THE ADVERSE EFFECT OF ASCORBIC ACID ON THE STABILITY OF ADRENALINE AND NORADRENALINE SOLUTIONS

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Although pro-oxidant as well as anti-oxidant effects have been attributed to vitamin C from the time of the first studies of this substance until the present, the anti-oxidant effect toward adrenaline[1–5] has been one of its most widely recognized effects. Statements such as the following[6] are readily found in the literature: "It is well known that ascorbic acid protects adrenaline from oxidation *in vitro*". The fact that it is possible to demonstrate such an effect only under certain limited conditions of pH and concentration has received little attention, although convincing demonstrations of such protective effect have almost always been performed in the presence of phosphate at a pH nearly neutral or slightly alkaline, and with a concentration of ascorbic acid no greater than 1 m*M*.

Since adrenaline and noradrenaline, in common with endiol compounds, are more stable in acid than in neutral or alkaline media, it was natural to attempt to enhance the stability of acid solutions by adding ascorbic acid. West[6, 7] has reported such an attempt which proved unsuccessful. However, the lack of success was not attributed to a destructive effect of ascorbic acid but rather to too low a concentration of ascorbic acid[6] or to the absorption of variable amounts of the anti-oxidants, sodium metabisulfite and ascorbic acid, by the rubber caps of the bottles during the heat sterilization[7].

Preliminary reports from this laboratory have indicated that rather than a preservative effect, ascorbic acid in an acid medium can have a definite destructive effect on adrenaline and noradrenaline[8, 9]. Furthermore, dehydroascorbic acid was found to have a similar but less pronounced effect. The details of these experiments are here recorded. Conditions which favor the pro-oxidant effects of this vitamin are distinguished from those that permit the anti-oxidant effect to be observed. The mechanism of the pro-oxidant effect is discussed.

MATERIALS AND METHODS

DL-Noradrenaline hydrochloride was used in the experiments (Table I) carried out in Prof. VON EULER's laboratory (see ACKNOWLEDGEMENTS). L-Adrenaline bitartrate and L-noradrenaline bitartrate monohydrate were used in the other experiments. All were crystalline synthetic products supplied by Winthrop-Stearns. The following description applies to the materials used in this laboratory (Tables II–IV and Figs. 1–3). Those used in Prof. VON EULER's laboratory were of corresponding purity.

L-Ascorbic acid was the U.S.P. crystalline product from Merck. L-Dehydroascorbic acid was a cream-colored powder from Nutritional Biochemicals Corp. Sodium acetate, glacial acetic acid, and $FeSO_4 \cdot 7H_2O$ were C.P. "Baker Analyzed" reagent grade. Cupric acetate monohydrate and toluene were Merck reagent grade. The hydrogen peroxide was Merck reagent Superoxol (30%).

The distilled water was collected directly from a Barnstead steam still and stored in a pyrex carboy. This water was analyzed by W. H. and L. D. Betz Chemical Engineers for soluble copper and total iron: the results in parts per million for iron: 0.00; for copper: 0.003. The pH 5 acetate buffer was also analyzed; the results for iron: 0.00; for copper: 0.012.

Glassware, except for the Stockholm experiments in which rubber-stoppered vaccine bottles were used, was pyrex throughout. The solutions of adrenaline or noradrenaline were kept in 125 ml glass-stoppered pyrex bottles and all contained 100 ml originally. Each day 2 or 3 ml were removed for analysis. A small drop of toluene was added to each bottle to prevent mold growth when observations were to be made for more than 5 days. Solutions were kept at room temperature not in the dark. The pH values were checked with a Beckman pH meter.

The noradrenaline determinations in Table I were carried out by the chemical method of VON EULER AND HAMBERG[10]. The pH was adjusted to 6 and the iodination was allowed to proceed for 3 minutes at room temperature. Appropriate dilutions and colorimeter readings were made immediately. A modification of this method has been used in subsequent work. Rather than adjusting the pH to 6, the time of oxidation is increased to that required for maximum red color development. A more stable color is obtained, since adrenochrome and noradrenochrome are more stable when the concentration of buffer salts during the oxidation is about $N/10$ rather than above $N/2$ as results in the original method. From Fig. 1 it is apparent that maximum red color development occurs with adrenaline at 30° C in 1 minute whether the pH is 4, 5, or 6, but that with noradrenaline maximum red color is developed in 1 minute only at pH 6; 5 minutes are required at pH 5; and 32 minutes at pH 4.

The modified VON EULER AND HAMBERG *method*

Reagents. Standard solutions containing 100 µg of adrenaline bitartrate or noradrenaline bitartrate monohydrate per ml were prepared immediately before using in $N/10$ acetate buffers of pH 4, 5, or 6. $N/10$ iodine was prepared by dissolving 12.7 g of iodine in about 150 ml of water containing 18 g of KI and diluting to 1 l. $N/20$ sodium thiosulfate was prepared by dissolving 12.41 g of $Na_2S_2O_3 \cdot 5H_2O$ plus 50 mg of Na_2CO_3 and diluting to 1 l.

Procedure. To 1 ml portions of the test solutions or standard solutions in 10 ml volumetric flasks placed in a water bath at 30° C 0.2 ml of $N/10$ iodine was added. The oxidation was thus carried out in the presence of 0.0167 N iodine. It was stopped after the following oxidation times by addition of 0.4 ml of $N/20$ thiosulfate with shaking.

	Minutes of oxidation		
	pH 4	pH 5	pH 6
Adrenaline	1	1	1
Noradrenaline	32	5	1

After diluting to the 10 ml mark, the contents of each flask were poured into matched Evelyn Colorimeter tubes and read with filter 520 after setting the colorimeter at 100% transmittance with distilled water. To correct for any color present before oxidation with iodine, a blank was prepared by diluting 1 ml to 10 ml with water. The optical density of the blank was subtracted from that of the sample oxidized with iodine.

The values for oxidized vitamin C of Fig. 2 were determined by subtracting the value for ascorbic acid, determined by the Bessey modification of the Evelyn, Malloy, and Rosen method as described by ROE[11], from the value for total vitamin C determined by the dinitrophenylhydrazine method as described by ROE[11].

Oxygen consumption studies were done with the conventional Warburg technique.

The bioassays were based on blood pressure response in the atropinized dog under phenobarbital anaesthesia.

RESULTS

In Table I are shown the results of a 5-day stability study on noradrenaline. The most marked destruction of this catechol amine occurred at pH 5 with an ascorbic acid concentration of 1 mM originally present; however definite destruction also occurred using this amount of ascorbic acid at pH 4 and 5.9. The stability of the compound is very nearly the same in acetate buffer of all three pH values over a 5 day period, although a trend toward lower stability is apparent with an increase in pH.

TABLE I

EFFECT OF ASCORBIC ACID ON THE STABILITY OF NORADRENALINE

N/10 Acetate	*Ascorbic* acid M*	*Noradrenaline***		*remaining after:*		
		1 day %	*2 days* %	*3 days* %	*4 days* %	*5 days* %
pH 4.0	0	100	100	100	100	100
	10^{-5}	97	100	99	97	97
	10^{-4}	99	99	96	95	96
	10^{-3}	94	92	85	79	77
pH 5.0	0	99	99	99	99	98
	10^{-5}	100	100	100	100	100
	10^{-4}	95	95	93	91	89
	10^{-3}	86	75	67	56	50
pH 5.9	0	98	98	98	97	96
	10^{-5}	100	100	100	97	99
	10^{-4}	97	99	97	96	97
	10^{-3}	88	87	82	82	80

* 10^{-2} *M* ascorbic acid exceeded the capacity of the buffer.

** Original concentration: 88 μg noradrenaline hydrochloride per ml ($4.28 \cdot 10^{-4}$ *M*).

Figs. 2 and 3 show the results of a similar study at pH 5 on both adrenaline and noradrenaline in which the effect of added copper as well as 1 m*M* ascorbic acid was measured. It is apparent that a somewhat greater destructive effect was produced by adding to the trace amounts of copper present as a contaminant. (Such amounts of copper are present in most solutions unless extremely stringent measures are employed to prevent this contamination[12]. The susceptibility of adrenaline[13, 14] and noradrenaline[15] as well as ascorbic acid[12] to oxidation by molecular oxygen is attributed to the effect of copper in initiating the oxidation when the pH is below that which would permit true autoxidation to occur readily[12, 16]. Samples of industrial distilled water have recently been reported[17] to contain 0.5 p.p.m. cupric ion.) However, the destructive effect of the copper alone was small at this pH compared with that of the ascorbic acid plus copper. The difficulty of controlling the amount of copper introduced as a contaminant in the reagents and distilled water is believed responsible for the variability in the amount of destruction from one series of experiments to another.

The curves in the lower part of Fig. 2 indicate the losses in oxidized vitamin C occurring simultaneously with the losses in noradrenaline. The vitamin did not remain in the reduced form for many hours and after one day almost all of it could be found as the oxidized vitamin, *i.e.* as dehydroascorbic acid and/or diketogulonic acid. It is possible that the oxidized vitamin then undergoes decarboxylation to form L-xylosone, since under similar conditions such a transformation has been observed[18, 19, 20]. The presence of noradrenaline caused only a small increase in the rate of this loss. Evidence that carbon dioxide was evolved under these conditions was obtained by oxygen consumption studies using a Warburg apparatus with and without KOH to absorb the carbon dioxide. In the Warburg flask were 5 ml of 1 m*M* ascorbic acid in *N*/10 acetate buffer, pH 5.12. The gas phase was air and the temperature 39° C. Without KOH the oxygen consumption reached a maximum of 63.8 μl

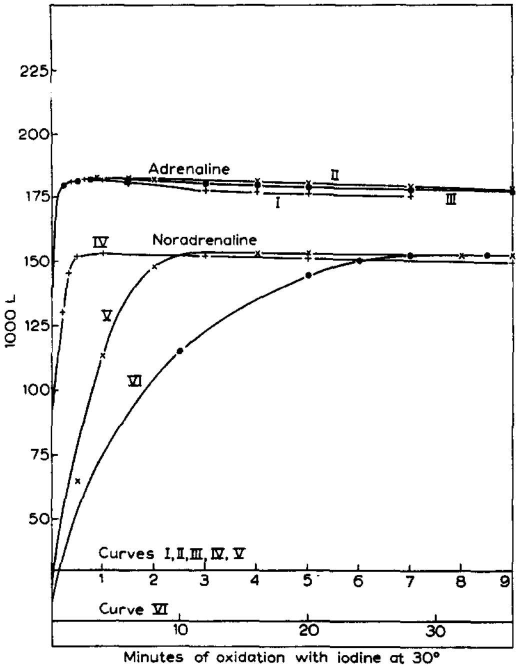

Fig. 1

Fig. 1. Oxidation rate curves for adrenaline ($3.00 \cdot 10^{-4}\,M$) and noradrenaline ($2.97 \cdot 10^{-4}\,M$) in $N/10$ acetate buffers of the following pH values: pH 6, I and IV; pH 5 II and V; pH 4, III and VI. Oxidizing agent: 0.0167 N iodine. See procedure for modified VON EULER AND HAMBERG method.

Fig. 2. Upper half of graph: noradrenaline rate of loss with and without added cupric acetate ($10^{-5}\,M$) and/or ascorbic acid ($10^{-3}\,M$). Lower half of graph: oxidized vitamin C (dehydroascorbic acid plus diketogulonic acid) rate of loss with and without noradrenaline. Solvent: $N/10$ acetate buffer pH 5.0. Room temperature.

Fig. 3. Adrenaline rate of loss with and without added cupric acetate ($10^{-5}\,M$) and/or ascorbic acid ($10^{-3}\,M$). Solvent: $N/10$ acetate buffer pH 5.0. Room temperature.

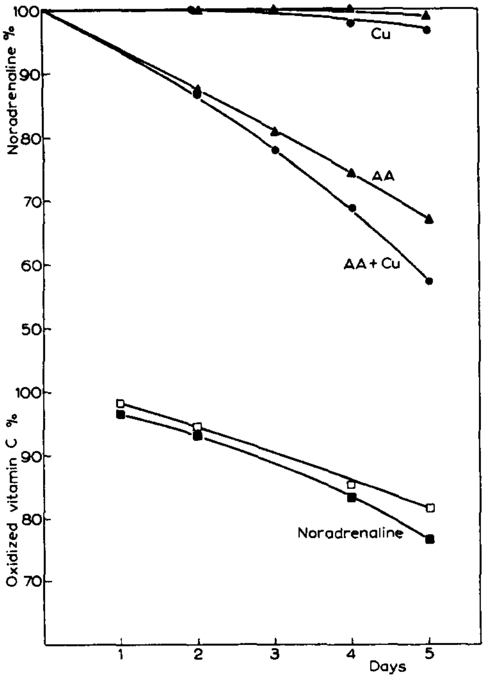

Fig. 2

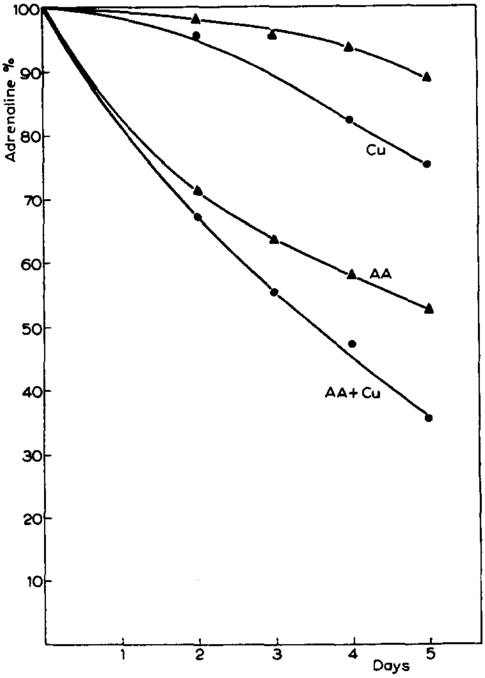

Fig. 3

in 1 hour, then appeared to drop, as would result from carbon dioxide evolution, to 58 μl in 10 hours. The theoretical oxygen consumption for 5 millimoles of ascorbic acid at 39° C is 64 μl, on the basis of 1 atom of oxygen per molecule of ascorbic acid. With KOH the oxygen consumption exceeded the above theoretical value within 1 hour and continued to increase. Removal of CO_2 would be expected to promote further decarboxylation and, since the product of the decarboxylation (L-xylosone) is a strong reducing agent, its formation and ready oxidation would be at least partially responsible for increasing the oxygen consumption above the theoretical value required for conversion of ascorbic acid to dehydroascorbic acid.

Table II shows that a similar destructive effect toward both adrenaline and noradrenaline occurs within 5 days at pH 5 when dehydroascorbic acid rather than ascorbic is added to the catechol amine solutions. At pH 4 very little, if any, destruction was observed after only 5 days, although it was readily apparent by 56 days with both adrenal hormones. At pH 6 only a slight destructive effect toward noradrenaline could be attributed to dehydroascorbic acid and none at all toward adrenaline. With the latter compound the presence of dehydroascorbic acid at this pH seems to protect to some extent against copper-catalyzed oxidation. It is obvious that adrenaline is considerably less stable than noradrenaline at this pH and that additional copper decreases the stability markedly. The destructive effect caused by ascorbic acid was greater than that caused by dehydroascorbic acid. This result is consistent with the mechanism described below.

TABLE II

DEHYDROASCORBIC ACID EFFECT WITH AND WITHOUT ADDED COPPER COMPARED WITH THE ASCORBIC ACID EFFECT ON STABILITY OF ADRENALINE AND NORADRENALINE

N/10 Acetate	*Substance added*	*Noradrenaline* remaining after*		*Adrenaline* remaining after*	
		5 days %	*56 days* %	*5 days* %	*56 days* %
pH 4.0	None	100.0	98.1	100.0	91.5
	$Cu(OAc)_2$ 10^{-5} *M*	100.0	98.1	100.0	81.3
	DHA 10^{-3} *M*	98.8	59.8	99.2	62.5
	DHA 10^{-3} *M* + $Cu(OAc)_2$ 10^{-5} *M*	97.8	53.8	95.6	56.2
	Ascorbic acid 10^{-3} *M*	86.5	37.1	76.5	34.3
pH 5.0	None	100.0		91.3	
	$Cu(OAc)_2$ 10^{-5} *M*	98.0		83.7	
	DHA 10^{-3} *M*	84.9		82.0	
	DHA 10^{-3} *M* + $Cu(OAc)_2$ 10^{-5} *M*	73.3		67.6	
	Ascorbic acid 10^{-3} *M*	62.3		64.3	
pH 6.0	None	92.2		65.7	
	$Cu(OAc)_2$ 10^{-5} *M*	79.1		4.0	
	DHA 10^{-3} *M*	88.0		82.5	
	DHA 10^{-3} *M* + $Cu(OAc)_2$ 10^{-5} *M*	75.8		58.6	
	Ascorbic acid 10^{-3} *M*	71.1		65.8	

* Original concentration:
100 μg adrenaline bitartrate per ml ($3.00 \cdot 10^{-4}$ *M*).
100 μg noradrenaline bitartrate monohydrate per ml ($2.97 \cdot 10^{-4}$ *M*).

Bioassays were done at 2 and 4 days on the solutions used in Figs. 2 and 3. These results were compared with those obtained by the chemical method and, as shown in Table III, there is good agreement within the error of the two methods. Therefore, the destructive effect of ascorbic acid cannot be attributed to an artifact of the method of analysis.

TABLE III

ASCORBIC ACID EFFECT ON THE STABILITY OF ADRENALINE AND NORADRENALINE
WITH AND WITHOUT ADDED COPPER
DETERMINED BY CHEMICAL METHOD AND BIOASSAY
(pH 5)

Ascorbic acid M	*Cupric acetate M*	*Method*	*Noradrenaline* remaining after*		*Adrenaline* remaining after*	
			2 days %	*4 days* %	*2 days* %	*4 days* %
0	0	Chem.	100	100	98	94
0	0	Biol.	100	100	100	91
0	10^{-5}	Chem.	100	98	96	82
0	10^{-5}	Biol.	100	89	98	80
10^{-3}	0	Chem.	87	74	71	59
10^{-3}	0	Biol.	93	80	70	57
10^{-3}	10^{-5}	Chem.	87	69	67	47
10^{-3}	10^{-5}	Biol.	78	57	60	42

* Original concentration:
100 μg adrenaline bitartrate per ml ($3.00 \cdot 10^{-4}$ *M*).
100 μg noradrenaline bitartrate monohydrate per ml ($2.97 \cdot 10^{-4}$ *M*).

DISCUSSION

Mechanism of the destructive effect

The development of oxidation power in definitely acidic solutions of ascorbic acid containing catalytic amounts of copper and exposed to atmospheric oxygen was reported in 1950 by KAUFFMANN[21]. The mechanism of the oxidation was believed to depend upon generation of free hydroxyl radicals by the action of cuprous ions on the hydrogen peroxide formed during copper-catalyzed oxidation of ascorbic acid[22, 23]. That cuprous ions react with hydrogen peroxide in a manner analogous to that of ferrous ions has been reported by BAXENDALE, EVANS AND PARK[24]. Although the actual oxidizing agent generated in this reaction may be tetravalent iron, trivalent copper, free hydroxyl radicals, or some as yet unpostulated reactor, it is agreed that oxidation power is produced. KAUFFMANN presents evidence that the true oxidizing agent in his reactions is the intermediate in the oxidation of ascorbic acid to dehydroascorbic acid which has lost only one electron and which is formed by the interaction of free hydroxyls (or tetravalent iron, trivalent copper, etc.) with ascorbic acid.

The same labile electron acceptor is formed during the course of copper-catalyzed ascorbic acid oxidation. According to WEISSBERGER AND LUVALLE[16], it is possible that an electron transfer within the complex of Cu^{++} with the monovalent ascorbate ion (AH^-) gives rise to Cu^+ and this very reactive intermediate (AH).

$$\mathrm{Cu(AH)_2} \longrightarrow \mathrm{Cu^+} + \mathrm{AH^-} + \mathrm{AH}$$

AH is believed to be readily oxidized by oxygen of the air[16], losing an electron followed by a proton.

$$2\ AH + \tfrac{1}{2}\ O_2 \longrightarrow 2\ A + H_2O$$

However, under certain conditions (*e.g.*, when less than one atom of oxygen is readily available per 2 molecules of AH), this very reactive intermediate may act as an oxidizing agent.

In the present experiments the formation of AH by both of these mechanisms is to be expected. Moreover, a similar intermediate, XH, would arise from xylosone in its endiol form. As indicated above, the latter compound is apparently formed, under the conditions of these experiments, as CO_2 is gradually evolved. Its formation is believed to account for the destructive effect observed with dehydroascorbic acid.

The mechanism of this destructive effect seems, therefore, to be oxidative. Further evidence that this is correct is provided by the results in Table IV. In this experiment no ascorbic acid is used, but hydrogen peroxide is added to make a concentration equivalent to that which is formed during the oxidation of a 1 m*M* ascorbic acid solution to dehydroascorbic acid, *i.e.* 1 m*M* H_2O_2. Very little destructive effect is observed toward adrenaline or noradrenaline at pH 4 within 70 minutes. However, in the presence of $10^{-4}\ M$ ferrous sulfate, rapid destruction occurs. This is an example of the well-known Fenton reaction, an oxidation brought about presumably by free hydroxyl radicals. In the previous experiments the source of ferrous or cuprous ions is reduction by endiol compounds of the traces which occur in the higher valence state as contaminants.

TABLE IV

EFFECT OF HYDROGEN PEROXIDE WITH AND WITHOUT FERROUS IONS ON STABILITY OF ADRENALINE AND NORADRENALINE

(pH 4)

Substance added	*Noradrenaline* remaining after*		*Adrenaline* remaining after*	
	10 min %	*70 min* %	*10 min* %	*70 min* %
None	100	100	100	100
$H_2O_2\ 10^{-3}\ M$	100	99	98	96
$H_2O_2\ 10^{-3}\ M$ $FeSO_4\ 10^{-4}\ M$	61	14	24	5

* Original concentration:
100 μg adrenaline bitartrate per ml ($3.00 \cdot 10^{-4}\ M$).
100 μg noradrenaline bitartrate monohydrate per ml ($2.97 \cdot 10^{-4}\ M$).

Conditions favoring protective effect compared with those favoring oxidative effect

It appears that the transformations undergone by dehydroascorbic acid are of prime importance in determining whether the presence of ascorbic acid in solutions which permit its conversion to dehydroascorbic acid will eventuate in pro-oxidant or antioxidant effects. Three main pathways of decomposition are known: (1) decarboxylation, (2) chain cleavage, (3) dismutation.

If conditions are such that the vitamin undergoes chiefly decarboxylation with attendant formation of a new endiol compound, pro-oxidant effects can be expected.

Unfortunately, the conditions that favor this reaction specifically have not been as well defined as the conditions favoring reactions 2 and 3. However, as in the present experiments, conditions can be carefully chosen to effectively rule out reactions 2 and 3.

Conditions favoring the rapid decomposition of dehydroascorbic acid by scission of the carbon chain between carbons 2 and 3 may promote the anti-oxidant effects of ascorbic acid. The products of this reaction are oxalyl threose or oxalyl threonic acid[25]. Such decomposition has been reported by ROSENFELD[25] to occur at a pH as low as 7 if there is phosphate present in a concentration at least 5 times that of the dehydroascorbic acid, *providing the concentration of dehydroascorbic acid does not exceed 1 mM*. Conditions conforming to these have been used in all the convincing demonstrations of the anti-oxidant effect of ascorbic acid toward adrenaline or noradrenaline that have come to the writer's attention. It is believed that this reaction does not occur appreciably in the present experiments because there is no phosphate present and the pH is below 7. In the absence of phosphate a pH of 11 is said to be required. The reaction is catalyzed by cyanide as well as by phosphate[25].

ROSENFELD[25] has pointed out that when the concentration of dehydroascorbic acid is as high as 4.3 mM another reaction supervenes which he designates "dismutative side reaction". In the course of this reaction a yellow-colored anion plus a strong reducing compound, which may be ascorbic acid itself, arise. These conditions seem to conduce to pro-oxidant effects and the mechanism discussed above should be applicable in view of the fact that a strong reducing agent is formed which is very likely an endiol. In fact, in the experiments of KAUFFMANN referred to above, the concentration of ascorbic acid was definitely above 4.3 mM and there was reducing power toward indophenol throughout the period of observation during which oxidation of methyl orange occurred. The pH in these experiments was definitely acid. Pro-oxidant effects have been attributed to ascorbic acid in a similarly high concentration but at a pH nearer neutrality[26, 27, 28]. The obvious interpretation to be placed on these results is that with concentrations of ascorbic acid of this magnitude the amount of highly reactive intermediate, HA, formed is so great that the ratio of atoms of available oxygen to molecules of HA is much less than 1:2. Therefore, HA is not readily oxidized and is available for promoting oxidations. In the present experiments the concentration of ascorbic acid is never greater than 1 mM, consequently the dismutative side reaction would not be expected to occur.

All of the aforesaid effects can be secured in the absence of any enzyme. In the presence of enzymes quite different results may be obtained. For example, according to VERLY[4], in the presence of catechol oxidase ascorbic acid does not protect adrenaline from oxidation under conditions which would otherwise afford such protection at least until the ascorbic acid is completely converted to dehydroascorbic acid. It seems that the specificity of enzymes in directing oxidations is accountable for the oxidation of substances in the presence of ascorbic acid which have higher oxidation-reduction potentials than ascorbic acid.

It is interesting to note that pro-oxidant effects toward reduced diphosphopyridine nucleotide[29, 30] in the presence of an enzyme sometimes called "ascorbic acid reductase", which occurs as an impurity in quinone reductase, have been attributed to the reactive intermediate herein designated "AH" under conditions that approach very closely those that usually favor anti-oxidant effects except that the pH is usually slightly below 7 and the molar ratio of phosphate to dehydroascorbic acid is less than 5:1. This enzyme is believed to catalyze transfer of electrons from DPNH to AH.

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SUMMARY

A convenient colorimetric analysis for adrenaline or noradrenaline based on the VON EULER AND HAMBERG method is described. Using this method it is shown that ascorbic acid at 10^{-3} M accelerates the rate of loss of adrenaline from acetate buffered solutions of pH 4 or 5 and of noradrenaline from such solutions of pH 4, 5, or 6. This destructive effect, which is greater at pH 5 than at pH 4 or 6, can be enhanced by addition of catalytic amounts of cupric acetate at pH 5. Addition of dehydroascorbic acid rather than ascorbic acid causes a similar but less pronounced destruction and the effect is also enhanced by addition of catalytic amounts of cupric acetate at pH 5.

The conditions under which this destructive effect is observed are such that dehydroascorbic acid undergoes slow decomposition with evolution of carbon dioxide rather than the rapid decomposition described by ROSENFELD which leads to cleavage between carbons 2 and 3. It appears that conditions which lead to this cleavage permit the anti-oxidant effects of ascorbic acid to be observed, whereas the conditions which lead to decarboxylation or to the dismutative side reaction described by ROSENFELD, favor the pro-oxidant effects.

The mechanism of this destructive effect is believed to be oxidative and to depend upon the generation of semiquinone-like free radicals by the removal of one electron from the endiolate group of ascorbic acid or endiol-containing decomposition products of dehydroascorbic acid such as L-xylosone. Such free radicals would be expected to arise during coppercatalyzed oxidation of endiol compounds. However, an additional source of these radicals, based upon the mechanism advanced by KAUFFMANN which involves a Fenton type of reaction, appears to exist under the conditions of these experiments.

REFERENCES

[1] R. D. H. HEARD AND A. D. WELCH, *Biochem. J.*, 29 (1935) 998.
[2] M. YAMAMOTO, *Z. physiol. Chem.*, 243 (1936) 266.
[3] K. M. DAOUD AND A. S. E. AYYADI, *Biochem. J.*, 32 (1938) 1424.
[4] W. VERLY, *Arch. intern. physiol.*, 56 (1948) 1.
[5] M. MANN, *J. Pharm. and Pharmacol.*, 5 (1953) 1024.
[6] G. B. WEST, *Quart. J. Pharm. and Pharmacol.*, 20 (1947) 541.
[7] G. B. WEST AND T. D. WHITTET, *ibid.*, 21 (1948) 225.
[8] M. J. OESTERLING, *19th Intern. Physiol. Congr. Montreal, 1953, Abstr. of Communs.*, p. 649.
[9] M. J. OESTERLING, *Federation Proc.*, 15 (1956) 322.
[10] U. S. VON EULER AND U. HAMBERG, *Acta Physiol. Scand.*, 19 (1949) 74.
[11] J. H. ROE, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 1, Interscience Publishers, New York, 1954, p. 115.
[12] E. S. G. BARRON, R. H. DEMEIO AND F. KLEMPERER, *J. Biol. Chem.*, 112 (1935) 625.
[13] P. CHAIX, J. CHAUVET AND J. JEZEQUEL, *Biochim. Biophys. Acta*, 4 (1950) 471.
[14] P. CHAIX, G.-A. MORIN AND J. JEZEQUEL, *ibid.*, 5 (1950) 472.
[15] P. CHAIX AND C. PALLAGET, *ibid.*, 10 (1953) 462.
[16] A. WEISSBERGER AND J. E. LUVALLE, *J. Am. Chem. Soc.*, 66 (1944) 700.

[17] A. J. ROSENBERG, *J. Biol. Chem.*, 219 (1956) 951.
[18] E. S. WEST AND R. E. RINEHART, *ibid.*, 146 (1942) 105.
[19] F. J. REITHEL AND R. P. WITHER, *J. Am. Chem. Soc.*, 71 (1949) 1879.
[20] R. MOUBASHER, *J. Biol. Chem.*, 176 (1948) 529.
[21] H. J. KAUFFMANN, *J. Am. Chem. Soc.*, 73 (1951) 4311.
[22] E. SILVERBLATT, A. L. ROBINSON AND C. G. KING, *ibid.*, 65 (1943) 137.
[23] H. G. STEINMAN AND C. R. DAWSON, *ibid.*, 64 (1942) 1212.
[24] J. H. BAXENDALE, M. G. EVANS AND G. S. PARK, *Trans. Faraday Soc.*, 42 (1946) 155.
[25] B. ROSENFELD, *J. Biol. Chem.*, 150 (1943) 281.
[26] P. HOLTZ AND G. TRIEM, *Naturwissenschaften*, 25 (1937) 251.
[27] K. H. BEYER, *J. Pharmacol. Exptl. Therap.*, 71 (1941) 394.
[28] S. UDENFRIEND, C. T. CLARK, J. AXELROD AND B. B. BRODIE, *J. Biol. Chem.*, 208 (1954) 731.
[29] M. B. MATHEWS, *ibid.*, 189 (1951) 695.
[30] A. NASON, W. D. WOSILAIT AND A. J. TERRELL, *Arch. Biochem. Biophys.*, 48 (1954) 233.

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A STUDY OF CARBON DIOXIDE FIXATION IN THE HEN OVIDUCT*

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The hen oviduct is a tissue uniquely versatile in its ability to utilize carbon dioxide. It has been shown that carbon dioxide is fixed efficiently into both carboxyl groups of glutamic acid[1] whereas in other tissues of higher organisms carbon dioxide goes almost exclusively to the α-carboxyl group. Carbon dioxide is fixed into the carboxyl groups of acetic acid and glycine under conditions where acetic acid itself is only poorly converted, if at all, to glycine[2]. The following paper describes a compound in which incorporated radioactive carbon dioxide is present in labile configuration. A preliminary report of this work has already appeared[3].

EXPERIMENTAL

The tissue system, medium, and conditions of incubation were the same as previously described[4]. After 2 hours of incubation the contents of the flask were centrifuged at 1000–2000 r.p.m. Two volumes of alcohol were added to the supernatant fluid and, after centrifugation, a few drops of 1 *N* NaOH to make the clear supernatant fluid alkaline to phenol red. This solution was concentrated nearly to dryness on the steam bath or *in vacuo* at about 50°.

Steam distillation and Duclaux procedures

The residue from the above treatment, in a volume of 1–2 ml, was placed in a Markham type steam-distillation apparatus[5]. 35 ml of distillate was collected and transferred to a Duclaux distillation apparatus of the KNETEMANN type[6], fitted with a 100 ml flask. Before distillation a 2.0 ml aliquot of the 35 ml was withdrawn for estimation of counts and acid equivalents and was replaced with 2.0 ml of distilled water. The first 30 ml of distillate were collected in three 10.0 ml volumetric flasks. The Duclaux constants represent the cumulative fraction of the material in these three successive samples, expressed as per cent of the total in the three fractions. A fast stream of N_2 was passed through all fractions for at least 2 minutes prior to neutralization in order to remove dissolved carbon dioxide.

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