HYDROGEN PEROXIDE STIMULATION OF THE FORMATION OF ASCORBIC ACID FROM L-GULONOLACTONE BY RAT LIVER PREPARATIONS

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The transformation of L-gulonolactone to ascorbic acid is catalyzed in the rat by an enzyme located in the liver microsomes. (Kanfer, Burns and Ashwell, 1959). Chatterjee et al. (1959) presented evidence that the overall transformation involved an oxidative step in which 2-ketogulonolactone is formed, followed by what is probably a spontaneous rearrangement to ascorbic acid. The enzyme is inactive in the absence of O_2 but in this condition can be reactivated by methylene blue or dichlorophenolindophenol.

Results in Table I show that the oxidation of L-gulonolactone by a suspension of liver microsomes from the rat or a soluble preparation obtained from those microsomes was stimulated by the addition of H_2O_2. The determination of the produced ascorbic acid was carried out by a modification of the dinitrophenylhydrazin procedure of Roe and Kuether (1943). In some cases the increase of synthesis of ascorbic acid in the presence of cyanide (added to prevent the oxidation to dehydr ascorbic acid) was corroborated by spectrophotometric studies in the U.V. region, in which ascorbic acid has a band of absorption with peak at 265 mμ. In other cases, the rates of synthesis with and without H_2O_2 were measured by spectrophotometric determination at 331 mu of the complex between o-phenylenediamine and dehydroascorbic acid (Chatterjee et al., 1959). Spectrofluorometric determination of the phenylenediamine complex showed similar activation and fluorescence spectra to that of synthetic dehydroascorbic acid.

Kanfer et al. (1959) state that the activity of gulonolactone oxidase under O_2 is twice as high as under air. The possibility was considered that the activation produced by H_2O_2 was due to the release of O_2 by catalase activity; it was

TABLE I

Effect of H_2O_2 on Ascorbic Acid Synthesis by Rat Liver Preparations

Addition µmoles	Incub. Time minutes	Gas Phase	Enzyme prep.	Ascorbic Acid Synthesis. µmoles
None	60	Air	Solubilized prep.(a)	.055
H_2O_2			Solubilized prep.(a)	.386
H_2O_2			Heated sol. prep. plus beef catalase(b)	.001
None	60	Air	Microsomal susp.	.392
H_2O_2 1.4				.554
H_2O_2 2.8				.681
H_2O_2 4.2				.847
None	5	Air	Microsomal susp.	.021
H_2O_2	5			.167
None	35			.068
H_2O_2	35			.285
None	60			.081
H_2O_2	60			.380
None	120			.115
H_2O_2	120			.390
None	15	Air	Microsomal susp.	.049
None		O_2		.140
None		O_2 (c)		.163
H_2O_2		Air		.444
None	60	Air	Microsomal susp.	.114
None		Vacuum		.023
H_2O_2		Air		.478
H_2O_2		Vacuum		.178
None	60	Air	Solubilized prep.(a)	.035
NaCN 1				.026
H_2O_2				.190
H_2O_2 plus NaCN 1				.150

a) Obtained according to Kitabchi et al., (1960).
b) 75 units of crystalline beef liver catalase (Worthington Biochemical Corp).
c) Incubated in a Warburg Apparatus.

INCUBATION SYSTEM: L-gulonolactone 2 µmoles; enzyme preparation .05 ml. of microsomes (equivalent to 50 mg. total liver) or solubilized preparation containing 1.2 mg. protein; Potassium phosphate buffer .1M pH 7.4; total volume, 1.0 ml. Unless otherwise specified H_2O_2 concentration was 2.4×10^{-2}M. Incubation at 37°C.

found, however, that ascorbate synthesis with H_2O_2 under air exceeded that obtain by incubation under O_2 (without H_2O_2) whether shaken or stationary (Table I).

Attempts to determine whether there is a specific enzyme responsible for the "peroxidase" effect separable from that responsible for the "oxidase" effect were carried out by determining the relative activities in the presence or absence of H_2O_2 for various preparations. Results showed that when these ratios were determined under standard conditions (1 hour at 37°C) they varied from 1.5 to 7.0 However, these results are not considered conclusive since determinations of a single preparation at different times of incubation (Table I) have shown greater differences in ratio than those from any two different preparations so far obtained, indicating that factors extraneous to the enzyme(s) itself may be governing the changes in ratio. The determination of H_2O_2 by the method of Satterfield and Bonnell (1955) in the same mixture used for the ascorbic acid synthesis, showed that H_2O_2 is destroyed at a rate many time faster than ascorbic acid is produced and this may be the main factor which determines changes in ratio of ascorbic acid produced in the presence or absence of added H_2O_2.

Hydrogen peroxide stimulates the synthesis of ascorbic acid under anaerobic conditions (in vacuum or N_2). When NaCN at a concentration of 1×10^{-3}M was added only a partial inactivation of the synthesis of ascorbic acid was observed both in the presence and absence of H_2O_2. In most experiments the increase by addition of H_2O_2 has been greater in the presence of air than under anaerobic conditions.

TABLE II

Ascorbic acid synthesis in the presence of H_2O_2 by liver preparations from rats fed tocopherol-free or tocopherol-sufficient diets

Addition to test	No. Animals	Nutritional State of Animals	
		Tocopherol-sufficient	Tocopherol-deficient
		µmoles/g/2 hrs.	µmoles/g/2 hrs.
None	2	5.30	0.88
H_2O_2	2	15.4	4.70

INCUBATION SYSTEM: 2 µmoles L-gulonolactone, 0.05 ml. microsome suspension (equivalent to 50 mg. total liver), phosphate buffer pH 7.4 to complete 1 ml. Hydrogen peroxide (when added), 24 µmoles. Incubation time 2 hrs. T. 37°C.

Gulonolactone oxidase was found by Kitabchi et al. (1959) to be inhibited in preparations from rats deprived of tocopherol. Table II shows that when the synthesis of ascorbic acid is carried out in systems activated with H_2O_2, the preparations from the tocopherol deprived animals had lower activities than the preparation obtained from liver of E-sufficient animals.

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