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Non-enzymatic Ethanol Oxidation in Biological Extracts

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Non-enzymatic ethanol oxidation was found to occur in fresh PCA-precipitated blood and tissue homogenate. The same reaction occurred in ascorbic acid but not in dehydroascorbic acid solutions. Hydrogen peroxide was completely inactive as a direct oxidizing agent in the absence of ascorbic acid. A semidehydroascorbate peroxy radical was hypothesized to act as an electron acceptor in the reaction.

Truitt [1] has found that varying amounts of acetaldehyde are produced when ethanol is added to blood precipitates *in vitro* and the sample is incubated at 55°C for 15 – 20 min. This artificially-produced acetaldehyde interferes with the determination of small amounts of acetaldehyde in biological samples.[1] It has been assumed that the source of the acetaldehyde is a protein from which an acetaldehyde group is split off, or a release of bound acetaldehyde by ethanol.[1–4] On the other hand, von Euler and Hasselquist [5] have assumed that the observed oxidation of ethanol is caused by hydrogen peroxide and is catalyzed by ascorbic acid and dehydroascorbic acid in the presence of ferric ions. The oxidative properties of ascorbic acid in the presence of oxygen have also been reported by other investigators.[6,7]

Fresh blood precipitates always contain small amounts of ascorbic acid and traces of iron ions; in addition, the autoxidation of ascorbic acid produces hydrogen peroxide.[8] Therefore, it is possible that the acetaldehyde found in blood precipitates might have been produced by this ascorbic acid system, rather than being released from a protein or macromolecule. This hypothesis requires that ascorbic acid is able to oxidize ethanol to acetaldehyde in biological extracts. The present studies have been carried out in an attempt to test this possibility.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical grade. Ethanol (AaS) from Alko, Helsinki, Finland; acetaldehyde, L(+)ascorbic acid, perhydrol, perchloric acid and riboflavin from E. Merck AG, Darmstadt, Germany; dehydro-L(+)-ascorbic acid from Fluka AG, Buchs, Switzerland.

Tissue materials. Rat and mouse livers were quickly removed from the animals after decapitation, then were weighed and homogenized in ice-cold 0.6 N perchloric acid (PCA). The precipitate was centrifuged, and 0.5 ml of the supernatant was incubated and analyzed by gas chromatography. In other experiments fresh bovine liver was used. The extract was filtered and the filtrate analyzed. Rat blood was also obtained by decapitation. The homogenate concentrations in the samples were reported as g tissue or ml blood per 100 ml sample.

Gas chromatography. The quantity of acetaldehyde formed was determined in a Perkin-Elmer automatic F 40 head-space gas liquid chromatograph with a hydrogen flame detector (hydrogen flow rate: 35 ml/min; air flow rate: 300 ml/min). The commercial column (15 % polyethylene glycol on celite 60/100) sold by the manufacturer was used. The column temperature was 75°C, and purified nitrogen was used as carrier gas, flow rate 35 ml/min. 0.5 ml of sample was added to each bottle in which the head-space was produced. The bottles were placed in a thermostatically controlled water bath at 65°C and kept at equilibrium for at least 15 min prior to the automatic analysis of the head-space gas. The highest sensitivity was used. The analytical procedure was standardized with samples of diluted acetaldehyde and ethanol. Standard and unknown samples were determined by identical procedures. 0.2 ‰ *tert*-butanol was used as internal standard in the ethanol determination. The acetaldehyde was determined by using fresh redistilled acetaldehyde (50 nmol/ml) as a reference standard. The acetaldehyde concentration in the standard was checked daily with 0.025 ‰ *tert*-butanol as the internal standard.

Gel filtration. The filtration experiments were done with columns packed with cross-linked polysaccharide Sephadex G-25 (manufactured by Pharmacia, Uppsala, Sweden). The characteristics of the bed material were: water regain = 2.5 g water/g dry gel; wet density = 1.13 g/ml. The packing of the column has been described by Gelotte.[9] V_0 necessary for the calculation of K_d was experimentally determined as the elution volume for bovine albumin (from Armour Pharmaceutical Company Ltd., Eastbourne, England) with 0.05 N NaCl as eluent.

Thin layer chromatography (TLC). Glass plates were coated with a thin layer of Silica Gel G (from E. Merck AG, Darmstadt, Germany) and dried at 110°C for 20 min. Samples of bovine liver extract (pH 5), eluted through Sephadex G-25 with distilled water and shaken with Florisil (magnesium trisilicate, 60 – 100 mesh, from Serva Feinbiochemica GmbH & Co., Heidelberg, Germany), were applied to the plate. The plate was developed in the solvent system 1-butanol : oxalic acid : water (4 : 1 : 5) and sprayed with a molybdate reagent that stains all reducing substances blue.[10]

RESULTS

Acetaldehyde formation in PCA-precipitated liver extract

Non-enzymatic ethanol oxidation was found to occur in biological extracts. The rate is dependent primarily on the homogenate concentration. Thus, it is possible to minimize the formation of acetaldehyde by using very diluted solutions. Compared with blood, liver tissue from rats and mice produced large amounts of acetaldehyde. Fig. 1 contains data which show that the amount of acetaldehyde formed was linearly proportional to the ethanol concentration.

Reaction time. The duration of incubation at 65°C had, as shown in Fig. 2, an obvious effect on the acetaldehyde production in liver extract. Compared with liver, PCA-precipitated blood produced only insignificant amounts of acetaldehyde. Blood cells supplied much more acetaldehyde than plasma in the presence of ethanol, which is in close agreement with the findings of Truitt.[1]

Effect of pH. As shown in Fig. 3 the pH of the analyzed solution had a clear effect on the acetaldehyde formation. At pH 1 the amount of acetaldehyde

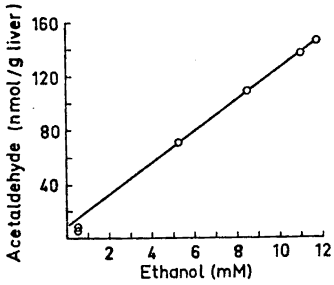


Fig. 1. Non-enzymatic ethanol oxidation in rat liver extract incubated at 65°C for 15 min with ethanol. The liver was homogenized in ice-cold 0.6 N PCA solution containing varying amounts of ethanol, centrifugated, and the supernatant was analyzed immediately. The amount of acetaldehyde formed is reported as nmol per g liver tissue.

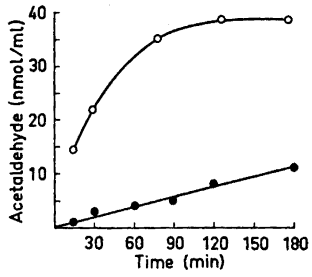
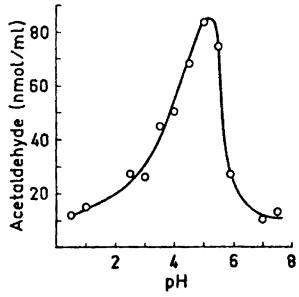


Fig. 2. The acetaldehyde formation in PCA-precipitated blood and liver extracts incubated with ethanol for different lengths of time at 65°C. (○) Rat liver homogenate (conc. 20 %) and (●) rat blood (conc. 25 %). The ethanol concentration was 20 mM in the former and 44 mM in the latter experiment.

formed was only 18 % of the maximal acetaldehyde concentration produced at pH 5.

Fig. 3. Effect of different pH on the acetaldehyde formation in PCA-precipitated bovine liver extract. The pH-value was gradually increased by adding NaOH to the sample; it was checked both before and after the analysis. The dilution of the extract can be disregarded because the homogenate concentration was decreased only about 3 % during the pH change from 1–7. The circles represent mean values obtained in two experiments. The samples were incubated for 60 min at 65°C before the analysis.



Effect of heating on the acetaldehyde formation. Bovine liver extract was heated in a covered test tube in a water bath at 80°C for different lengths of time. The result of this experiment showed that heating liver extract (pH < 1) in the water bath for 90 min had no effect on the acetaldehyde formation. At higher pH, the heating of the extract before the addition of ethanol greatly reduced the acetaldehyde production.

Effect of the redox system, ascorbic acid – dehydroascorbic acid, on the ethanol oxidation

Ascorbic acid was found to oxidize ethanol to acetaldehyde when a 0.5 mM ascorbic acid solution was incubated in the presence of 20 mM ethanol and Cu^{2+} for 15 min at pH 5 and 7 (Table 1).

Table 1. Ethanol oxidation in ascorbic acid and dehydroascorbic acid solutions. The ethanol concentration was 20 mM and the samples were incubated for 15 min at 65°C. The acetaldehyde formed is given as nmol per ml incubation solution.

Solution	pH	$CuSO_4$ (μM)	Acetaldehyde concentration (nmol/ml)
Ascorbic acid (0.5 nM)	5	10	55
	7	10	15
	5	–	72
Dehydroascorbic acid (0.5 mM)	5	10	0
	7	10	0
	5	–	0
Ascorbic acid (0.25 mM) + dehydroascorbic acid (0.25 mM)	5	10	28
	7	10	4

Ascorbic acid is easily oxidized to dehydroascorbic acid, especially at pH > 4.[11] In order to test if dehydroascorbic acid is also able to oxidize ethanol the same experiment was repeated with the former compound. As Table 1 illustrates, there was no acetaldehyde formed in this case. If equivalent amounts of 0.5 mM ascorbic acid and 0.5 mM dehydroascorbic acid were then mixed together and the mixture was analyzed, there was only a quarter as much acetaldehyde produced at pH 7 as in the first experiment. Even a suspension of dehydroascorbic acid did not produce any acetaldehyde after an incubation time of 15 min.

Effect of varying concentrations of ascorbic acid on the reaction at different pH

As shown in Fig. 4A, the amount of acetaldehyde produced was a direct function of the ascorbic acid concentration. Very little ascorbic acid is needed for the reaction; even a 0.2 mM solution (pH4) produced 50 nmol acetaldehyde per ml after incubation with 20 mM ethanol for 15 min. At pH 7, greater amounts of acetaldehyde were formed only when the ascorbic acid concentration was over 0.6 mM. In a strong acid PCA-solution (pH < 1), an ascorbic acid concentration of 1.2 mM produced only insignificant amounts of acetaldehyde after an incubation time of 15 min (Fig. 4A).

Fig. 4B illustrates that the acetaldehyde formation in a solution of 0.25 mM ascorbic acid and 20 mM ethanol has a maximum at pH 4. No measurable acetaldehyde was formed at pH 7 – 8.

To check if ascorbic acid has the same oxidizing capacity in PCA-precipitated liver homogenate, varying amounts of ascorbic acid were added to liver extract (Fig. 5). Compared with the reaction in a pure ascorbic acid

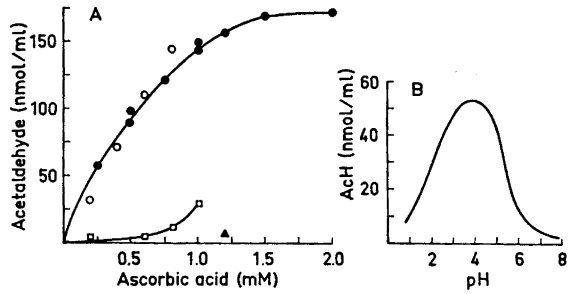


Fig. 4. A. Effect of different ascorbic acid concentrations on the acetaldehyde formation. The ascorbic acid was dissolved in distilled water and the pH of the solution regulated with HCl or NaOH, respectively. The samples were incubated at 65°C in the presence of 20 mM ethanol: (●) 15 min at pH 4, (○) 60 min at pH 4, (□) 60 min at pH 7, and (▲) 15 min at pH < 1 (in the latter experiment, the ascorbic acid was dissolved in a 0.6 N PCA solution). B. Acetaldehyde formed at different pH values. The concentration of the ascorbic acid solution was 0.25 mM and that of the ethanol concentration 20 mM. The samples were incubated for 60 min.

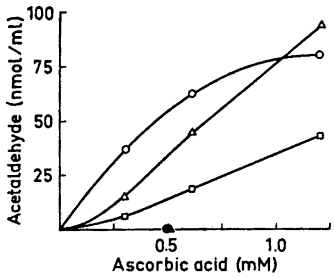


Fig. 5. Effect of ascorbic and dehydroascorbic acid at different pH on the non-enzymatic ethanol oxidation in PCA-precipitated rat liver extract (homogenate conc. 8 %). The samples were incubated for 15 min at 65°C in the presence of 20 mM ethanol before the analysis. Ascorbic acid: (△) ph < 1, (○) pH 5, (□) pH 7; dehydroascorbic acid: (▲) pH < 1, (●) pH 5. pH was regulated with NaOH. The acetaldehyde values given were subtracted from the acetaldehyde formed in a sample without added ascorbic acid at respective pH.

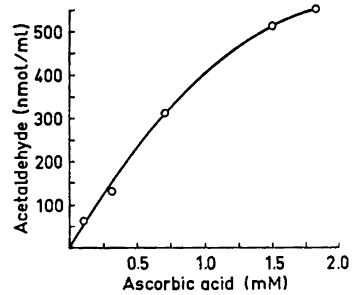


Fig. 6. Effect of hydrogen peroxide and ascorbic acid on the acetaldehyde formation. Varying amounts of ascorbic acid were added to a solution containing 20 mM ethanol and 1 mM H_2O_2. The samples were incubated for 15 min at 65°C and the acetaldehyde formed was analyzed.

solution, the liver extract gave much less acetaldehyde at pH 5, especially at high ascorbic acid concentrations. The reason for this may be that liver extract contains redox compounds such as glutathione and cysteine which have a strong inhibitory effect on the autoxidation of ascorbic acid.[12] On the other hand, in a strong acid solution (pH < 1) a much greater amount of acetaldehyde was

formed in the liver extract than in the pure ascorbic acid solution. When dehydroascorbic acid was added instead of ascorbic acid to the PCA-extract no acetaldehyde was produced.

Effect of hydrogen peroxide on the reaction

Hydrogen peroxide is generated by the autoxidation of ascorbic acid.[8,13] To investigate the effect of H_2O_2 as a direct oxidizing agent on the reaction, varying amounts of hydrogen peroxide were added to a 20 mM ethanol solution and the sample was incubated for 15 min at 65°C. H_2O_2 concentrations up to 1.0 mM gave only insignificant amounts of acetaldehyde (< 5 nmol/ml). If, on the other hand, 1.0 mM hydrogen peroxide was added to a solution containing ascorbic acid and ethanol, the acetaldehyde formation increased considerably (Fig. 6). This catalytic effect of hydrogen peroxide on the non-enzymatic ethanol oxidation on fresh liver extract is illustrated in Fig. 7.

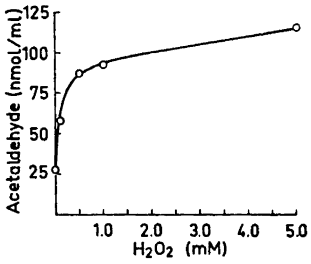


Fig. 7. Effect of hydrogen perioxide on the non-enzymatic ethanol oxidation in rat liver extract (pH < 1). The homogenate concentration was 20 %, and the samples (○) were incubated with 20 mM ethanol for 15 min at 65°C. (●) Control: acetaldehyde formed in extract without added H_2O_2.

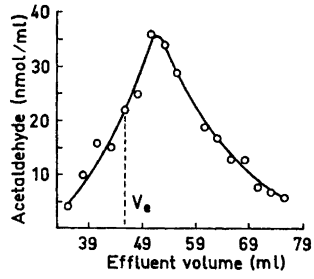


Fig. 8. 2 ml concentrated liver extract (pH 5) was applied on a Sephadex G-25 column and eluted by 0.05 N NaCl. Fractions of 2.5 ml were collected and their activity (capacity for acetaldehyde formation in the presence of ethanol) measured by GLC, after incubation at 65°C for 60 min in the presence of 20 mM ethanol. V_e for the active compound was 46.5 ml.

Ethanol-induced release of "bound" acetaldehyde

In order to determine whether acetaldehyde is liberated from some macromolecules by ethanol the macromolecules in the liver extract were separated by gel filtration. Bovine liver extract was eluted through a Sephadex G-25 column. The effluent volume (V_e) was determined by measuring V_e from the point at which the extract was added to the point at which the activity of the eluted substance was optimal (Fig. 8). There was no activity in the first 16 fractions (no acetaldehyde was formed when ethanol was added to the fractions), but it rapidly increased to maximum at an effluent volume of about 52 – 53 ml. From the data obtained, the distribution coefficient (K_d) of the active compound was calculated to be 0.6. The single maximum found shows

that there was probably only one agent present in the extract capable of oxidizing ethanol to acetaldehyde.

The high K_d-value of the active compound makes it unlikely that the observed acetaldehyde had been released from a macromolecule by ethanol. If riboflavin (Mw 367) is eluted under the same conditions with 0.05 N NaCl, both it and the active compound have the same K_d-value.

The active fractions collected had a strong yellow color. To test whether these pigments have a direct effect on the acetaldehyde production, florisil was added to the fractions and the mixture was shaken until the yellow color was adsorbed on the florisil. The yellow florisil extract had no activity at all, while the colorless liver extract had a high activity. Active, colorless liver extract, analyzed by TLC, showed the presence of ascorbic acid.

DISCUSSION

The results show that ethanol is oxidized to acetaldehyde in the presence of ascorbic acid. This reaction was found to take place also in biological extracts. Ascorbic acid is widely distributed throughout the animal, including the liver.[14] Thus it seems probable that the ascorbic acid present in fresh liver tissue homogenate gave rise to the non-enzymatic acetaldehyde formation in the biological extracts. The results obtained by gel filtration and TLC of liver extract strongly support this hypothesis by showing first that the acetaldehyde is not released from a protein or another macromolecule by ethanol, and then that the active compound contained ascorbic acid.

The heat stability of ascorbic acid in strong acidic solutions is well known.[15] If ascorbic acid is involved in the acetaldehyde formation it would be logical to expect that the active liver extract could be heated without loss of activity. This is, in fact, what happened. It was possible to heat acidic liver extract without any loss of activity. At higher pH (> 3) ascorbic acid loses its heat stability;[12] consistently, the activity of liver extracts at pH > 3 is eliminated by heating.

The fact that the active compound had no absorbancy in the visible area excludes free flavins or other redox pigments, but not ascorbic acid as the actual oxidizing agents.

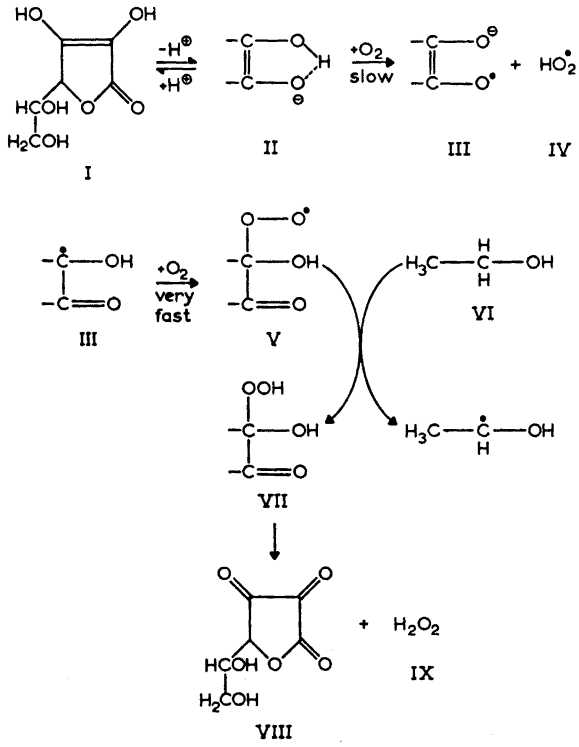
From the data given in Fig. 2, it was possible to calculate that 1 g fresh liver from a rat could non-enzymatically oxidize 25 nmol of ethanol per min after 15 – 30 min at 65°C. Compared with liver homogenate, blood from the rat gives only insignificant amounts of acetaldehyde (Fig. 2). The low ascorbic acid content in whole blood may be the reasons for this finding.[14]

The maximum pH for acetaldehyde formation in both an ascorbic acid solution (Fig. 4B) and crude liver extract (Fig. 3) are approximately the same. The reason that the pH-maximum is somewhat higher in crude extract may be that ascorbic acid is more stable in it than in a water solution at pH > 6.[11]

The observation that small amounts of hydrogen peroxide were completely inactive as direct oxidizing agents in the absence of ascorbic acid is in agreement with the results obtained by Udenfriend *et al.*[6] and Kersten *et al.*,[7] but contrary to the hypothesis that H_2O_2 itself is the oxidizing agent at physiologi-

cal concentration. In the presence of ascorbic acid, however, the hydrogen peroxide had an obvious effect on the oxidation reaction.[5]

A semiquinone like intermediate between ascorbic acid and dehydroascorbic acid is hypothesized to be the electron acceptor in the ethanol oxidation. The autoxidation of ascorbic acid (I; Scheme 1) to dehydroascorbic acid (VIII) is generally assumed to proceed by a free radical mechanism, and free radicals have also been detected in solutions of ascorbic acid.[16,17] The reaction is catalyzed by Cu^{2+} and Fe^{3+} but also proceeds without a catalyst.[18]



Scheme 1.

An unstable ascorbate-oxygen complex is formed in the presence of molecular oxygen, and the subsequent decomposition of this complex yields a free radical of semiquinone type and a reactive perhydroxyl radical $HO_2^{\cdot}$ (IV). The rate-determining step is the transference of one electron from an ascorbate monoanion (II) to molecular oxygen. This implies that the rate of the reaction is dependent on the pH of the solution (Figs. 3, 4B).

As shown in Scheme 1, the semidehydroascorbate free radical (III) reacts rapidly with molecular oxygen to form a semidehydroascorbate peroxy radical (V)[19] which is able to oxidize ethanol (VI) to acetaldehyde. It is also possible

that the free radical can directly oxidize ethanol to acetaldehyde, and is itself reduced to ascorbic acid.

Khan and Martell [18] have suggested that ascorbic acid at low oxygen concentrations may be directly oxidized by the $HO_2\cdot$ formed. It is possible that, under the same conditions, ethanol can be oxidized by this reactive radical.

The hydrogen peroxide (IX) formed has a catalytic effect on the non-enzymatic acetaldehyde formation (Figs. 6, 7), probably by accelerating the autoxidation of ascorbic acid, or by assisting in the formation of the reactive peroxy radical. In the presence of ferrous and cuprous ions, H_2O_2 decomposes very rapidly, giving $HO_2\cdot$ and a new reactive radical, $OH\cdot$, which is able to oxidize ethanol to acetaldehyde. The existence of such an active semidehydroascorbate radical has been demonstrated by many investigators.[16,20] Schneider and Staudinger [21] have postulated that this radical can be formed by "comproportionation" between ascorbic acid and dehydroascorbic acid.

The oxidation of ethanol into acetaldehyde in the microsomal fraction of liver has been reported by Lieber and De Carli,[22] Lieber *et al.*[23] and Khanna *et al.*[24] The so-called microsomal ethanol oxidizing system (MEOS) which they describe requires NADPH and O_2, and they considered that ADH, the principal ethanol oxidizing enzyme *in vivo*, was not involved in this reaction. A similar ethanol oxidation system was found in adipose tissue from rat.[25]

The activity of the MEOS has been expressed as the acetaldehyde formed per unit of time. Using the method described by Burbridge *et al.*[26] this acetaldehyde concentration has been analyzed by microdiffusion separation and the subsequent spectrophotometric measuring of acetaldehyde semicarbazone formed. Truitt [1] has reported that both the spectrophotometric method of Burbridge *et al.*[26] and the gas chromatographic method developed by Duritz and Truitt [27] produce high levels of non-enzymatically formed acetaldehyde. Thus, it is possible that a part of the acetaldehyde which was thought to have been produced by the MEOS in fact was formed by the ascorbic acid system.

Non-enzymatic ethanol oxidation also occurs at physiological temperature (37°C), but its physiological role has not yet been determined.

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