LXVII. THE INFLUENCE OF ANIMAL TISSUES ON THE OXIDATION OF ASCORBIC ACID.

By COLIN ASHLEY MAWSON.

*From the John Burford Carlill Laboratories, Westminster Hospital
Medical School, S.W. 1.*

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It has usually been considered that ascorbic acid cannot be kept under aerobic conditions except in the solid form unless in relatively strongly acid solution. Green [1933] suggested that under the conditions of p_H and oxygenation of living tissues the existence therein of reduced ascorbic acid might be explained by the presence of some anti-oxidative mechanism. St Huszák [1933] showed that a substance occurred in the suprarenal medulla which prevented the reduction of silver nitrate by the ascorbic acid of suprarenal medulla and cortex. This protective substance was precipitated by lead acetate. Further work by de Caro [1934] and by de Caro and Giani [1934] which appeared during the course of this investigation led to the conclusion that in most animal tissues a protective mechanism existed of the kind postulated by Green. This work showed that ascorbic acid in phosphate buffer (p_H 7.5) would remain unoxidised in presence of air for several hours if mixed with small amounts of liver, muscle, blood, ovary, nerve or suprarenal gland from normal or scorbutic guinea-pigs. The protective material could be extracted by phosphate buffers, trichloroacetic acid or alcohol and was not precipitated by lead acetate or mercury. Although extracts deprived of reduced glutathione by mercury treatment still possessed protective power, de Caro and Giani considered that traces of oxidised glutathione had escaped precipitation and would account for the protection, but they pointed out that in this case the substance responsible differed from that observed by St Huszák, which was precipitated by lead acetate.

In the present paper it has been shown that although no protective mechanism exists in the lemon apart from the acidity of the juice, small traces of animal tissues or tissue extracts will prevent the oxidation of ascorbic acid in $M/30$ phosphate buffer at p_H 7.4 when incubated at 37° . This protective effect is most powerful in the presence of whole tissues, but extracts filtered through Bechhold collodion membranes (0.7μ pores) still contain the protective mechanism. Active extracts have been made from the liver, spleen, brain and muscle of rats.

The protective effect of tissues can be closely imitated by cysteine, cystine, H_2S and glutathione. Extracts treated with mercuric acetate retain only about 1/100 of their original activity and at the same time lose most of their glutathione, but by heating liver extract for one hour in a boiling water-bath the protective activity can be reduced to about 1/10 of its original value without any change in the glutathione content. Addition of iodoacetate has no effect on protection by liver extract and only slightly reduces protection by glutathione when present in excess. Quantitative experiments showed that far more glutathione, as the pure substance, was necessary to prevent the oxidation of ascorbic acid than could be found in extracts giving optimum protection, and dialysed extracts containing practically no reduced glutathione had exactly the same protective power as undialysed extracts. The various pure substances used did

not give as high a degree of protection as liver extract, the presence of which, at optimum concentration, would usually preserve 90 % of the original concentration of ascorbic acid for an hour at 37° in *M*/30 phosphate (p_H 7.4).

Ascorbic acid is comparatively stable at 37° if dissolved in water twice redistilled from a glass apparatus, but the addition of minute traces of copper caused rapid oxidation. Ferrous and ferric ions catalysed the oxidation to a less extent, while mixtures of iron and copper caused oxidation to take place more rapidly than addition of either metal alone. It is considered likely that the rapid oxidation of ascorbic acid solutions made from ordinary laboratory distilled water is due to contamination with traces of copper and iron.

Copper in fairly high concentration catalysed the aerobic oxidation of glutathione, but iron had no appreciable effect, confirming the results of Pirie [1931]. Addition of iron to liver extracts did not reduce their power to protect ascorbic acid from oxidation until a concentration of 10^{-3} mg. per ml. was reached, and iron had little effect on the protective power of glutathione. A reduction in the protective power of glutathione was effected by 4×10^{-4} mg. Cu per ml., but that of liver extract containing the same amount of glutathione was only reduced by copper concentrations greater than 2×10^{-3} mg. per ml. The amounts of free copper and iron in the serum, and especially in the liver, are so large that without some anti-oxidative mechanism the oxidation of ascorbic acid would be very rapid indeed. Tompsett [1934] gives the copper content of blood serum as 2×10^{-3} mg. per ml. and Fowweather [1934] states that the iron content of serum is about 1.6×10^{-3} mg. per ml. At the copper concentration of serum a 0.005 % solution of ascorbic acid in glass-distilled water was almost completely oxidised in one hour without the addition of any iron at all.

It has not been found possible to decide upon the nature of the undialysable part of the protective mechanism, but several results suggest that in the fresh extract the effect is due to several substances, some of which can be removed by dialysis and some precipitated by mercury or trichloroacetic acid or by boiling, but whichever of these processes is used there is always sufficient of the protective mechanism left behind to be easily detectable. It seems unlikely that the protective effect can be wholly due to the same substance or group of substances in each case.

EXPERIMENTAL.

Ascorbic acid has been estimated by titration with 0.01 % 2:6-dichlorophenolindophenol [Harris and Ray, 1933] in 4 % trichloroacetic acid solution. Glutathione has been estimated in 4 % trichloroacetic acid solutions containing ascorbic acid by oxidation of the ascorbic acid with the dye, addition of starch and titration with *N*/500 iodine. Using mixtures of glutathione and ascorbic acid, it was found that these substances could be accurately estimated by this method.

Where tissues or tissue extracts containing "ascorbic acid" or glutathione have been used, appropriate corrections have been applied. Unless otherwise stated, reagents and extracts have been made up with water distilled through a tinned copper coil with a solid tin nozzle and this has been referred to as "ordinary" distilled water. All metallic salt solutions have been made up in glass-distilled water.

In an attempt to decide whether the reducing substance which occurs in normal animal tissues was identical with that of tumour tissue [Boyland, 1933], and whether these substances were indeed ascorbic acid, an investigation was made of the effect of exposure to a temperature of 37° on the reducing values of various tissues. It was found that whether the tissue were whole or minced, alone or in presence of phosphate (*M*/30, p_H 7.4), or with phosphate + glucose, the amount of reducing substance remained unchanged, at any rate for periods

up to 4 hours at 37° (Table I). These results confirm a recent observation by Guha and Ghosh [1934] on normal rat tissues.

Table I. "Ascorbic acid" in tissues incubated at 37°.

Tissue 1 g. minced	Duration of incubation mins.	ml. dye	mg. "ascorbic acid" per g.
Rat liver	0	7.40	0.33
	90	7.77	0.35
Rat testes	0	7.52	0.34
	90	7.77	0.35
Rous chicken tumour	0	1.48	0.07
	60	1.32	0.06

Table II. Ascorbic acid incubated at 37° with and without minced rat liver.

All experiments had total volume of 4.5 ml. (phosphate $M/34$, p_H 7.4).

Ascorbic acid alone					
Incubation time (hrs.) ...	0	$\frac{1}{2}$	1	2	24
ml. dye	3.82	0.30	0.32	0.20	0.16
ml. iodine ($N/500$)	0.10	0.10	0.10	0.10	0.10
Liver alone					
Incubation time (hrs.) ...	0	$\frac{1}{2}$	1	2	24
ml. dye	4.31	4.30	4.54	4.28	2.14
ml. iodine ($N/500$)	2.05	2.22	2.13	2.08	1.63
Ascorbic acid + liver					
Incubation time (hrs.) ...	0	$\frac{1}{2}$	1	2	24
ml. dye	8.07	8.25	8.11	8.14	5.55
ml. iodine ($N/500$)	2.18	2.14	2.07	2.20	1.78

Blank on reagents: 0.19 ml. dye, 0.10 ml. iodine.

The instability of ascorbic acid in air at p_H 7.4 is well known, so it seemed doubtful whether the reducing substance could really be ascorbic acid. In order to check this point ascorbic acid solution (B.D.H. commercial) was kept at 37° with and without minced rat liver tissue in presence of $M/30$ phosphate, p_H 7.4. Although the ascorbic acid without additions had almost completely disappeared in half an hour at 37°, the same amount of ascorbic acid added to 0.5 g. minced liver was not oxidised at all after 2 hours. Even after 24 hours a large proportion still remained, though autolytic changes probably made the estimations in the latter case somewhat unreliable (Table II, Fig. 1).

In this experiment, and in subsequent experiments of a similar nature, the iodine titre obtained subsequent to oxidation with 2:6-dichlorophenolindophenol has been assumed to represent glutathione, and it is evident that under the conditions of this experiment the glutathione content of the incubated tissue remained constant at 37° for at least 2 hours whether added ascorbic acid were present or not.

Fig. 1. Stabilisation of ascorbic acid by minced liver.

The stability of the reducing substance of liver, and the exact similarity of behaviour of ascorbic acid added to liver, are in such marked contrast to the rapid disappearance of ascorbic acid in absence of added tissue that this experiment tends to confirm the view that these substances are identical. The stability of the reducing substance (subsequently referred to as "ascorbic acid") is not confined to short periods of incubation at 37°, for if an animal was killed and kept at 4° for 24 hours, or if 5 % trichloroacetic acid extracts of the tissues were kept under similar conditions, the amount of "ascorbic acid" present did not decrease to any great extent.

Since tissues maintained their "ascorbic acid" content equally well whether minced or whole, the behaviour of extracts and suspensions was investigated. In these and subsequent experiments rat liver was generally used because of the ease with which it could be ground up and the large amount available in each animal, but similar results were obtained with other tissues. The suspensions were made by grinding the tissue with *M*/30 phosphate, p_H 7.5, and the extracts by centrifuging such suspensions for 15 minutes at 3000 r.p.m., pouring off, and re-centrifuging the supernatant liquid for the same time.

Both "ascorbic acid" and glutathione disappeared more rapidly in extracts than in suspensions, and both were more stable in the more concentrated preparations. In each case the "ascorbic acid" and glutathione occurring naturally in these preparations were estimated; no additions were made (Table III). The

Table III. "Ascorbic acid" and glutathione in liver extracts and liver suspensions after various periods of incubation at 37°.

		Blanks on reagents subtracted.									
		% remaining after x hours									
Preparation	mg. tissue per ml.	"Ascorbic acid"					Glutathione				
		0	$\frac{1}{2}$	1	2	4	0	$\frac{1}{2}$	1	2	4
*Suspension	200	100	103	105	101	87	100	97	96	90	87
*Extract	200	100	107	107	106	93	100	92	86	76	64
Suspension	100	100	99	99	98	87	100	99	98	71	48
Extract	100	100	93	86	80	68	100	70	52	32	14
*Suspension	5	100	87	87	76	51	100	36	30	28	29
*Extract	5	100	88	83	64	34	100	1.7	1.7	7.4	8.4
†Glutathione alone	1 mg. per ml.	—	—	—	—	—	100	57	30	Nil	Nil

* Mean of two experiments.

† B.D.H. glutathione in *M*/30 phosphate, p_H 7.4.

"ascorbic acid" in liver extracts and suspensions is more stable than the glutathione, but the decrease in amount of these substances during incubation does to some extent run a parallel course. A comparison was therefore made between the protective properties of rat liver extract and those of B.D.H. glutathione. In both cases an optimum concentration could be determined at which the maximum anti-oxidative effect was operative and the curves showing the variation of the protective effect with glutathione concentration were very similar in general appearance (Table IV, Fig. 2). In a series of ten experiments with centrifuged liver extracts the amount of liver necessary to give optimum protection varied considerably (from 0.13 mg. per ml. of ascorbic acid solution to 1.70 mg. per ml.), the mean value being 0.6 mg. per ml. The latter amount of rat liver would contain about 1.5×10^{-3} mg. glutathione, but the actual amount of reduced glutathione in the extracts was only of the order of 5×10^{-4} mg per ml. owing to the rapid oxidation of dilute solutions of liver glutathione.

Table IV. *Comparison of protective powers of centrifuged liver extract and glutathione.*

2 ml. liver extract (or 2 ml. glutathione solution) in $M/30$ phosphate p_H 7.4 + 2 ml. ascorbic acid in same buffer. Incubated in open tubes for one hour at 37° . Unincubated control 2.87 ml. dye. Blanks on reagents subtracted.

Liver dilution	1/40	1/400	1/2000	1/4000	1/8000	1/16,000	1/32,000
Containing mg. G.SH per ml.	3×10^{-2}	3×10^{-3}	6×10^{-4}	3×10^{-4}	1.5×10^{-4}	8×10^{-5}	4×10^{-5}
ml. dye	2.34	2.80	2.65	0.85	0.05	0	0
% of control	82	98	93	30	1.8	0	0
mg. B.D.H. glutathione per ml.	1.8×10^{-1}	1.8×10^{-2}	9×10^{-3}	4.4×10^{-3}	1.1×10^{-3}	2.2×10^{-4}	2.2×10^{-5}
ml. dye	1.58	1.78	1.17	0.74	0	0	0
% of contro	55	62	41	26	0	0	0

Fig. 2. Stabilisation of ascorbic acid by liver extract and glutathione.

Fig. 3. Stabilisation of ascorbic acid by liver extract and glutathione, inhibition by copper.

The amount of B.D.H. glutathione necessary to give optimum protection was of the order of 3.3×10^{-2} mg. per ml. At the optimum liver concentration the protection afforded during 1 hour at 37° was usually about 90 %, but the protection given by glutathione seldom rose above 75 %. Nevertheless some of the pure glutathione might have been oxidised before addition to the ascorbic acid, although the glutathione and ascorbic acid solutions were both made in air-free phosphate and the tubes set up as rapidly as possible. Dr E. Boyland suggested that the commercial glutathione might contain traces of copper which

Table V. *Effect of copper on protective properties of liver extract and glutathione.*

Liver extract and B.D.H. glutathione (each 0.02 mg. G.SH per ml.) for 1 hour at 37° with ascorbic acid and CuSO_4 . Unincubated control 11.75 ml. dye. Blanks on reagents subtracted.

mg. Cu per ml.	0	4×10^{-5}	8×10^{-4}	2.4×10^{-3}	4×10^{-3}	8×10^{-3}	8×10^{-2}
ml. dye with liver	10.53	10.48	10.45	10.62	9.88	5.08	0.10
% of control	90.0	89.3	89.1	90.5	84.2	43.3	0.85
ml. dye with G.SH	9.81	9.80	9.05	4.41	2.25	1.05	0.78
% of control	83.6	83.6	77.1	37.6	19.2	9.0	6.6

might act as a positive catalyst in the oxidation of ascorbic acid. Investigation of this suggestion led to several interesting results.

A rat liver extract was prepared so as to contain 0.10 mg. liver glutathione per ml., and the protective effect of this material against oxidation of added ascorbic acid was compared with that of a solution containing 0.10 mg. B.D.H. glutathione per ml. Various amounts of copper sulphate were added to each, and the mixtures incubated for 1 hour at 37° in a total volume of 5 ml. *M*/30 phosphate p_H 7.4, containing 0.5 mg. ascorbic acid (Table V, Fig. 3). The addition of copper has very similar inhibitory effects upon the protective properties of liver and glutathione, but the suppression of the protective effect of glutathione commences at a copper concentration of 4×10^{-4} mg. per ml., whereas in the case of liver extract containing the same amount of glutathione a copper concentration of 2.3×10^{-3} mg. per ml. is necessary. At the latter concentration the protection by glutathione is inhibited to the extent of 50 %.

A somewhat similar experiment was done using ferrous sulphate, in which the glutathione and liver concentrations were so arranged as to have equal anti-oxidative effects, and under these conditions ferrous sulphate had little effect on protection by glutathione but at concentrations higher than 10^{-3} mg. Fe^{++} per ml. the protection by liver was considerably decreased (Table VI).

Table VI. *Effect of Fe^{++} on anti-oxidative powers of liver extract and glutathione.*

2 ml. extract (1 part in 1000) or 2 ml. B.D.H. glutathione (0.05 mg. per ml.) for 1 hour at 37° with 2 ml. ascorbic acid. Unincubated control 4.17 ml. dye.

mg. Fe^{++} per ml.	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
ml. dye with liver	3.50	3.46	3.60	3.13	0.70	1.05
ml. dye with G.SH	3.53	3.62	3.54	3.44	2.72	3.53

These experiments suggested the possibility that the disappearance of ascorbic acid in aqueous solution might be connected with catalysis by heavy metals, and a preliminary experiment showed that ascorbic acid was remarkably stable in solution in water which had been twice re-distilled from an all-glass apparatus. Table VII gives the rates of oxidation in glass-distilled, ordinary

Table VII. *Oxidation of ascorbic acid in various types of water.*

3.7 mg. ascorbic acid in 20 ml. glass-distilled water. 1 ml. incubated with 4 ml. tap, ordinary distilled or glass-distilled water. Blanks on reagents subtracted.

Duration of incubation (mins.) ...		0	4	6	10	20	40
ml. dye {	London tap-water	2.85	1.05	0.77	0.19	0.05	0.05
	Ordinary distilled water	3.33	2.56	2.12	1.66	0.33	0.00
	Glass-distilled water	3.38	3.39	3.76	2.95	2.74	2.40

distilled and London tap-water. The ordinary distilled water, which has been used throughout these experiments unless otherwise stated, was prepared by means of a tinned copper coil with a solid tin nozzle. It is quite evident from these results that something is present in ordinary distilled water and in tap-water which very greatly accelerates oxidation of ascorbic acid.

The amount of copper which must be added to glass-distilled water in order to cause an appreciable increase in the rate of oxidation of ascorbic acid is less than 2×10^{-6} mg. Cu per ml. (Table VIII (a)). 2×10^{-4} mg. Fe^{++} or Fe^{+++} have a similar effect, but even at higher concentrations of iron the catalytic effect of this metal is not so great as that of copper. If copper and iron are present

Table VIII.

(a) *Influence of CuSO_4 on aerobic oxidation of ascorbic acid and glutathione.*2 ml. ascorbic acid (or G.SH) + 2 ml. CuSO_4 incubated 1 hour at 37° .

Blanks on reagents subtracted.

mg. Cu per ml.	0	2×10^{-6}	2×10^{-5}	2×10^{-4}	2×10^{-3}	2×10^{-2}	2×10^{-1}
ml. dye	4.48	3.16	3.06	2.46	0.11	0.08	0.04
Ascorbic acid:							
% of control	71	50	48	39	1.8	1.3	0.6
mg. Cu per ml.	0	6×10^{-7}	6×10^{-6}	6×10^{-5}	6×10^{-4}	6×10^{-3}	6×10^{-2}
ml. N/500 iodine	3.01	3.03	3.05	3.02	2.99	2.13	1.57
Glutathione:							
% of control	100	101	102	101	99	71	52

(b) *Influence of FeSO_4 on aerobic oxidation of ascorbic acid and glutathione.*2 ml. ascorbic acid (or G.SH) + 2 ml. FeSO_4 incubated 1 hour at 37° .

mg. Fe^{++} per ml.	0	2×10^{-6}	2×10^{-5}	2×10^{-4}	2×10^{-3}	2×10^{-2}	2×10^{-1}
ml. dye	4.48	3.95	4.31	3.66	3.39	1.95	1.58
Ascorbic acid:							
% of control	73	64	70	59	55	32	26
ml. N/500 iodine	2.77	2.73	2.71	2.72	2.76	2.76	2.77
Glutathione:							
% of control	99	98	98	98	99	99	99

(c) *Influence of FeCl_3 on aerobic oxidation of ascorbic acid.*2 ml. ascorbic acid + 2 ml. FeCl_3 incubated 1 hour at 37° .

mg. Fe^{+++} per ml.	0	1.8×10^{-6}	1.8×10^{-4}	1.8×10^{-3}	1.8×10^{-2}	1.8×10^{-1}
ml. dye	3.92	4.10	3.84	2.41	1.86	0.05
Ascorbic acid:						
% of control	72	75	71	44	34	0.1

(d) *Influence of FeCl_3 + CuSO_4 on aerobic oxidation of ascorbic acid.*2 ml. metal solution + 2 ml. ascorbic acid incubated 1 hour at 37° .

mg. Cu^{++} per ml.	0	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-5}
mg. Fe^{+++} per ml.	0	4×10^{-4}	4×10^{-7}	4×10^{-4}	4×10^{-7}	4×10^{-4}	4×10^{-7}
ml. dye	3.92	0.04	0.10	0.61	1.19	1.09	2.43
Ascorbic acid:							
% of control	72	0.1	0.2	11	22	20	45

together, however, the catalytic effect is greater than that due to either metal alone (Table VIII (b), (c) and (d)). Fe^{++} has no effect on the rate of aerobic oxidation of glutathione, which is, however, increased by copper at concentrations greater than 6×10^{-4} mg. per ml. (Table VIII (a) and (b)). Addition of tin has no effect on the aerobic oxidation of either ascorbic acid or glutathione.

Since serum contains about 2×10^{-3} mg. Cu per ml. [Tompsett, 1934] and 1.6×10^{-3} mg. Fe per ml. [Fowweather, 1934] it is evident that the relative stability of ascorbic acid in tissues cannot be explained by absence of a metallic catalyst, and we must look for a special protective mechanism. De Caro and Giani [1934] stated that cysteine and cystine both had effects similar to that of glutathione in the protection of ascorbic acid, and this has been confirmed. It has also been found that very minute traces of H_2S have a similar effect (Table IX). From the results with cysteine and cystine it seemed probable that the sulphhydryl group played no part in the phenomenon, and this conclusion was supported by the observation that the protective power of liver was unaffected

Table IX. *Prevention of aerobic oxidation of ascorbic acid by cysteine, cystine and H₂S.*

4 ml. *M*/30 phosphate *p_H* 7.4 containing ascorbic acid and added compound, incubated 1 hour at 37°. Blanks on reagents subtracted.

mg. cysteine per ml.	3.7×10^{-2}	3.7×10^{-3}	3.7×10^{-4}	3.7×10^{-5}	3.7×10^{-6}
% of control	66	65	40	1.1	0
mg. cystine per ml.	5.2×10^{-2}	5.2×10^{-3}	5.2×10^{-4}	5.2×10^{-5}	5.2×10^{-6}
% of control	65	65	51	1.1	0
mg. H ₂ S per ml.	1.5×10^{-2}	1.5×10^{-3}	1.5×10^{-4}	1.5×10^{-5}	1.5×10^{-6}
% of control	108	90	82	20	1.8

by iodoacetic acid. Glutathione protection is only slightly reduced by considerable excess of iodoacetate (Table X).

Table X. *Effect of iodoacetate on protection of ascorbic acid by liver and glutathione.*

Blanks on reagents subtracted.

- (a) Dilutions made from 10 ml. 1/4 liver extract + 1.0 mg. iodoacetic acid at 18° for 30 minutes or 10 ml. extract untreated under same conditions. Incubated 1 hour at 37° in *M*/30 phosphate *p_H* 7.4.

Liver dilution	...	...	1/40	1/400	1/4000	1/40,000
ml. dye	{ No iodoacetate		3.76	4.32	4.51	0.01
	{ Iodoacetate		3.62	4.38	4.35	0.03

- (b) 2 ml. 1/1000 liver extract + 0.25 ml. iodoacetate + 2 ml. ascorbic acid. Incubated 1 hour at 37° in *M*/30 phosphate *p_H* 7.4. Control 4.00 ml. dye.

mg. iodoacetate per ml.	1.3×10^{-1}	2.5×10^{-2}	2.5×10^{-3}	1.5×10^{-5}
ml. dye	3.62	3.76	3.65	3.85

- (c) 2 ml. ascorbic acid, 1 ml. glutathione (0.1 mg. G.SH), 1 ml. iodoacetate *M*/30 phosphate *p_H* 7.4. Incubated 1 hour at 37°. Control 3.40 ml. dye.

mg. iodoacetic acid	0	0.001	0.01	0.03	0.06	0.15	0.30
ml. dye	2.61	2.70	2.72	2.50	2.49	2.40	2.27

0.1 mg. glutathione is equivalent to 0.06 mg. iodoacetic acid.

It was found that if liver extract was kept in a boiling water-bath for one hour with the tube loosely corked its glutathione content remained practically unchanged but its protective power was reduced to one-tenth of normal. If reducing substances were removed from the extract by mercury precipitation [Emmerie and Van Eekelen, 1934] the glutathione content was considerably reduced and the protective power became only 1/100 that of untreated extract (Table XI). Trichloroacetic acid extracts of liver retained a large proportion of the protective properties of the untreated tissue.

Table XI. *Effect of boiling and mercury treatment on anti-oxidative properties of liver extract.*

2 ml. extract + 2 ml. ascorbic acid in *M*/30 phosphate *p_H* 7.4. Incubated 1 hour at 37°.

Blanks on reagents subtracted.

Liver dilution added	...	...	1/20	1/200	1/2000	1/20,000	mg. G.SH per ml. 1/20
Ascorbic acid:	{ Boiled		73	89	17	0	0.07
% of control	{ Mercury-treated		96	59	1.5	0	0.01
	{ Untreated		83	95	99	0	0.07

The evidence presented up to this point tends to suggest that, although the effect of glutathione on the stability of ascorbic acid is very similar to that of liver extract, the effect of liver cannot wholly be accounted for by its glutathione content. Nor can any possible cysteine or cystine present be responsible as the amount necessary would be far too large. Since mannitol in minute traces is known to inhibit the aerobic oxidation of sulphite [Bigelow, 1898] this substance was tried, but neither mannitol nor inositol had any inhibitory effect on the aerobic oxidation of ascorbic acid. Indeed it seemed likely that the properties of liver extract depended upon the combined protective effect of traces of several reducing substances, until an experiment was carried out on the protective powers of dialysed and undialysed liver extracts. Suspensions of liver tissue in phosphate or distilled water were dialysed in collodion sacs or by means of cellophane membranes kindly supplied by the Cellophane Co., Ltd. During dialysis the liquid within the dialyser was stirred by means of a fine stream of nitrogen. Dialysis was continued for $3\frac{1}{2}$ – $4\frac{1}{2}$ hours against running tap-water, and then for one hour against 100 volumes of glass-distilled water. The suspension so obtained was centrifuged and was usually found to contain no detectable iodine-reducing substances. These extracts, however, retained unimpaired their power to prevent the aerobic oxidation of ascorbic acid. The dialysed extracts were compared with extracts which had been kept in stoppered tubes in the dialysis bath and which, though they usually lost a considerable proportion of their reduced glutathione, always retained a comparatively large amount of this substance. The facts that cysteine and cystine possess protective powers of the same order of magnitude and that glutathione loses little of this property in presence of iodoacetate suggest that a change from reduced to oxidised glutathione would have little effect on the protective power of this compound. In a series of ten experiments with dialysed materials, not all of which were quite free from reduced glutathione, no difference could be found between the protective powers of dialysed and undialysed extracts. The protocol of one experiment is given in Table XII.

Table XII. *Effect of dialysis on protective power of liver extract.*

1/10 extract dialysed $4\frac{1}{2}$ hours in tap-water and 1 hour in glass-distilled water. Ascorbic acid in *M*/30 phosphate (p_H 7.5) made up with ordinary distilled water. 5 ml. dialysed extract required 0.12 ml. dye and 0.13 ml. *N*/500 iodine. 5 ml. undialysed extract required 2.09 ml. dye and 0.63 ml. iodine.

Blanks: 0.15 ml. dye; 0.13 ml. iodine.

1 ml. dye $\equiv$ 0.050 mg. ascorbic acid. 1 ml. iodine $\equiv$ 0.60 mg. glutathione.

2 ml. liver extract + 2 ml. ascorbic acid solution.

Liver dilution added ...	1/10	1/100	1/1000	1/3000	1/7000	1/10,000
Dialysed extract:						
G.SH (mg.)	0	0	0	0	0	0
ml. dye	2.53	2.77	2.30	0.19	0.15	0.18
Undialysed extract:						
G.SH (mg.)	6×10^{-2}	6×10^{-3}	6×10^{-4}	2×10^{-4}	9×10^{-5}	6×10^{-5}
ml. dye (corr.)	2.75	2.75	2.30	0.25	0.22	0.20

It was of interest to find whether any protective mechanism existed in vegetable material similar to that found in animal tissues, and for this purpose 10 ml. of lemon juice were brought to p_H 7.5 by addition of 5 ml. of dilute ammonia. 10 ml. of untreated juice were diluted to 15 ml. and 5 ml. of each sample diluted to 50 ml. with *M*/30 phosphate, p_H 7.5. 5 ml. portions were

incubated at 37°, and it was found that the ascorbic acid in the neutralised material disappeared at the same rate as ascorbic acid in phosphate solution, while that in the diluted juice which had not been treated with ammonia was relatively stable. The p_H of the un-neutralised juice was less than 3.6 (Table XIII).

Table XIII. *Incubation of lemon juice.*

Blanks on reagents subtracted.										
Time (mins.)	...	...	...	0	5	15	39	60	120	240
Untreated	{ ml. dye			5.83	5.74	5.57	4.99	4.02	2.47	0.79
	{ % of control			100	99	96	86	70	42	14
Neutralised	{ ml. dye			4.58	3.64	2.47	0.63	0.05	0.03	0.06
	{ % of control			79	62	42	11	0.9	0.5	1.0

It is evident, therefore, that the stability of ascorbic acid in the lemon is entirely due to the low p_H of the juice and not to any protective mechanism such as that found in animal tissues.

DISCUSSION.

Although it has not been found possible to identify all the constituents of the mechanism which prevents the spontaneous oxidation of ascorbic acid in the animal body, it has been shown that glutathione is probably one of the factors involved. De Caro and Giani [1934] concluded that glutathione was the substance which caused stabilisation, but it cannot be wholly responsible for the phenomenon since liver extracts from which glutathione has been removed by prolonged dialysis retain the protective properties of fresh extract. The protective properties of boiled liver extract and extract which has been treated with trichloroacetic acid are likely to be due to their glutathione content, but the main part of the protective mechanism is of an undialysable nature. Although certain sulphydryl compounds when present in small traces have the property of preventing the oxidation of ascorbic acid, their effectiveness in this direction does not necessarily lie in the —SH group. De Caro and Giani found considerable differences in the protective properties of cysteine and cystine, although the curves were along the same general lines, but we found that equal amounts of these compounds had practically equal protective powers over wide ranges of concentration. This might be explained by a condition of equilibrium between cysteine and cystine, but this is made less likely by the fact that iodoacetate only slightly inhibits the protective properties of glutathione even when present in more than equimolecular proportions. Dickens [1933] has shown that iodoacetic acid condenses with sulphydryl compounds to give substances of the type $RS.CH_2.COOH$.

It is likely that a chain of protective mechanisms of a similar kind will be found to occur in animal tissues. Abderhalden [1934] states that tyrosine, 3:4-dihydroxyphenylalanine, and more especially adrenaline, are stabilised against oxidation by traces of ascorbic acid, and in a private communication Dr Heard states that work done in Toronto confirms Abderhalden's results.

The instability of aerobic solutions of ascorbic acid can be explained by the presence of small traces of copper and iron, which act as very powerful catalysts in the oxidation of ascorbic acid. The protective properties of tissue extracts and of the pure compounds investigated are probably due to their inhibition of metallic catalysts, which are present in body fluids in such a concentration that without a protective mechanism of some kind ascorbic acid could never exist in them at all.

The stability of the reducing substance of tumour tissue when in contact with tissue and the fact that its concentration in tissue is neither increased nor decreased by periods of incubation with or without glucose or phosphate are in such close agreement with the similar properties of ascorbic acid as strongly to suggest that these substances are identical. Before this can be stated with confidence, however, more biological and chemical work is necessary.

SUMMARY.

1. The ascorbic acid-like reducing substances of normal and tumour tissue are unaltered in amount by incubation of whole or minced tissue with or without phosphate or glucose for periods up to 4 hours at 37°.

2. Small amounts of animal tissues or tissue extracts prevent the aerobic oxidation of ascorbic acid solutions.

3. Glutathione, cysteine, cystine and H_2S prevent the aerobic oxidation of ascorbic acid, but protection by tissue extracts cannot wholly be explained by these facts.

4. Dialysed tissue extracts retain their anti-oxidative properties in full. These properties are also present, in reduced amount, in extracts which have been boiled or precipitated with mercuric acetate or trichloroacetic acid.

5. Ascorbic acid is relatively stable in glass-distilled water. Copper, and to a less extent iron, and especially mixtures of these two metals act as positive catalysts in the aerobic oxidation of ascorbic acid.

6. Lemon juice contains no protective mechanism other than the low p_{H} .

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