Inhibition of Urease by Copper Salts in the Presence of Ascorbic Acid and Related Substances

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(*Received* 21 *December* 1945)

Elson (1943) found that urease is inhibited by small amounts of ascorbic acid and that this inhibition is eliminated in the presence of cysteine. Quastel (1943) suggested that the inhibition was due to a reaction between dehydroascorbic acid and an —SH group of the enzyme, and that the protective effect of thiol compounds was due to the fact that they reduced dehydroascorbic acid. Giri & Seshagiri Rao (1944), however, did not find that dehydroascorbic acid inhibited urease, and since they observed that there was no inhibition of the enzyme by ascorbic acid in the presence of substances which form complexes with copper (8-hydroxyquinoline and diethyl-dithio-carbamate), they suggested that the inhibition might be due to the formation of intermediary substances like Cu_2O, produced during the oxidation of the vitamin. Our experiments show that neither ascorbic, nor its oxidation products, dehydroascorbic acid or 2:3-diketo-gulonic acid, themselves inhibit urease. Ascorbic acid and 2:3-diketo-gulonic acid, however (but not dehydroascorbic acid), act as inhibitors in the presence of Cu^{++}. Cu^{+} inhibits urease to a much greater extent than does Cu^{++}, and the increased inhibition observed when ascorbic acid is added to a solution of the enzyme containing Cu^{++} is due, it is therefore concluded, to the reduction of Cu^{++} to Cu^{+}.

METHODS

Reagents. Salts were A.R. and glass-distilled water was used for all solutions. Copper was added either as chloride or sulphate.

Urease. Two types of enzyme preparation were used. The first was crystalline urease prepared according to the method of Sumner (1926). The second was made by extracting 1 g. of Arlco jack bean meal with 40 ml. of glass-distilled water; insoluble material was separated by centrifuging and the centrifugate dialyzed for 24 hr. against glass-distilled water at 0°. The former preparation is referred to in the text as crystalline urease and the latter as dialyzed enzyme. No major differences were observed in the behaviour of these two enzyme solutions.

Urease activity. The activity of the enzyme was tested by its reaction with urea in phosphate buffer (pH 6·8), containing 5·4% (w/v) Na_2HPO_4 and 4·2% (w/v) KH_2PO_4. Each 10 ml. of reaction mixture contained 5 ml. of buffer solution, the appropriate amount of enzyme, and 1% (w/v) of urea. Samples for analysis were removed at intervals over a period of 20 min., the reaction was arrested by the addition of HCl, and the ammonia formed estimated either by Van Slyke & Cullen's method of aeration or by nesslerization. The concentration of enzyme used was adjusted so that within a period of 20 min. about 1–1·4 mg. of NH_3/ml. of reaction mixture was formed.

Ascorbic acid and related substances were added to a solution of total volume 9 ml., containing 5 ml. of the phosphate buffer and 1 ml. of a suitable concentration of urease solution. The enzymic activity was then tested as described, at any given time, after the addition of 1 ml. of a 10% urea solution. All experiments were carried out at 20°. The values in the tables under the heading 'inhibition of urease activity' refer, unless otherwise stated, to the suppression of NH_3 formation over a period of 20 min.

Dehydroascorbic acid was prepared by shaking ascorbic acid, dissolved in 0·05 M-phosphate-citric acid buffer (McIlvaine), pH 3·5, with activated charcoal. Before use the charcoal was extracted with boiling N-HCl, re-extracted with distilled water and washed with water to remove metallic impurities. Five mg. of ascorbic acid, dissolved in 50 ml. of the buffer solution, were shaken for 3–5 min. with 0·4 g. of the charcoal and the latter removed by filtration. The concentration of dehydroascorbic acid so formed was determined by reduction with H_2S and titration against indophenol in the usual way. For studying the effect of this dehydroascorbic acid solution on the enzyme the concentration of the phosphate-citric acid buffer solution added to the control was equal to that used in the preparation of the dehydroascorbic acid solution.

2:3-*Diketo-gulonic acid* was used in the form of the mono-sodium salt, kindly given by Messrs Roche Products, Ltd., and as the calcium salt, isolated according to the method described by Penney & Zilva (1943).

RESULTS

Exp. 1. *Influence of copper.* Ascorbic acid had no inhibitory influence on urease in the absence of ionized copper. The results of experiments (Table 1) with a dialyzed enzyme preparation showed no inhibitory effect of ascorbic acid in concentrations of 20–200 μg./ml. in the absence of copper, but marked inhibitory effects when it was added. An apparently anomalous finding was that when a more purified crystalline enzyme preparation was used, ascorbic acid caused some inhibition even in the absence of added copper. This inhibition was, however, due to traces of copper in the reagents used,* for the inhibition was abolished in the presence of

* Phosphate buffer-urea solution used in these experiments contained approximately 0·05–0·1 μg. of copper/ml. as estimated by sodium diethyl-dithio-carbamate reagent.

Table 1. *Influence of copper on the inhibition of urease by ascorbic acid*

Test no.	Enzyme preparation used	Final concentration of copper added (μg./ml.)	Final concentration of ascorbic acid added (μg./ml.)	Inhibition of urease activity (%)
1	Dialyzed	0	2·5	0
		0	5	0
		0	10	0
		0	20	1
		0	200	0
2	Dialyzed	0·2	0	5
		0·2	2·5	17
		0·2	5	26
		0·2	10	36
		0·2	20	42
3	Dialyzed	1	0	24
		1	10	96
		1	20	100
4	Crystalline	0*	2·5	10
		0	5	25
		0	10	32
		0	20	44
		0†	2·5	0
		0†	5	0
		0†	10	0
		0†	20	0
		0‡	5	0
		0‡	10	0
5	Crystalline	0·1	0	7
		0·1	10	86
		1	0	39
		1	10	100

* Solution contained approx. 0·05–0·1 μg./ml. of copper as impurity.
† Solutions contained 0·01 M-pyrophosphate.
‡ Solutions contained 0·01% (w/v) sodium diethyl-dithio-carbamate.

pyrophosphate, or sodium diethyl-dithio-carbamate, reagents which are known to form complexes with copper. The most probable explanation of this apparent anomaly is that, on purification of the enzyme, substances like thiol compounds and proteins, which are able to form complexes with copper, are removed. Thus with the crude enzyme any traces of copper present in the reagents are rendered inactive, but this is not so in the case of the pure enzyme, and the copper is thus able to exert its full effect.

The results also indicate that the magnitude of the inhibitory action of ascorbic acid is conditioned by, and increases with, the concentration of Cu^{++} (Table 1, tests 2, 3 and 5).

Exp. 2. *Inhibition under anaerobic and aerobic conditions.* On the basis of the suggestion advanced by Quastel the function of copper would be to catalyze the aerobic production of dehydroascorbic acid from ascorbic acid. The results of experiments (Table 2) suggest that such an explanation is unlikely. Ascorbic acid was added to urease in the presence of Cu^{++}, and the solutions left for periods varying from 0 to 90 min. before testing the activity of the enzyme. The inhibitory effect was found to be as marked under anaerobic as under aerobic conditions, despite the fact that the oxidation of ascorbic acid and formation of dehydroascorbic acid was largely prevented in the former case. However, anaerobic conditions will not prevent the reduction of Cu^{++} by ascorbic acid and the possibility existed that the small amounts of dehydroascorbic acid ($< 0{\cdot}025$ μg./ml.) so formed might have been sufficient to account for the inhibition.

Exp. 3. *Inhibitory effect of substances formed from dehydroascorbic acid.* This possibility was disproved

Table 2. *Inhibition of urease by ascorbic acid in presence of copper under anaerobic and aerobic conditions*

Test no.		Enzyme preparation used	Time elapsing between addition of Cu and ascorbic acid to enzyme and test of activity (min.)	Cu present (μg./ml. of soln.)	Ascorbic acid initially present (μg./ml. of soln.)	Inhibition due to ascorbic acid as percentage of controls* (%)	Ascorbic acid oxidized (%)
1	Anaerobic	Crystalline	<1	0·1	5	64	2
			30	0·1	5	61	4
			60	0·1	5	62	3
	Aerobic	Crystalline	<1	0·1	5	67	—
			30	0·1	5	62	40
			60	0·1	5	68	60
2	Anaerobic	Dialyzed	2	1	10	100	5
	Aerobic		2	1	10	100	20
3	Aerobic	Dialyzed	10	0·2	20	53	12
			30	0·2	20	50	28
			60	0·2	20	51	50
			90	0·2	20	53	66

* Control solutions of the enzyme were treated in the same way, but without the addition of ascorbic acid.

on finding that when a solution containing dehydroascorbic acid was added to urease in the absence of copper there was no inhibition, even after the dehydroascorbic acid had been left in contact with the enzyme for periods up to 2 hr. before the test of activity (Table 3). In the presence of copper,

Table 3. *Influence of copper and time of standing on the inhibition caused by a solution which initially contained dehydroascorbic acid*

Test no.	Enzyme used	Time elapsing between addition of copper and/or D.H.A.* solution to enzyme and test of activity (min.)	Copper present (μg./ml. of soln.)	D.H.A.* initially present (μg./ml. of soln.)	Inhibition due to D.H.A. as percentage of controls†
1	Crystalline	5	0	10	0
		30	0	10	0
		60	0	10	0
		2	1	10	24
		30	1	10	72
		60	1	10	88
2	Dialyzed	2	0	10	0
		15	0	10	0
		60	0	10	0
		120	0	10	0
		2	1	10	8
		15	1	10	28
		60	1	10	50
		120	1	10	51

* D.H.A. = dehydroascorbic acid.

† Control solutions of the enzyme were similarly treated, but without the addition of the solution of dehydroascorbic acid.

however, inhibition increased with the length of time the enzyme had been previously left in contact with the dehydroascorbic acid. With ascorbic acid under similar conditions the maximum inhibition was attained after a period of less than 1 min. (cf. Table 3). This delay in obtaining maximum inhibition with solutions of dehydroascorbic acid might be attributed to either a slow reaction between the acid and the reactive group of the enzyme, or the slow production of an inhibitor from it. The delay could not be explained by the former hypothesis, for the inhibition was almost as great when the dehydroascorbic acid solution, after standing 1 hr., was added to the enzyme, as when the enzyme was left in contact with the dehydroascorbic acid solution for 1 hr. (Table 4, test 1). These results suggested that dehydroascorbic acid itself, even in the presence of copper, does not inhibit urease, but a product formed from it is responsible for the effect.

Copper was not necessary for the formation of the inhibitor from the dehydroascorbic acid, although it was necessary for the reaction between the inhibitor and the enzyme (Table 4, test 2).

Exp. 4. *Correlation between rate of decomposition of dehydroascorbic acid and development of inhibition.* The conclusions reached in the last section were confirmed by an experiment which established that there was a close parallel between the extent of decomposition of dehydroascorbic acid and the development of inhibitory power.

10 ml. of a solution of dehydroascorbic acid in 0·05 M-phosphate-citric acid buffer, pH 3·5, were added to 90 ml. of a solution containing 50 ml. of phosphate buffer, pH 6·8, to give a final concentration of dehydroascorbic acid of 5 mg./100 ml. and a pH value of 6·4. This solution was allowed to stand at room temperature and at intervals 1 ml. was added to the enzyme solution in phosphate buffer (pH 6·8) containing copper (1 μg./ml.). After 2 min. urea was added and the enzymic activity determined. Samples of the dehydroascorbic acid solution were also analyzed by reduction with H_2S, to determine the rate of decomposition of the dehydroascorbic acid.

The results (Fig. 1) show that a substance is formed from dehydroascorbic acid which, in the presence of copper, causes inactivation of the

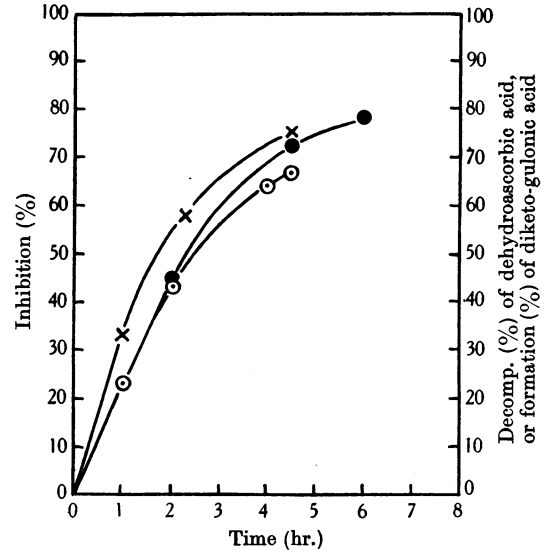

Fig. 1. Correlation between development of inhibition, decomposition of dehydroascorbic acid and formation of 2:3-diketo-gulonic acid. ×—× Inhibition of urease, ⊙—⊙ decomposition of dehydroascorbic acid, •—• formation of diketo-gulonic acid.

enzyme. It was concluded that this substance was 2:3-diketo-*l*-gulonic acid (which is formed from dehydro-*l*-ascorbic acid in aqueous solution by mutarotation), for determinations of 2:3-diketo-

Table 4. *Conditions affecting the development of inhibition by a solution initially containing dehydroascorbic acid*

(*Test no.* 1. To 1 ml. of a solution of dehydroascorbic acid were added 2 ml. of a solution containing copper, 5 ml. of phosphate buffer (pH 6·8) and 1 ml. of a urease solution. Allowed to stand 1 hr. at 20°. Another 8 ml. was allowed to stand 1 hr. before the addition of the urease. The activity of the enzyme in both solutions was then determined. A control was similarly treated with copper, but without dehydroascorbic acid.)

Test no.	Enzyme used	Copper present (μg./ml. of soln.)	D.H.A.* present initially (μg./ml. of soln.)	Treatment	NH_3 produced (μg./ml.)	Inhibition of urease activity (%)
1	Dialyzed	1	10	Enzyme in contact with D.H.A. soln. for 1 hr.	0·10	91·5
		1	10	D.H.A. soln. after standing 1 hr. added to enzyme	0·18	85
		1 (control)	0	—	1·20	—

(*Test no.* 2. Two solutions of dehydroascorbic acid in phosphate buffer were prepared as described above, one containing copper and one without. After standing 1 hr. at 20° urease was added to each of these solutions, and to a further sample of the dehydroascorbic acid solution without copper both urease and copper were added. The activity of the enzyme in each of these solutions was then determined. Controls without dehydroascorbic acid, but with and without added copper, were similarly prepared.)

Test no.	Enzyme used	Copper present (μg./ml. of soln.)	D.H.A. present initially (μg./ml. of soln.)	Treatment	NH_3 produced (μg./ml.)	Inhibition of urease activity (%)
2	Dialyzed	0	10	D.H.A. soln. after standing 1 hr. added to enzyme	1·30	6
		0 (control 1)	0	—	1·38	—
		1	10	D.H.A. soln. after standing 1 hr. added to Cu and enzyme	0·16	87
		1	10	D.H.A. soln., which contained Cu and had stood for 1 hr., added to enzyme	0·20	83·5
		1 (control 2)	0	—	1·21	—

* D.H.A. = dehydroascorbic acid.

gulonic acid in the solution by the method of Penney & Zilva (1943) showed a close correspondence between the production of this acid, the extent of the decomposition of dehydroascorbic acid and the rise in inhibition.

Exp. 5. *Correlation of inhibitory activity with changes occurring in a solution of ascorbic acid during its oxidation in the presence of* Cu^{++}. The results previously recorded indicated that, in the presence of Cu^{++}, inactivation of urease can result from either the addition of ascorbic acid, or its degradation product, 2:3-diketo-gulonic acid, but not of dehydroascorbic acid. Further evidence in favour of this view was obtained by following the changes in inhibitory effect of ascorbic acid undergoing oxidation in the presence of Cu^{++} (Fig. 2). Samples of a solution of ascorbic acid in phosphate buffer (pH 6·8) containing copper, after standing for varying periods of time, were added to urease; after a further period of 2 min. urea was added and the enzymic activity determined. At the start of the experiment the inhibition obtained was nearly complete, but

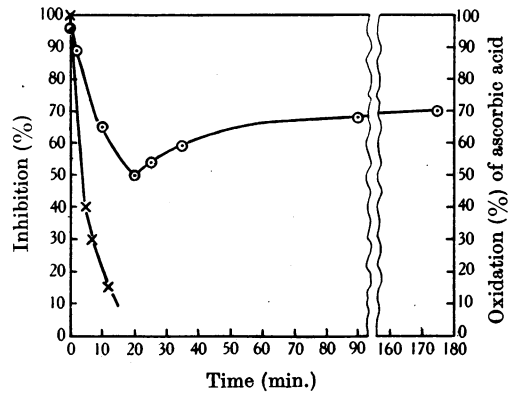

Fig. 2. Correlation of inhibitory activity with changes occurring in a solution of ascorbic acid during its oxidation in presence of Cu^{++}. Urease (dialyzed) was added to a solution of ascorbic acid (10 μg./ml.) in phosphate buffer (pH 6·8) with Cu^{++} (1 μg./ml.) after different time intervals. ⊙—⊙ Inhibition of urease, ×—× oxidation of ascorbic acid.

Table 5. *Inhibition of urease by 2:3-diketo-gulonic and 2-keto-gulonic acids*

Test no.	Enzyme	Copper present (μg./ml. of soln.)	2:3-Diketo-gulonic acid present (μg./ml. of soln.)	Inhibition of urease activity (%)
1	Dialyzed	0 (control)	0	—
		0	2·5	1
		0	5	4
		0	10	0
		1 (control)	0	—
		1	2·5	58
		1	5	74
		1	10	81
2	Dialyzed	0 (control)	0	—
		0	5	0
		0	10	0
		0	20	0
		1 (control)	0	—
		1	5	70
		1	10	81
		1	20	89
			2-Keto-gulonic acid present (μg./ml. of soln.)	
3	Dialyzed	0 (control)	0	—
		0	5	1·5
		0	10	2
		0	20	3
		0	100	2
		1 (control)	0	—
		1	10	20
		1	20	27
		1	100	37
		0·5 (control)	0	—
		0·5	5	6
		0·5	10	9·5
		0·5	20	15·5
		0·5	100	26

Table 5*a*. *Comparison of effect on urease of ascorbic acid and 2:3-diketo-gulonic acid*

Test no.		Copper present (μg./ml. of soln.)	Addition	Concentration (mM)	Inhibition (%)
4	Crystalline enzyme	0 (control)	0	—	—
		0·3	0	—	28
		0·3	Ascorbic acid	0·011	90
		0·3	2:3-Diketo-gulonic acid	0·011	68

In tests 1 and 4, 2:3-diketo-gulonic acid added as Na salt; in test 2, as Ca salt.

as the ascorbic acid was converted to dehydro-ascorbic acid the inhibition first diminished, but subsequently increased. As the previous experiment showed, this increase could be correlated with the formation of 2:3-diketo-gulonic acid.

Exp. 6. *Inhibition by 2:3-diketo-gulonic acid and 2-keto-gulonic acid.* Confirmation that the oxidation product of ascorbic acid, which caused the inhibition, was 2:3-diketo-gulonic acid was obtained in experiments in which pure preparations of this acid were used (Table 5). Diketo-gulonic acid only acts as an inhibitor in the presence of copper but is less effective than ascorbic acid in an equivalent amount (Table 5*a*). With the related compound, 2-keto-gulonic acid, there was appreciable inhibition only in relatively high concentration.

Exp. 7. *Role of copper, and comparison of inhibition by* Cu^{++} *and* Cu^{+}. Since ascorbic acid readily reduces $Cu^{++} \rightarrow Cu^{+}$ it seemed worth while to ascertain how far the inhibitory action of ascorbic acid could be correlated with this reaction. The effect of equimolecular amounts of Cu^{++} and Cu^{+} salts was, therefore, examined.

All solutions were first freed of O_2 by the passage of O_2-free N_2. Stock solutions of $CuCl_2$ and CuCl were prepared by dissolving the required amount of the salts in 2N-HCl. To a solution containing 5 ml. of phosphate buffer (pH 6·8) and 1 ml. of enzyme solution, made up with water to a volume of 8·9 ml., 0·1 ml. of either the Cu^{++} or Cu^{+} solution was added. Parallel experiments were also set up in which ascorbic acid was present. To the control solutions 0·1 ml. of 2N-HCl was added in place of the copper solution. The enzyme was left in contact with the Cu^{+} or Cu^{++} and ascorbic acid for 1 min. before the addition of urea. The reaction period in these experiments was 5 min. O_2-free N_2 was passed through the solution throughout the period of test.

The results (Table 6) indicate (1) that Cu^{+} ions, in equivalent concentration, have a much greater

Table 6. *Comparison of effect of* Cu^{++} *and* Cu^{+} *on urease*

Test no.	Enzyme	Cu^{++} present (μg./ml. of soln.)	Cu^{+} present (μg./ml. of soln.)	Ascorbic acid present (μg./ml. of soln.)	Inhibition of urease activity (%)
1	Dialyzed	0·5	—	—	35
		0·5	—	5	95
		0·5	—	10	97
		—	0·5	—	89
2	Dialyzed	0·2	—	—	7
		0·2	—	10	61
		—	0·2	—	48
3	Dialyzed	0·5	—	—	34
		0·5	—	5	88
		0·5	—	10	88
		—	0·5	—	91
4	Crystalline	0·5	—	—	50
		—	0·5	—	100
		0·2	—	—	25
		—	0·2	—	94
5	Crystalline	0·2	—	—	20
		0·2	—	5	98
		0·2	—	10	98
		—	0·2	—	97

inhibitory action on urease than Cu^{++} ions, and (2) that the maximum inhibition obtained by the addition of ascorbic acid to urease solutions containing Cu^{++} was identical with that obtained with Cu^{+} alone. Experiments of a similar nature (Fig. 3)

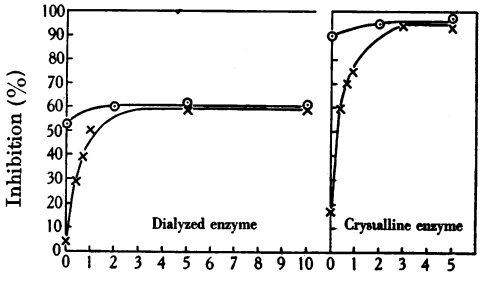

Fig. 3. Effect of ascorbic acid in varying concentration on inhibition of urease by Cu^{++} or Cu^{+} (concentration of Cu 0·2 μg./ml.). ⊙—⊙ Cu^{+}, ×—× Cu^{++}.

also show that ascorbic acid has no additional inhibitory influence when added to a solution containing urease and a cuprous salt.

These facts are most simply explained on the assumption that the inhibitory action of ascorbic acid in the presence of Cu^{++} is due to the reduction of $Cu^{++} \rightarrow Cu^{+}$, with a consequent increase in inhibitory power.

Exp. 8. *Protection and reactivation of urease by thiol compounds.* Both cysteine and glutathione protected the enzyme against inactivation by either Cu^{++} plus ascorbic acid, or by Cu^{+} alone (Table 7*a*). This effect is no doubt due to a reaction between the copper salt and thiol group, whereby the —SH group of the enzyme is protected.

Hellerman, Perkins & Clark (1933) showed that urease inactivated by Cu_2O could be reactivated by treatment with H_2S. We have found that the inactivation of urease, either by Cu^{+} or by Cu^{++} plus ascorbic acid, can be reversed by H_2S.

Solutions of the enzyme in phosphate buffer (pH 6·8) were first freed of O_2 and the enzyme inactivated by the addition of either $CuCl_2$ plus ascorbic acid, or CuCl. After standing 2 min. at 20°, H_2S was passed through a sample of the inactivated enzyme solution for 5 min. Urea was added at once and the enzymic activity determined. A control solution was treated with H_2S: there was no increase in the activity as a result of the H_2S treatment.

The results show that complete reactivation in both cases was obtained. Cysteine in place of H_2S gave a similar result (Table 7*b*).

Exp. 9. *Influence of other metallic salts.* The effect on urease of ascorbic acid in the presence of small amounts of Ag^{+}, Au^{+++}, Hg^{++} or Fe^{+++} ions is shown in Table 8. Except in the case of Au^{+++} the addition of ascorbic acid caused little or no increase in the degree of inhibition. Although the inhibition of the enzyme by Au^{+++} alone was equal to that caused by Cu^{++} (cf. Table 1, tests 2 and 3), the increase in the inhibition on addition of ascorbic acid was less than the corresponding effect with Cu^{++}. The toxicity of Hg^{++} was reduced by the addition of ascorbic acid. In equivalent concentrations Hg^{++} inhibited more than Hg^{+} (Table 8*a*); the reduced toxicity of Hg^{++} salts in the presence of ascorbic acid was presumably due to the reduction of $Hg^{++} \rightarrow Hg^{+}$. Other metallic salts were tested, and the results showed that in solutions containing Zn^{++}, Sn^{++++}, Pb^{+} or Co^{++} in a concentration of 0·016 mM., ascorbic acid had no inhibitory action on urease.

Table 7*a*. *Protection of urease by thiol compounds*

Enzyme	Cu^{++} present (μg./ml. of soln.)	Ascorbic acid present (μg./ml. of soln.)	Cu^{+} present (μg./ml. of soln.)	Cysteine present (mM)	Glutathione present (mM)	Inhibition (%)
Crystalline	0·5	3	—	—	—	100
	0·5	3	—	1	—	5
	0·5	3	—	—	1	0
	—	—	0·5	—	—	100
	—	—	0·5	1	—	0
	—	—	0·5	—	1	0

Table 7*b*. *Reactivation of urease by thiol compounds*

Enzyme	Cu^{++} present (μg./ml. of soln.)	Ascorbic acid present (μg./ml. of soln.)	Cu^{+} present (μg./ml. of soln.)	Inhibition (%)	Reactivation. Inhibition (%) after treatment with H_2S	Reactivation. Inhibition (%) after treatment with Cysteine ($M \times 10^{-3}$)
Crystalline	0·2	3	—	99	0	—
	—	—	0·2	97	0	—
	0·5	3	—	100	—	4
	—	—	0·5	100	—	6

Table 8. *Effect of ascorbic acid on the inhibition of urease in the presence of* Ag^+, Au^{+++}, Hg^{++}, *or* Fe^{+++}

(In these experiments a dialyzed preparation of urease was used.)

Salt	Concentration (mM)	Ascorbic acid present (μg./ml. of soln.)	Inhibition of urease activity (%)
$AgNO_3$	0·00475	0	54
		5	62
		10	62
		20	57
$AgNO_3$	0·0032	0	47
		20	54
$HAuCl_4$	0·00475	0	24
		5	45
		10	51
		20	55
$HAuCl_4$	0·0009	0	5
		5	7
		10	8
		20	8
$Hg(CH_3COO)_2$	0·0064	0	65
		5	56
		10	43
		20	36
$Hg(CH_3COO)_2$	0·0032	0	33
		5	26
		10	10
$Hg(CH_3COO)_2$	0·0016	0	15
		20	0
$FeCl_3$	0·016	0	11
		10	16
$FeCl_3$	0·016	0	2
		10	0

Table 8*a*. *Comparison of effect of* Hg^{++} *and* Hg^+ *on urease*

$Hg(CH_3COO)_2$	0·0032	0	36
$Hg(CH_3COO)$	0·0032	0	7
$Hg(CH_3COO)_2$	0·0126	0	92
$Hg(CH_3COO)$	0·0126	0	63

Exp. 10. *Tests with other substances.* Hydroxytetronic, reductic and dihydroxymaleic acids, which all contain a dienol group, increased the inhibitory effect of Cu^{++} on urease to a degree comparable with that observed with ascorbic acid. Adrenalin behaved likewise, though the inhibition was much less marked than with ascorbic acid.

Quinone, in contrast to these substances, inhibited in the complete absence of Cu^{++}, for its action was not reduced when substances such as pyrophosphate were present. The inhibition in this case is no doubt due to a reaction between quinone and an essential —SH group of the enzyme (cf. Quastel, 1932).

DISCUSSION

There has been a general consensus of opinion that the inhibitory action of many different substances on urease can be most simply explained on the assumption that they react as oxidants, or that they form derivatives with at least one of the enzyme's —SH groups, the integrity of which is essential for activity (Hellerman, 1937, 1939: Hellerman, Chinard & Dietz, 1943).

Hopkins (1929) showed that Cu_2O converts mercaptans (RSH) to the corresponding mercaptides (RSCu). With glutathione he found that the reaction could be initiated by the addition of a cupric salt, the Cu^{++} ion being first reduced to Cu^+ by the thiol group, and the resulting Cu^+ ion reacting to form the insoluble copper derivative of glutathione. Hellerman *et al.* (1933) noted the inhibitory action of Cu_2O on the activity of urease, which was attributed to the formation of a cuprous mercaptide. They also showed that Cu^{++} salts did not rapidly inactivate urease; only after aeration of the solution for 2–3 hr. was the enzymic activity destroyed. They considered the toxic effect of the Cu^{++} was due not to the ion *per se*, but to its ability to accelerate catalytically the oxygenation of urease.

The experiments reported in this paper have emphasized the comparative non-toxicity of Cu^{++} compared with Cu^+ ions. The simplest explanation of the toxic action of ascorbic acid on urease is that it is able to reduce $Cu^{++} \rightarrow Cu^+$. It was expected, therefore, that other compounds containing the dienol group, e.g. reductic, hydroxytetronic and dihydroxymaleic acids would exert a similar effect, and this has indeed been found to be so. Of interest too is the fact that adrenalin mildly inhibits urease in the presence of Cu, but not in its absence.

The inhibitory action of 2:3-diketo-gulonic acid is probably due to its ability to reduce Cu^{++}. In fact this substance acts as a reducing agent at pH values above 4·0 (Borsook, Davenport, Jeffreys & Warner, 1937), and it reduces methylene blue in the presence of phosphate (Frankenthal, 1944). It is conceivable that the reductant in such solutions is the enolized form of the keto acid.

Whether ascorbic acid acts as an inhibitor of urease activity *in vivo* is more doubtful. The present work has emphasized that Cu^{++} ions must be present if ascorbic acid is to act as an inhibitor. Whether the mechanism of the inhibitory action of ascorbic acid on plant β-amylase (Purr, 1934; Seshagiri Rao & Giri, 1942), which is reported to require an —SH group for its activity, is the same as with urease remains to be determined. It is, however, suggestive that the latter workers found that the action of ascorbic acid was increased in the presence of copper.

It seems unlikely that the inhibition of such enzyme systems can be considered as an essential role of ascorbic acid in the cell, in view of the well-marked inhibitory properties of other dienols which are biologically inactive.

SUMMARY

1. Evidence is presented which shows that Cu^{+} is a much more effective agent than Cu^{++} in inhibiting urease, and that ascorbic acid acts as an inhibitor only in so far as it reduces $C^{++} \rightarrow Cu^{+}$. The experimental findings on which this evidence is based are as follows:

(*a*) Ascorbic acid does not inhibit urease when copper is absent, but does so when copper is present.

(*b*) The inhibition by Cu^{++} plus ascorbic acid is of the same magnitude as with Cu^{+} alone. The inhibition produced by Cu^{+} is not increased by the addition of ascorbic acid.

(*c*) Other reducing substances containing a dienol group and capable of reducing $Cu^{++} \rightarrow Cu^{+}$, e.g. hydroxytetronic, reductic and dihydroxymaleic acids, resemble ascorbic acid in their power to inactivate urease in the presence of copper.

(*d*) Urease inactivated either by Cu^{++} plus ascorbic acid or by Cu^{+} alone may be reactivated by H_2S, or by cysteine. It is postulated that the Cu^{+} inactivates by forming a derivative with an —SH group of the enzyme, and that substances like H_2S reactivate by removing the bound copper and regenerating this —SH group.

2. Dehydroascorbic acid does not inhibit urease, even in the presence of copper. 2:3-Diketo-gulonic acid (produced by mutarotation when solutions of dehydroascorbic acid are left standing) inhibits only in the presence of copper, but to a less extent than does ascorbic acid.

3. Of the metallic salts tested, Cu^{++} and Au^{+++} alone had their toxicity increased by the addition of ascorbic acid. With Hg^{++} salts the toxicity was reduced by ascorbic acid.

I wish to thank Dr L. J. Harris for his valuable criticism and advice, and Mr B. J. Constable for his technical assistance.

The work described in this paper was carried out as part of a programme of the Food Investigation Board of the Department of Scientific and Industrial Research, by arrangement with the Medical Research Council.

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Blood Haemoglobin: the Relationship between Oxygen Capacity and Iron Content of Blood in Men and Women

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(*Received* 22 *December* 1945)

In a recent paper (Gibson & Harrison, 1945) we described the preparation of a stable artificial standard solution which matched the absorption spectrum of blood in 0·1N-NaOH over a considerable range of the visible spectrum. In order to assign a haemoglobin value to this solution for use in the estimation of blood haemoglobin, we standardized it against samples of normal male blood of known oxygen capacity and iron content. It was found that the haemoglobin values of the blood samples determined by oxygen capacity measurement were slightly, but consistently, lower than those obtained by iron estimation, a finding which agrees with results which have been obtained by others, for example, Macfarlane & O'Brien (1944). The latter workers, however, obtained results with blood samples from women which suggested that in them this relationship is reversed, the haemoglobin value derived from oxygen being higher than that from iron. They pointed out, however, that this result was unexpected and that their investigation had not been planned to determine the extent of any such sex difference.

It seemed quite possible that if there were a systematic sex difference in the iron:oxygen ratio, there might also be a sex difference in the iron:colour