and corpuscles. This impermeability of the red blood corpuscle to ascorbic acid recalls the observation of Olmsted (34) that red blood corpuscles, particularly when the blood is not oxalated, are impermeable to glucose.

Reduction of Dehydroascorbic Acid in Tissues—Although dehydroascorbic acid is neither reduced to ascorbic acid, nor protected from its irreversible transformation to diketogulonic acid

in the blood, it is rapidly reduced in the tissues. The evidence supporting this conclusion is 3-fold. First, when dehydroascorbic acid is ingested, there are an increase in the ascorbic acid content of the plasma and a very large increase in the ascorbic acid excreted in the urine. On the other hand there are only negligible increases in the dehydroascorbic acid content of the plasma and

TABLE VI

Concentration of Ascorbic Acid in Plasma after Its Addition to Whole Blood and Incubation for 1 Hour at 37.5°

Species	Anesthetic used	Preliminary treatment	Concentration in whole blood of added ascorbic acid	Ratio of serum volume to total blood volume × 100	Ascorbic acid concentration in plasma		Degree of hemolysis
					Calculated on complete impermeability of corpuscles and no oxidation	Observed	
			mg. per cent		*mg. per cent*	*mg. per cent*	
Beef	None	Defibrinated	0			0.4	0
"	"	"	15	65	23.5	20.6	0
Cat	Ether	"	0			0.3	0
"	"	"	10	69	14.7	13.3	0
"	"	Oxalated	0			0.2	0
"	"	"	10	69	14.6	12.9	0
Dog	None	Defibrinated	0			0.5	++
"	"	"	15	58	26.0	22.8	++
Human	"	Oxalated	0			1.2	0
"	"	"	12	55	23.1	21.0	0
Pig	"	Defibrinated	0			0.6	+
"	"	"	15	50	30.6	24.8	+
Rat	Chloroform	Oxalated	0			0.2	+++
"	"	"	10	63	15.6	12.8	+++
Sheep	None	Defibrinated	0			0.9	0
"	"	"	15	49	31.5	23.6	0

urine. Second, dehydroascorbic acid added to minced or intact isolated tissues is rapidly reduced. This finding stands in marked contrast to the findings in blood. Third, the reducing mechanism in the tissues capable of effecting a rapid reduction of dehydroascorbic acid under physiological conditions was demonstrated to be probably glutathione.

Changes in Ascorbic Acid and Dehydroascorbic Acid Content of Blood and Urine after Ingestion of Large Quantities of These Substances—Typical results of one of a number of such experiments are given in Table VII.

Both the ascorbic acid and the dehydroascorbic acid were taken in the form of orange juice. The oxidized form was prepared by oxidizing the ascorbic acid in orange juice with charcoal (35). The amounts of ascorbic acid in the ordinary daily diets of the two subjects in this experiment were different. The only citrus fruits or juices in the diet of the subject who drank the untreated orange juice was one grapefruit every other day. The other subject took 300 to 400 cc. of orange juice daily. This dietary difference is reflected in the difference in the urinary excretion of ascorbic acid of the two subjects both before and after ingestion of the orange juice.

The figures in Table VII show that in both cases, *i.e.* after the ingestion of the reversibly oxidized and of the reduced forms of the vitamin, there were definite increases in the ascorbic acid in the plasma and urine, and only insignificant changes in the concentrations of oxidized ascorbic acid. Johnson and Zilva (36) had previously found increased amounts of reduced and no dehydroascorbic acid in the urine of human subjects after the ingestion of a large quantity of dehydroascorbic acid. The group of experiments of Table VII were carried out prior to our having seen the paper by these authors.

These experiments demonstrate the rapid reduction of dehydroascorbic acid in the animal body; and that only a very small fraction, if any, of the total ascorbic acid is transported as dehydroascorbic acid. Taken in conjunction with those in blood described above these findings indicate that the reduction of dehydroascorbic acid occurs in the tissues.

Reduction of Dehydroascorbic Acid by Minced and Intact Isolated Tissues—Table VIII shows that when dehydroascorbic acid is added to minced tissues it is rapidly reduced.

The procedure in this experiment was as follows: Rat kidney and liver were frozen with solid CO_2, pulverized in the frozen state, triturated with an equal volume of phosphate buffer at pH 7.0, and then squeezed through cheese-cloth. To each 5 cc. of the juice 0.5 mg. of dehydroascorbic acid (in solution) was added. The

TABLE VII

Concentrations of Reduced and Reversibly Oxidized Ascorbic Acid in Human Plasma and Total Urinary Excretion after Ingestion of 450 Cc. of Plain Orange Juice, and of Orange Juice in Which the Ascorbic Acid Was Oxidized with Charcoal

		Time after ingestion			
		0 hr.	1 hr.	2 hrs.	3 hrs.
Plain orange juice					
Plasma	Ascorbic acid	*mg. per cent* 1.5	*mg. per cent* 2.1	*mg. per cent* 1.0	*mg. per cent* 2.0
	Dehydroascorbic acid	0.2	0.2	0.15	0.1
	Total	1.7	2.3	2.0	2.1
		Urine excretion in 1 hr. intervals ending at following hrs. after ingestion			
	Urine volume	0.5 hr. before 141 cc.	0.5 hr. 125 cc.	1.5 hrs. 270 cc.	2.5 hrs. 68 cc.	3.5 hrs. 58 cc.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*
Urine	Ascorbic acid	2.3	2.4	25.3	61.9	21.6
	Dehydroascorbic acid	0.2	0.4	0.8	0.4	0.8
	Total	2.5	2.8	26.1	62.3	22.4
Orange juice in which ascorbic acid was oxidized					
		Time after ingestion			
		0 hr.	1 hr.	2 hrs.	3 hrs.
Plasma	Ascorbic acid	*mg. per cent* 1.6	*mg. per cent* 2.2	*mg. per cent* 2.35	*mg. per cent* 2.3
	Dehydroascorbic acid	0.3	0.3	0.4	0.3
	Total	1.9	2.5	2.75	2.6
		Urine excretion in 1 hr. intervals ending at following hrs. after ingestion			
	Urine volume	0 hr. 250 cc.	1 hr. 340 cc.	2 hrs. 118 cc.	3 hrs. 75 cc.
		mg.	*mg.*	*mg.*	*mg.*
Urine	Ascorbic acid	26	76	112	139
	Dehydroascorbic acid	0	0	3	4
	Total	26	76	115	143

juice was then incubated at 37.5° in air or *in vacuo* for 15 minutes. At the end of this time 2 cc. of 10 per cent metaphosphoric acid were added for each 5 cc. of juice. The ascorbic acid and glutathione content of the filtrate were then measured—the glutathione by the method of Quensel and Wacholder (37). The glutathione values obtained, referred to the original weight of tissue, are of the

TABLE VIII

Reduction of Dehydroascorbic Acid by Minced Rat Tissues in Air and in Vacuo, at pH 7.0, and 37.5°, in 15 Minutes

Tissue	Experiment carried out in air or *in vacuo*	Ascorbic acid in 5 cc. solution		Reduced glutathione found in solution	Added dehydroascorbic acid recovered as ascorbic acid	
		Added as dehydroascorbic acid	Found as ascorbic acid		Observed	Calculated (see Tables X and XII)
		mg.	*mg.*	*mg. per cent*	*per cent*	*per cent*
Kidney	Air	0	0.27	59		
	"	0.5	0.53	60	52	35
	Vacuum	0	0.22	76		
	"	0.5	0.51	59	59	40
Liver	Air	0	0.35	74		
	"	0.5	0.63	70	60	50
	Vacuum	0	0.32	80		
	"	0.5	0.58	70	52	50
Intestinal mucosa thoroughly macerated	Air	0	0.33	66		
	"	0.5	0.58	44	50	30
	Vacuum	0	0.38	58		
	"	0.5	0.64	44	52	30
Intestinal mucosa not ground up, not diluted	Air	0	0.43	105		
	"	0.5	0.75	105	64	55

same order of magnitude as those determined by Benet and Weller (38) with their cadmium-iodate method. With intestinal mucosa one experiment was carried out with the tissue macerated as with liver and kidney; and a second in which the mucosa after being scraped off the muscular wall was left intact.

Table VIII shows that the reduction of dehydroascorbic acid by minced tissues is as rapid in air as *in vacuo*. This finding and

the rates of reduction observed indicate (anticipating here the results in Tables X and XII) that the reducing agent is glutathione. The figures for the calculated amounts given in Table VIII were obtained from rate studies with glutathione (see below). The reduction obtained in excess of these calculated amounts may be ascribed to the fixed —SH groups. A slow reduction of oxidized ascorbic acid by washed, minced muscle was observed by Szent-Györgyi (2). He ascribed this reducing action to the fixed —SH groups. The smallness of the excess of the observed over the calculated rate of reduction argues against the existence of an enzyme for this reaction. If there is an enzyme its potency or concentration is low.

Table IX shows the reduction of dehydroascorbic acid by slices or sections of surviving scorbutic guinea pig tissues in an atmosphere of 95 per cent oxygen and 5 per cent CO_2.

The technique of preparing and handling the tissue slices has been described in a previous communication (39). The figures in Table IX are those obtained after subtracting the quantities found in the parallel controls, where the tissues were incubated without added dehydroascorbic acid. The small amounts of dehydroascorbic acid found may have been formed during the extraction of the tissues with acid water.

The reducing capacity of the tissues for dehydroascorbic acid is greater than the bare figures in Table IX indicate. In interpretation of these figures it must be borne in mind that the irreversible change in dehydroascorbic acid in Ringer's solution at the pH and temperature of these experiments is very rapid, and the volume of solution (5 cc.) in which the tissue slices were suspended was quite large. Unlike a minced tissue in which the reducing mechanism is uniformly distributed throughout the solution, in these experiments with tissue slices the reducing mechanism was concentrated in not more than 20 per cent of the volume, and was effective against only that small fraction of the dehydroascorbic acid which had succeeded in penetrating into the interior of the cell before undergoing irreversible change in the external solution.

Protection of Ascorbic Acid from Oxidation by Minced and Intact Tissues—De Caro (33), Mawson (40), and Kellie and Zilva (28) have shown that minced tissues and tissue extracts protect ascorbic acid from oxidation. Quastel and Wheatley (41) have observed a

similar protective action exerted by rat liver slices, and we have observed it with sections or slices of scorbutic guinea pig tissues—intestine, liver, and kidney.

The explanation given has been that the tissues combine with the heavy metals, iron and copper, which catalyze the oxidation of ascorbic acid. The experiments in Tables VIII and IX indicate that an additional protective mechanism, and possibly a more important one, is reduction of the dehydroascorbic acid formed by any oxidation of the ascorbic acid which may have occurred.

TABLE IX

Reduction of Dehydroascorbic Acid (10 Mg. Per Cent) by Scorbutic Guinea Pig Tissue Sections in Ringer's Solution under 95 Per Cent Oxygen and 5 Per Cent CO_2 in 2 Hours, at pH 7.4, and 37.5°

Tissue	Dry weight	Amount recovered		Recovered, reduced + oxidized
		As ascorbic acid	As dehydro-ascorbic acid	
	mg.	*mg. per cent*	*mg. per cent*	*per cent*
Ringer's solution		0.038	0.30	3.5
Liver	112	1.24	0.38	16
Kidney	122	1.36	0.06	14
Intestine	323	2.09	0.16	23

Reduction of Dehydroascorbic Acid by Glutathione

In his first full account of hexuronic acid Szent-Györgyi (2) reported that when the reversibly oxidized substance was added to a suspension of minced kidney, after 15 minutes a qualitative test for the reduced form was obtained, and at the same time the nitroprusside test had become weaker. Also when oxidized hexuronic acid was incubated *in vacuo* in neutral solution with glutathione, the latter substance was oxidized. The method used for reduced ascorbic acid, when applied to a minced tissue, is questionable. No mention was made of the ascorbic acid in the minced kidney preparation alone. It was possible that the oxidation of glutathione observed was the result of interaction with one of the products of the irreversible change in dehydroascorbic acid which is very rapid at pH 7.0. Later Pfankuch *et al.* (42) and Bersin and his collaborators (43) stated that neither glutathione alone nor

cysteine alone is able to reduce oxidized ascorbic acid. Pfankuch adduced some evidence for the existence of an enzyme in the potato which makes this reaction possible; and by implication Bersin and his collaborators postulated the existence of such an enzyme in animal tissues—at least in the guinea pig adrenal.

Preliminary to an investigation of this postulated enzyme we reexamined the reaction between glutathione and dehydroascorbic acid. We found that if a sufficiently high concentration of glutathione is used, the reduction of dehydroascorbic acid is very rapid,

TABLE X

Reduction of Dehydroascorbic Acid, Initial Concentration 4 Mg. Per Cent, by Different Concentrations of Glutathione, in Vacuo, at pH 7.0, and 37.5°, in 4 Hours

Glutathione	Oxidizing agent for ascorbic acid	Reduction of oxidized ascorbic acid added
mg. per cent		*per cent*
4	I_2	3
20	"	13
60	"	40
100	"	57
150	"	72
200	"	88
200	Br_2	96
200	Air + $CuCl_2$ (36 mg. per liter)	90
250	I_2	96
300	"	95
350	"	100
400	"	97

even in air. The reduction of this substance by minced tissues can be nearly quantitatively accounted for by the acid-soluble —SH groups (*i.e.*, mainly glutathione) on the assumption that no enzyme intervenes.

Rate of Reduction of Dehydroascorbic Acid by Different Concentrations of Glutathione—Table X shows the effect of different glutathione concentrations on the degree of reduction of the dehydroascorbic acid. The figures in Tables X to XIV require little additional comment.

The experimental and analytical technique used was as follows:

10 mg. of dry glutathione and 4 cc. of phosphate-citrate buffer (McIlvaine's series) were transferred to the lower part of a Thunberg tube. 1 cc. of 20 mg. per cent ascorbic acid oxidized by means of iodine in unbuffered aqueous solution was pipetted into the overhang. After evacuation with an oil pump the two solutions were mixed and the vessel then immersed in a water bath at 37.5°. At the end of 4 hours the volume of the solution was marked, the vacuum broken, 2 cc. of 10 per cent metaphosphoric acid added immediately after, and then aliquots of the solution titrated with dye in the usual manner. The glutathione present did not interfere with the end-point until its concentration was 300 mg. per cent. With this and higher concentrations of glutathione the end-points were uncertain. Parallel experiments with glutathione alone, at all the concentrations used, and dehydroascorbic acid alone were also carried out. The blanks in every case except with the two highest concentrations of glutathione were negligible.

We examined the possibility that this reaction between glutathione and dehydroascorbic acid was peculiar to solutions containing HI; *i.e.*, resulting from the oxidation of the ascorbic acid by iodine. This possibility was mentioned by Pfankuch in explanation of the discrepancy between his negative results and the positive results reported by Szent-Györgyi. Accordingly the experiment of Table X with 200 mg. per cent of glutathione was repeated with ascorbic acid oxidized in one case, by bromine (the oxidizing agent used by Pfankuch), and in another by oxygen catalyzed by copper. The figures show that somewhat higher yields were obtained with these oxidizing agents than with iodine.

We also investigated at this stage the effect, if any, of the composition of the buffer solution. Different concentrations of the same buffer salt mixture and none at all (in which the pH was maintained by addition of alkali), were tried. In four such solutions differing with respect to the amount of salt present the same degree of reduction was obtained with 200 mg. per cent of glutathione and an initial concentration of 4 mg. per cent of oxidized ascorbic acid.

We have concluded therefore that neither the oxidizing agent used in the oxidation of the ascorbic acid nor the composition of the buffer solution is significant in the reduction of dehydroascorbic acid by glutathione.

A much shorter time than 4 hours is required for the attainment of a nearly maximum reduction of added dehydroascorbic acid. Thus with 4 mg. per cent of dehydroascorbic acid and 200 mg. per cent of glutathione at pH 7.0 and 37.5°, *in vacuo*, the following degrees of reduction were found: in 5 minutes 52 per cent, in 10 minutes 73 per cent, in 15 minutes 80 per cent, in 120 minutes 86 per cent, and in 240 minutes 87 per cent. The failure to obtain 100 per cent reduction we ascribe mainly to the irreversible change in dehydroascorbic acid before it is reduced by the glutathione. At this pH and concentrations of glutathione and dehydroascorbic acid the interaction between the reductant and oxidant is very rapid; but a small fraction of the latter does undergo its irreversible

TABLE XI

Effect of pH on Rate of Reduction of Dehydroascorbic Acid (10 Mg. Per Cent) by Glutathione (200 Mg. Per Cent) at 37.5°, in Vacuo

pH	Reduction of ascorbic acid in	
	½ hr.	20 hrs.
	per cent	*per cent*
4	<1	13
5	6	62
6	49	79
6.5	71	82
7.0	85	86
8.0	91	91

change. After this change it loses the property of reduction to the original form by glutathione in neutral solution (Table III).

Effect of pH on Rate of Reduction of Dehydroascorbic Acid by Glutathione—The higher the pH the faster is the rate of reduction of dehydroascorbic acid by glutathione. This rate increases faster with increasing pH than the rate of the irreversible change in dehydroascorbic acid. Hence the highest yields of the series were obtained at pH 8.0.

Effect of Concentration of Dehydroascorbic Acid on Its Rate of Reduction by Glutathione—The degree of reduction of dehydroascorbic acid at any given pH and temperature is dependent mainly on the concentration of glutathione.

Table XII shows that with 200 mg. per cent of glutathione

nearly the same percentage reduction was obtained in 15 to 30 minutes with concentrations of ascorbic acid varying from 2 to 50 mg. per cent. This relation together with the speed at which dehydroascorbic acid is reduced by glutathione suggests that there is some preliminary compound formation between it and gluta-

TABLE XII

Rate of Reduction of Different Concentrations of Dehydroascorbic Acid by 200 Mg. Per Cent of Glutathione at pH 7.0, and 37.5°, in Vacuo

Dehydro-ascorbic acid	Reduction of added dehydroascorbic acid in different time intervals				
	5 min.	15 min.	30 min.	120 min.	240 min.
mg. per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2		85		91	
4	66	76	86	85	87
10	65	83	86	86	88
25	57	84	81		
50	48		72		
100	32		57		

TABLE XIII

Rate of Reduction of Dehydroascorbic Acid in Vacuo and in Air with Different Concentrations of Glutathione and Ascorbic Acid in 15 Minutes, at pH 7.0, and 37.5°

Glutathione	Per cent dehydroascorbic acid reduced			
	Initial concentration, 4 mg. per cent		Initial concentration, 10 mg. per cent	
	In vacuo	In air	*In vacuo*	In air
mg. per cent				
20	12	10	14	9
60	35	32	30	33
100	54	56	56	54
150	69	72	70	73
200	80	80	81	78

thione. The rate is much too rapid for a third order reaction; *i.e.*, between 2 separate molecules of glutathione and 1 of dehydroascorbic acid. Bersin and his collaborators found some evidence of compound formation between glutathione and reduced ascorbic acid.

Reduction of Dehydroascorbic Acid by Glutathione in Vacuo and

in Air—Table XIII shows that the rate of reduction of dehydroascorbic acid by glutathione is as rapid in air as *in vacuo*. This holds true over a wide range of glutathione concentrations, and probably, though here only two concentrations have been tested, also over a wide range of dehydroascorbic acid concentrations.

TABLE XIV

Recovery of Ascorbic Acid from Dehydroascorbic Acid (10 Mg. Per Cent) in Vacuo and in Air at 37.5° after Different Time Intervals in Solutions Containing Glutathione (200 Mg. Per Cent) and Different Metallic Impurities, and at Different Hydrogen Ion Concentrations

Variety of glutathione	pH	*In vacuo* or in air	Per cent of original dehydroascorbic acid recovered in reduced form after different time intervals							
			0.25 hr.	0.5 hr.	1 hr.	2 hrs.	4 hrs.	5 hrs.	7 hrs.	20 hrs.
Crude	7	Air		64	60	48				
"	7	Vacuum		73			76			
"	8	Air		81	66	11				
"	8	Vacuum		85			90			
Cadmium	7	Air		54	48	31				
"	7	Vacuum		63			65			
"	8	Air		82	66	5				
"	8	Vacuum		80			79			
Alcohol	6	Air		52	50			53	48	34
" + Cu	6	"			70*			23*	3	
"	6	Vacuum		49						79
"	7	Air	78	85	75	69	53			0
" + Cu	7	"		101*	99*	90*	4*			
"	7	Vacuum	82	85	86		86			86
"	8	Air		92	87	64	0			0
" + Cu	8	"		91*	108*	24*	0			0
"	8	Vacuum	91	91				91		91

Crude designates glutathione prepared and purified by the usual copper precipitation; cadmium is the crude material subjected to further purification by way of the cadmium salt; and alcohol the crude material precipitated from aqueous solution by alcohol.

* Denotes that the end-point was fugitive and arbitrarily chosen. Only part of the titration value is to be attributed to ascorbic acid, the remainder probably to cysteine arising from the hydrolysis of the glutathione.

This can be observed only if the period of incubation is relatively short—15 minutes to ½ hour. In a longer time air oxidation of both the ascorbic acid formed and the glutathione, accompanied

by hydrolysis of the latter, supervenes (Table XIV). The figures show that the yields obtained in air are affected by metallic impurities present. The addition of copper (182 mM of $CuCl_2$ per liter) accelerated, in air, the destruction of the ascorbic acid and the glutathione. The figures marked with an asterisk were those in which the behavior of the end-point in the dye titration indicated the presence of cysteine, arising from the hydrolysis of the glutathione. Titrations for glutathione indicated that in the air experiments a large fraction (50 per cent or more) had disappeared in 7 hours, and none remained after standing overnight.

Three preparations of glutathione were used in order to examine the effect of impurities. One preparation, designated as crude in Table XIV, was prepared and purified by the usual copper precipitation. Some of the crude material was purified further by way of the cadmium salt; it is designated as cadmium. A third alcohol product was prepared by precipitating the crude material from aqueous solution by alcohol. The crude material contained 91 per cent of the theoretical quantity of—SH groups, the other two preparations 97.5 per cent. Table XIV shows that impurities are less significant *in vacuo* than in air. The rates of reduction *in vacuo* with the three preparations were in the inverse order of their destruction in air. Judged by these two criteria the cadmium preparation contained the most impurity, the alcohol preparation the least. In all the other experiments recorded in this paper the alcohol preparation was used.[1]

We have compared the following twenty-nine substances with glutathione with respect to their ability to reduce dehydroascorbic acid: *dl*-alanine, *l*-asparagine, *l*-aspartic acid, *l*-cysteine, lithium formate, fructose, sodium fumarate, sodium glycerophosphate, galactose, glucose, *d*-glutamic acid, glycine, calcium hexosediphosphate, *l* histidine, sodium *l*(+)-lactate, *l*-leucine, *d*-lysine, sodium *l*-malate, sodium maleate, *dl*-methionine, *d*-ornithine, *dl*-phenylalanine, *l*-proline, lithium pyruvate, *dl*-serine, sodium succinate, *l*-tryptophane, *l*-tyrosine, and *d*-valine. The experiments were carried out in air, at pH 7.0 and 37.5°. The initial concentration of dehydroascorbic acid was 10 mg. per cent, that of the other substances 0.01 M.

[1] We are indebted for these preparations of glutathione to Mr. S. W. Fox.

Except with cysteine no reduction was found with any of these substance in 15 minutes. In 60 minutes reduction equivalent to 3 per cent was found with lithium pyruvate, 2 per cent with calcium hexosediphosphate, and 1 per cent with sodium *l*-malate. The remainder gave less than 1 per cent or none.

Cysteine was conspicuous in this group as the only substance capable of reducing dehydroascorbic acid at a significant rate. Compared with glutathione, at the same molal concentration the amino acid was one-half as effective as the tripeptide. In other respects its action was similar. In the first 15 minutes the same degree of reduction was obtained in air and *in vacuo*. On longer incubation in air a lower yield was obtained on account of the oxidation of the cysteine and of the ascorbic acid formed.

Von Euler, Karrer, and Zehender (44) reported that dehydroascorbic acid may oxidize leucine, with the formation of ammonia and reducing substances. Similarly Heard and Welch (17) stated that the primary oxidation product of ascorbic acid is capable of oxidizing pyruvate and amino acids. Our observations indicate that none of the amino acids (except cysteine) nor pyruvate is capable of reducing dehydroascorbic acid at a significant rate. It would seem a safe generalization that only the —SH group is physiologically significant in this respect.

It is interesting that glucose, glucose dehydrogenase plus its coenzyme, glutathione, a hemochromagen, ascorbic acid, and oxygen constitute a complete respiratory system in which a metabolite is burned and oxygen used. The system is a simple one involving only one enzyme. All its components have been found in the tissues. It is probably this system which is responsible for the higher oxygen consumption observed by Quastel and Wheatley (41) when ascorbic acid is added to liver slices.

The authors wish to thank Mr. A. J. Lorenz, Research Director of the California Fruit Growers Exchange, for his helpfulness during the course of this work, and for the fellowships which made the participation of two of the authors possible. We are indebted to Dr. A. G. Foord and Miss L. Randall, of the Huntington Memorial Hospital, for their cheerful assistance on numerous occasions.

SUMMARY

1. Data are presented on the reversible potentials of the first and second oxidation stages of ascorbic acid.

2. These data and other experiments account for most of the previous discrepancies in the determination of the reversible oxidation-reduction potential of the first oxidation stage.

3. Proof is presented that only the first oxidation, ascorbic acid $\rightleftharpoons$ dehydroascorbic acid, is physiologically reversible and significant in its antiscorbutic action.

4. An irreversible non-oxidative change in dehydroascorbic acid intervenes between the first and second oxidation stages. This irreversible change underlies the following phenomena: the negative drift in the oxidation-reduction potential measurements, the loss of reversibility of the first oxidation stage in the electrometric and colorimetric potential measurements, the increase in reducing power, the increase in strength of the dissociation constant of the ionizable hydrogen, the inability of H_2S in dilute acid solution and of glutathione in neutral and alkaline solutions to restore ascorbic acid from solutions of the oxidized vitamin, and the loss in antiscorbutic potency.

5. All of these manifestations of the irreversible change in dehydroascorbic acid exhibit the same dependence on the hydrogen ion concentration.

6. They are similarly independent of the presence of air or oxidizing agents (where air does not interfere with the reaction employed to show the irreversible change).

7. They are all similarly in accord that the change is an irreversible one; *i.e.*, that the two forms of the first oxidation product are not in equilibrium in dilute solution.

8. Ascorbic acid is oxidized very slowly in whole blood (human). It remains in the reduced state much longer in whole blood than in plasma.

9. The red blood corpuscles of beef, cat, dog, human, pig, rat, and sheep are nearly, if not absolutely, impermeable to added ascorbic acid.

10. There is no mechanism in human blood for reducing dehydroascorbic acid, or for retarding its irreversible change.

11. The antiscorbutic potency of dehydroascorbic acid is the

same as that of ascorbic acid, whether administered *per os* or subcutaneously. Yet *in vitro* at the pH and temperature of the tissues, dehydroascorbic acid quickly undergoes an irreversible change, whereby it loses its antiscorbutic potency.

12. This difference in its behavior *in vivo* and *in vitro* is the result of the rapid reduction of dehydroascorbic acid in the tissues. This reduction does not occur in the blood.

13. Evidence is presented that glutathione is probably the principal reducing agent here.

14. Some of the conditions are described affecting the rate of the reaction between glutathione and dehydroascorbic acid.

BIBLIOGRAPHY

1. Borsook, H., and Jeffreys, C. E. P., *Science*, **83**, 397 (1936).
2. Szent-Györgyi, A., *Biochem. J.*, **22**, 1387 (1928).
3. Georgescu, I. D., *J. chim. physiq.*, **29**, 217 (1932).
4. Karrer, P., Schwarzenbach, K., and Schöpp, G., *Helv. chim. acta*, **16**, 302 (1933).
5. Laki, K., *Z. physiol. Chem.*, **217**, 54 (1933).
6. Green, D. E., *Biochem. J.*, **27**, 1044 (1933).
7. Borsook, H., and Keighley, G. L., *Proc. Nat. Acad. Sc.*, **19**, 875 (1933).
8. Wurmser, R., and de Loureiro, J.-A., *J. chim. physiq.*, **31**, 419 (1934).
9. Fruton, J. S., *J. Biol. Chem.*, **105**, 79 (1934).
10. Herbert, R. W., Hirst, E. L., Percival, E. G. V., Reynolds, R. J. W., and Smith, F., *J. Chem. Soc.*, 1270 (1933).
11. Borsook, H., and Schott, H. F., *J. Biol. Chem.*, **92**, 535 (1931).
12. Birch, T. W., and Harris, L. J., *Biochem. J.*, **27**, 595 (1933).
13. Ball, E. G., *Proc. Am. Soc. Biol. Chem.*, **8**, vii (1936) (*J. Biol. Chem.*, **114** (1936)).
14. Barron, E. S. G., deMeio, R. H., and Klemperer, F., *J. Biol. Chem.*, **112**, 625 (1935–36).
15. Bessey, O. A., and King, C. G., *J. Biol. Chem.*, **103**, 687 (1933). Harris, L. J., and Ray, S. N., *Biochem. J.*, **27**, 303 (1933). Birch, T. W., Harris, L. J., and Ray, S. N., *Nature*, **131**, 273 (1933).
16. Tillmans, J., *Z. Untersuch. Lebensmittel*, **40**, 34 (1930).
17. Heard, R. D. H., and Welch, A. deM., *Biochem. J.*, **29**, 998 (1935).
18. Shwachman, H., Hellerman, L., and Cohen, B., *J. Biol. Chem.*, **107**, 257 (1934).
19. Demole, I., *Z. physiol. Chem.*, **217**, 83 (1933).
20. Zilva, S. S., *Biochem. J.*, **21**, 689 (1927); **22**, 779 (1928).
21. Fox, F. S., and Levy, L. F., *Biochem. J.*, **30**, 211 (1936).
22. Sherman, H. C., and Smith, S. L., The vitamins, American Chemical Society monograph series, New York, 2nd edition, 148 (1931).
23. Roe, J. H., and Barnum, G. L., *J. Nutrition*, **11**, 359 (1936).

24. Emmerie, A., and van Eekelen, M., *Biochem. J.*, **28**, 1153 (1934).
25. Linderstrom-Lang, K., and Holter, H., *Ergebn. Enzymforsch.*, **3**, 309 (1934).
26. Fujita, A., and Iwatake, D., *Biochem. Z.*, **277**, 293 (1935).
27. Barrenscheen, H. K., and Popper, L., *Biochem. Z.*, **161**, 210 (1925). Barrenscheen, H. K., and dell'Acqua, G., *Biochem. Z.*, **219**, 297 (1930). Barrenscheen, H. K., and Prinz, H., *Biochem. Z.*, **284**, 99 (1936).
28. Kellie, A. E., and Zilva, S. S., *Biochem. J* , **29**, 1028 (1935).
29. van Eekelen, M., *Nature*, **136**, 144 (1935).
30. Kellie, A. E., and Zilva, S. S., *Biochem. J.*, **30**, 361 (1936).
31. Farmer, C. J., and Abt, A. F., *Proc. Soc. Exp. Biol. and Med.*, **34**, 146 (1936).
32. Plaut, F., and Bülow, M., *Z. physiol. Chem.*, **236**, 241 (1935).
33. De Caro, L., *Z. physiol. Chem.*, **223**, 229 (1933–34). De Caro, L., and Giani, M., *Z. physiol. Chem.*, **228**, 13 (1934).
34. Olmsted, J. M. D., *Am. J. Physiol.*, **3**, 551 (1935).
35. Fox, F. W., and Levy, L. F., *Biochem. J.*, **30**, 207 (1936).
36. Johnson, S. W., and Zilva, S. S., *Biochem. J.*, **28**, 1393 (1934).
37. Quensel, W., and Wacholder, K., *Z. physiol. Chem.*, **231**, 65 (1934–35).
38. Benet, L., and Weller, G., *Bull. Soc. chim. biol.*, **18**, 358 (1936).
39. Borsook, H., and Jeffreys, C. E. P., *J. Biol. Chem.*, **110**, 495 (1935).
40. Mawson, C. A., *Biochem. J.*, **29**, 569 (1935).
41. Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, **28**, 1014 (1935).
42. Pfankuch, E., and Lindau, G., *Biochem. Z.*, **277**, 129 (1935); **279**, 115 (1935).
43. Bersin, T., Köster, H., and Jusatz, H. J., *Z. physiol. Chem.*, **235**, 12 (1935).
44. von Euler, H., Karrer, P., and Zehender, F., *Helv. chim. acta*, **17**, 157 (1934).