HYDROXYLATION OF AROMATIC COMPOUNDS BY A SYNTHETIC ANALOG OF AN ENZYME SYSTEM

by

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Alec E. Kelley — March 21, 1957

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Date

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INTRODUCTION

The fundamental biological modes of action of ascorbic acid (I)* are: (1) its ability to keep other substances in the reduced state due to its low oxidation potential, and (2) its function as an oxidation catalyst, because it can be readily dehydrogenated (oxidized) and the resulting product reversibly reduced (II). Its biological oxidation requires the enzyme ascorbinase. Ascorbic acid has been reported as a reagent to bring about the hydroxylation of the aromatic nucleus.

In 1941 Rothman (1) reported that ascorbic acid, in the presence of ultraviolet light, transforms tyrosine (III) to dihydroxyphenylalanine (IV), and synepherine (V) into adrenaline (VI).

Ekman (2,3) felt that hydrogen peroxide formed from the oxidation of ascorbic acid caused the oxidation of aromatic nuclei, since he found no reduction of ascorbic acid and the oxidation was not a dehydrogenation. Benzene-poisoned guinea pigs responded to ascorbic acid treatment, and a marked increase in polyphenols was found, especially if the enzyme ascorbic acid oxidase (ascorbinase) was present in the diet. In his studies of the *in vitro* oxidation of benzene by ascorbic acid or by hydrogen peroxide, he found

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* Roman numerals refer to formulas as given on pp. 5-6.

the same products as in the *in vivo* oxidation, namely, polyphenols and urochrome-A.

Earlier evidence for the existence of hydrogen peroxide resulting from the oxidation of ascorbic acid was reported by Barron and co-workers (4). Along this line Calcutt (5) in 1951 identified hydrogen peroxide as a product of ascorbic acid autoxidation. He offers this as an explanation for ascorbic acid acting as an oxidizing agent under certain conditions.

In the field of dye chemistry it was reported (6) that ascorbic acid in the presence of cupric or ferric salts will accelerate the catalytic oxidation of dyes with hydrogen peroxide.

An intensive study of the use of ascorbic acid in aromatic hydroxylation was begun at the National Institutes of Health at Bethesda, Maryland (7). They found that hydroxylation of tyramine (VII) to hydroxytyramine (VIII) by homogenates of adrenal medulla was enhanced by the addition of ascorbic acid. They dispensed with the adrenal tissue when they discovered that ascorbic acid and oxygen alone produced the hydroxylation. However, metal ions were needed to catalyze the reaction. Ferrous or ferric ion served equally well, while cuprous, cupric, or cobaltous ion were about 5 to 10 per cent as effective as iron. They also report that ethylenediaminetetraacetic acid (IX) enhanced

the reaction rate, though it was not essential for oxidation of the substrate.

The reaction is pH sensitive with the optimal pH depending upon the individual compound. As the temperature increases oxidation increases, but the phenolic product disappears more rapidly at elevated temperatures; 37° was the temperature at which they carried out most of their reactions.

They reached the following conclusions concerning the reaction as a result of their findings:

(1) Ascorbic acid is not the hydroxylating agent since toward the end of the reaction, when the ascorbic acid concentration had become negligible, the substrate is still oxidized.

(2) Hydrogen peroxide was ruled out as the direct hydroxylating agent because they failed to detect any hydrogen peroxide in solution. In addition, substitution of hydrogen peroxide for ascorbic acid gave negligible oxidation.

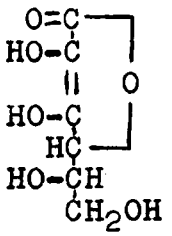
(3) They believe that hydroxylation occurs through a reaction product of hydrogen peroxide with ascorbic acid (though not a hydroperoxide, since a synthetic hydroperoxide (X) possessed no oxidative activity).

Using the system developed at Bethesda, Keston and Carsiotis (8) reported that they were able to hydroxylate steroids.

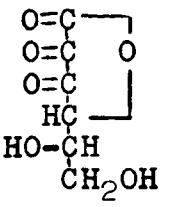
The mechanism of the detoxicating activity of ascorbic

acid may not be in a state of general agreement, but the evidence that hydroxylation occurs is substantial. This investigation was begun to see if a laboratory scale synthetic process could be devised using the model ascorbic acid system. Compounds were treated using this system under a variety of conditions to test the practicability of the process and to see if adequate yields could be obtained.

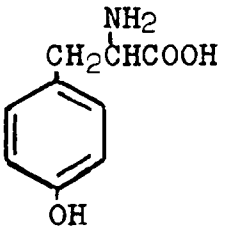
FORMULAS



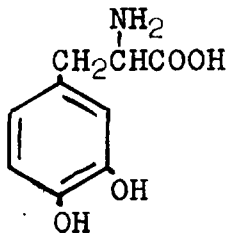
(I)
Ascorbic acid



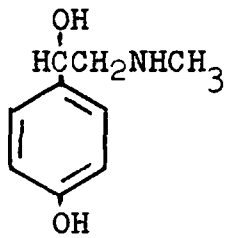
(II)
Dehydroascorbic acid



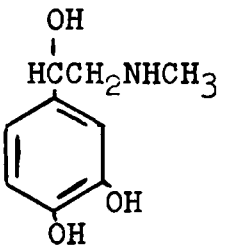
(III)
Tyrosine
(p-hydroxy-phenylalanine)



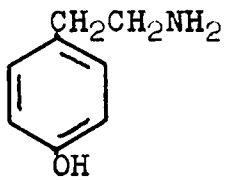
(IV)
dihydroxy-phenylalanine



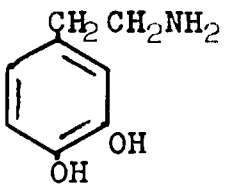
(V)
Synepherine



(VI)
Adrenaline

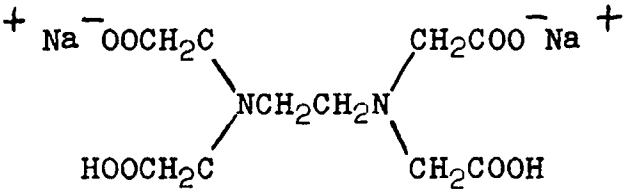


(VII)
Tyramine

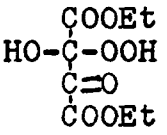


(VIII)
Hydroxytyramine

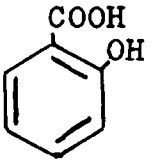
FORMULAS
(cont'd)



(IX)
Ethylenediaminetetraacetic acid (EDTA) disodium salt



(X)
Diketosuccinic acid ethyl ester hydroperoxide



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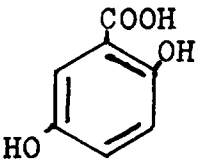
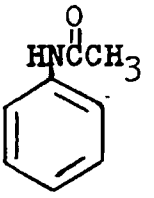
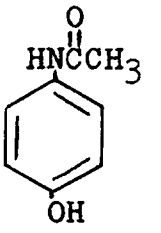
Salicylic acid



(XII)
Gentisic acid



(XIII)
Acetanilide



(XIV)
p-Hydroxyacetanilide

DISCUSSION

In a synthetic problem of this type various conditions are studied in an attempt to find the set of conditions which give the best results insofar as yield and practicability are concerned. The results of this investigation for the hydroxylation of salicylic acid are summarized in Table I.

From our findings it appears that the reaction will occur in buffered and non-buffered media in the presence of oxygen. However, the buffered solutions lend to greater ease of handling when working up the product, since no separation of solid material results, even if the reaction mixture has been allowed to stand overnight. In buffered media the solution remains homogeneous throughout the course of the run.

It was observed that separation of a solid complex occurred in non-buffered solutions, implying that excess of the reagents were present. Comparison of Run H with earlier runs indicates that lower concentrations of the reagents are permissible. This may be a lead to possible work in the future, since there will be obvious benefit from using less reagents, if yields can be maintained.

Recovery of the unreacted salicylic acid (substrate) is an important factor, since this permits recycling the substrate and further increases the yield. This would be of special importance if the starting material is available only in small quantities, and a hydroxylated derivative is desired.

Repeated runs could be carried out on the recovered material to give ultimately a high overall yield.

The reaction does not occur to a reasonable extent in the presence of hydrogen peroxide alone (Run E), or in the presence of air alone (Run G). The best oxidant appears to be a combination of hydrogen peroxide and oxygen, as seen by the yield obtained for Run D. Run F was run under non-homogeneous conditions since great excess of substrate was used. The per cent yield was less than Run D; however, the actual weight of product obtained was greater.

It appears that in some cases, as with acetanilide, isomeric hydroxylated modifications may occur as a result of the reaction. If one modification only is desired and the isomers cannot be separated easily, the hydroxylated derivative will be impure. It may turn out that compounds to be hydroxylated by the ascorbic acid system will require having only one site for possible hydroxylation to ensure purity of products without resort to tedious separation procedures.

From our studies it is apparent that hydroxylation of the aromatic nucleus can be carried out using ascorbic acid on a laboratory scale.

TABLE I

Hydroxylation of Salicylic Acid under Various Conditions

0.2 M ascorbic acid, 0.02 M $Fe(NH_4)_2(SO_4)_2$, 0.1 M ethylenediaminetetraacetic acid

Run	Temp. in °C	Reaction Time, Hrs.	Solvent Used	Moles of Substrate	Oxidant Added	% Salicylic Acid Recovered	% of Gentisic Acid Conversion	% of Gentisic Acid Yield
A	36.4	0.5 1.0 1.5 2.0	Buffer[a]	0.02	O_2	--	8 24 19 27	--
B	36.4	2.0 4.0 6.0	Buffer[a]	0.02	O_2	53[b]	39 41 40	85[b]
C	36.4	5.0 6.0 7.0	Water	0.02	O_2	53[b]	35 32 43	81[b]
D	36.4	2.0 4.0	Water	0.02	H_2O_2 + O_2	38[b]	36 46	67[b]
E	36.4	2.5	Water	0.02	H_2O_2	33	8	12
F	36.4	2.0	Buffer[a]	0.2	O_2	70	16	53
G	36.4	2.0	Buffer[a]	0.02	Air	29	8	12
H[c]	50.0	2.5	Buffer[a]	0.02	O_2	33	23	36

[a] Na_2HPO_4 buffer according to McIlvaine (9) to give pH 6.4; see Experimental.
[b] These figures represent percentages of the total reaction, rather than individual aliquots.
[c] Half the quantities of ascorbic acid, $Fe(NH_4)_2(SO_4)_2$ and EDTA used.

EXPERIMENTAL

Materials. The l-ascorbic acid, ethylenediaminetetraacetic acid disodium salt (EDTA), p-aminophenol, and the gentisic acid (XII) were Eastman Kodak materials. Hydrogen peroxide, citric acid, acetanilide (XIII), and ferrous ammonium sulfate were manufactured by Baker's. The disodium hydrogen phosphate was obtained from Merck. Salicylic acid (XI) was a Mallinckrodt product.

Apparatus. All reactions were carried out in a one liter round-bottom, three-neck flask with ground glass joints fitted with a Teflon stirrer, a Friedrich condenser, and a gas dispersion tube (sintered glass head) placed below the surface of the reaction mixture to deliver the air or oxygen into the medium. In reactions where hydrogen peroxide and air or oxygen were used, a ground glass dropping funnel delivered the peroxide solution dropwise, and after all the peroxide was added (15-20 minutes), the gas delivery tube replaced the dropping funnel. Reactions which were carried out at other than room temperature were performed in a constant temperature water bath.

Preparation of McIlvaine's Buffer Solution (9). To prepare the buffered reaction medium 35.8 g. of $Na_2HPO_4 \cdot 12H_2O$ was dissolved in a 500 ml. volumetric flask with distilled water to form solution A. Solution B consisted of 10.5 g. of citric

acid monohydrate dissolved in a 500 ml. volumetric flask with distilled water. Just before starting the reaction 346 ml. of solution A was mixed with 154 ml. of solution B to give a total volume of 500 ml. of solution, from which portions were withdrawn to dissolve the various reactants, then recombined to initiate the reaction. Although the calculated pH of the buffer alone was 6.4, the acidity of the relatively large quantities of reactants used decreased this value appreciably. Upon measurement of the pH throughout the run and to its end the pH was found to be around 5-5.5. Since no difficulty resulted using this pH, all runs made with buffer were in this pH range.

Preparation of p-hydroxyacetanilide (XIV) for standard curves. For the preparation of p-hydroxyacetanilide a standard procedure (10) was used as follows: Into 500 ml. of water was poured 16.6 ml. (0.2 mole) of concentrated hydrochloric acid; 21.8 g. (0.2 mole) of p-aminophenol was added, and the mixture was warmed to 50° to dissolve the amine. A solution of 30 g. (0.22 mole) of sodium acetate in 100 ml. of water was prepared. Acetic anhydride (23.3 ml., 0.24 mole) was added to the p-aminophenol hydrochloride solution and stirred until dissolved; the sodium acetate solution was then added immediately. The mixture was stirred, cooled well in an ice bath overnight, and the crystals of p-hydroxyacetanilide removed by filtration.

Standard curves. Standard solutions at various concentrations

were made and absorbances measured in the ultraviolet range from 220 to 310 millimicrons on the Beckman model D. U. quartz spectrophotometer.

A. Standard curve for p-hydroxyacetanilide. To identify the product of the reaction the $\Delta\varepsilon$ method (11) was to be used. This method is based on the idea that the spectrum obtained for the unionized phenol is different from that for the ionized form of the phenol. Thus, if samples are run both at pH of 10 and of 1, there can be obtained the absorbances for the ionized and unionized forms. The differences of these absorbances at various wavelengths divided by the concentration is equal to $\Delta\varepsilon$. Within experimental error the curve has the same value for various concentrations. The absorbances, however, do differ with concentration. Therefore, knowing the $\Delta\varepsilon$ values (from standards) and the ΔA values (from run) the concentrations of the samples can be calculated.

B. Standard curves for gentisic acid and salicylic acid. Unfortunately, the $\Delta\varepsilon$ method was not applicable here since reproducible readings of the absorbance could not be obtained for gentisic acid in the basic pH range. This was believed to be due to the oxidation of the phenolate ion to a quinoid type structure. No stabilization of readings occurred even after one hour standing. Therefore, the method was abandoned and the U. V. absorbances of gentisic acid and salicylic acid at pH of 1 were used directly. The absorbances were taken and plotted from 220 to 310 millimicrons. A Beer's law plot of

absorbance against concentration at various wavelengths was made and a straight line obtained, showing that Beer's law was valid over the range studied.

Extraction and analysis of products. A. p-hydroxyacetanilide. The reaction mixture was first extracted with ethyl acetate using several small (50 ml.) portions to remove the unreacted acetanilide and the product, p-hydroxyacetanilide. It should be noted that in a trial run with a known amount of p-hydroxy-acetanilide a 72% recovery was obtained using this method. Other solvents which were tried, but found ineffective, were benzene, chloroform, benzyl alcohol, and petroleum ether. The ethyl acetate was then extracted with base to remove the base soluble potassium salt of p-hydroxyacetanilide. In the early runs 20% potassium hydroxide was used as the base and a black color resulted in the basic extract which was believed to be due to polyphenol oxidation. Therefore, a weaker basic solution, 0.1 N, was used and found to be successful. Samples were drawn from this basic solution and the spectra obtained compared to the standard curves.

B. Gentisic acid. The reaction mixture was acidified and then shaken with small portions of chloroform to remove unreacted salicylic acid. The water layer was then extracted with ether several times to remove the gentisic acid and the ether allowed to evaporate. A known volume of 0.1 N hydrochloric acid was added and heated to dissolve the product; an aliquot was withdrawn and diluted in a volumetric flask. The spectrum

was taken at a pH of approximately one and compared with the standard curves. The chloroform solution of the unreacted salicylic acid was evaporated to dryness, the residue was taken up in 0.1 N hydrochloric acid, and the amount of salicylic acid was measured spectrophotometrically.

Description of runs. A. Attempted preparation of p-hydroxy-acetanilide. At the beginning little success was found in attempting to prepare this compound by the hydroxylation of acetanilide. In general the runs were carried out as follows: 6.5 g. (0.1 mole) acetanilide was dissolved in 250 ml. of 95% alcohol. In another beaker 18.5 g. (0.05 mole) of EDTA was dissolved in 250 ml. of distilled water warmed to about 50°. In the EDTA solution 18.5 g. (0.1 mole) of ascorbic acid was dissolved and both solutions were mixed. At once there was added in one portion to the mixed solution 3.9 g. (0.01 mole) of ferrous ammonium sulfate. The solution turned a dark purple color immediately. This mixture was then poured into the reaction flask and the reaction allowed to proceed.

In the early experiments air was drawn through the solution by an aspirator and the reaction allowed to continue for 6, 8, or 12 hours and even allowed to stand overnight to no avail. Runs were also made using hydrogen peroxide with no apparent success. During all runs a white solid separated from the solution, apparently a complex formed during the reaction.

Other attempts were tried, and finally some product was formed. The reaction was run at 36.4° in 250 ml. of buffered alcoholic solution as follows: 9.25 g. (0.025 mole) of EDTA was dissolved in 125 ml. of phosphate buffer. There was added 9.25 g. (0.05 mole) of ascorbic acid, and this solution was added to a 125 ml. solution of 95% alcohol containing 3.2 g. (0.05 mole) of acetanilide. A 1.95 g. (0.005 mole) portion of ferrous ammonium sulfate was added, and oxygen was circulated through the mixture. Aliquots of 50 ml. were withdrawn after thirty minutes, one hour, and two hours, and the remaining 100 ml. of solution was extracted at the end of eight hours. The extraction process used was the one described above using 0.1 N potassium hydroxide. From the spectra obtained there appeared to be the two isomeric forms, o- and p-hydroxyacetanilide. Since separation of these isomers was difficult, and since the ascorbic acid system appeared successful, hydroxylation of a compound which would yield only one hydroxylated product was attempted. For this reason salicylic acid was chosen.

B. Preparation of gentisic acid by hydroxylation of salicylic acid. The reactions, with the variations of conditions indicated by Table I, were run in the following manner: 18.5 g. (0.05 mole) of EDTA was dissolved in 400 ml. of warm phosphate buffer solution. The solution was cooled to room temperature, and 18.5 g. (0.1 mole) of ascorbic acid was then dissolved to form solution A. At the same time in 100 ml. of

phosphate buffer 3.9 g. (0.01 mole) of ferrous ammonium sulfate was dissolved to form solution B. Salicylic acid (2.8 g., 0.02 mole) was dissolved in solution A at 36.4°. Solution A and solution B were mixed and oxygen passed into the mixture for two hours. A brown solution was formed immediately. In some runs aliquots were withdrawn and the reaction quenched by acidifying it. Extraction of these aliquots was made using chloroform and ether as described above. The remaining reaction mixture was acidified at the end of the two hours, and extracted with chloroform and ether as given above.

In the runs with hydrogen peroxide 45.3 ml. (2 moles) of 30% hydrogen peroxide solution was added dropwise during fifteen to twenty minutes.

SUMMARY

The use of ascorbic acid as a synthetic hydroxylating agent for aromatic compounds, in the presence of oxygen, has been shown to work for some compounds. From the results obtained the ideal reaction conditions appear to be the use of a buffered medium to ensure homogeneous conditions, a temperature of 36.4°, a constant supply of oxygen to the reaction mixture, and perhaps a ten-fold molar excess of hydrogen peroxide.

The way is open to test other conditions including: the temperature, pH, solvent systems and a variety of compounds to see the scope and limitations of the ascorbic acid scheme for the hydroxylation of ring systems.

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