with cold 6 *N* hydrochloric acid, extracted with chloroform, dried and concentrated, leading to the monopyrrolidide, 0.155 g. (17% yield), m.p. 107–108° (from ethyl acetate), mixed m.p. with an authentic sample, 107–108°.

Optically Active β-Phenylglutar-monopyrrolidide. (a).—Pyrrolidine was distilled from sodium. Lithium, 0.1 g. (0.03 mole), was boiled in 15 g. of this pyrrolidine under reflux with exclusion of moisture for 40 hours. *l*-Menthyl-β-phenylglutarate (IVA), 1.5 g. (0.0043 mole), was added and the solution was allowed to stand at room temperature for 6 hours. The solution was cooled, acidified to *p*H 1 with 6 *N* hydrochloric acid and filtered; menthol was collected and purified by sublimation, 0.36 g. (54% yield), m.p. 35–36°. The filtrate was extracted with eight 15-ml. portions of chloroform, which were dried and concentrated. The residue was crystallized from ethyl acetate, 0.47 g. (41% yield), m.p. 128.5–129.5°, $[\alpha]^{30}D$ −13.6° (*c* 2.18, absolute ethanol). *Anal.* Calcd. for $C_{15}H_{19}O_3N$: C, 68.94; H, 7.33; N, 5.36. Found: C, 68.62; H, 7.23; N, 5.37. In two other experiments run under similar conditions, the yields were lower, 16 and 19%.

(b).—*l*-Menthyl-β-phenylglutarate, diastereomer IVB was treated with lithium pyrrolidide in essentially the same way, leading to menthol and to the enantiomorphic pyrrolidide, 0.175 g. (17% yield), m.p. 128–129°, $[\alpha]^{30}D$ +11.8 (*c* 2.34, absolute ethanol). The compound was crystallized a second time from ethyl acetate and analyzed. Found: C, 68.54; H, 7.24; N, 5.57; a mixed m.p. with an approximately equal amount of the first enantiomorph was 108–110°; the racemic material synthesized from the anhydride melted at 108–109°.

Monomethyl-β-phenylglutarate.—β-Phenylglutaric anhydride, 2 g., (0.01 mole), was boiled under reflux for 6 hours in 25 ml. of dried methanol. The methanol was removed *in vacuo*, the residue was crystallized from cyclohexane, 2.19 g. (94% yield), m.p. 96–97°. *Anal.* Calcd. for $C_{12}H_{14}O_4$: C, 64.85; H, 6.35; neut. equiv., 222. Found: C, 65.09; H, 6.21; neut. equiv., 220, 220.

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[CONTRIBUTION FROM THE WESTERN UTILIZATION RESEARCH BRANCH][1]

Effect of Ascorbic Acid on Polyphenol Oxidase

BY LLOYD L. INGRAHAM

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A study is reported of the effect of ascorbic acid concentration on polyphenol oxidase. This study involves measurement of initial rates of oxidation, amount of ascorbic acid oxidized in 5 minutes, and limiting amount of ascorbic acid oxidized. The conclusion is drawn that ascorbic acid does not activate or inhibit either the rate of oxidation of catechol or the reaction–inactivation of the enzyme.

In many researches on polyphenol oxidase[2–4] ascorbic acid has been assumed to serve only to keep the substrate reduced and not to affect the enzyme directly. However, other papers have reported that ascorbic acid activates[5] polyphenol oxidase (from mushrooms) and also that it inhibits[6] polyphenol oxidase (from potatoes).

Baruah and Swain[6] have carefully studied the effect of copper on the reaction and have come to the conclusion that the apparent activation by ascorbic acid is the result of traces of copper present in solution which catalyzes the aerobic oxidation of ascorbic acid.[7,8]

Baruah and Swain[6] have reported that polyphenol oxidase is inhibited by reaction for two hr. with ascorbic acid in the absence of substrate under nitrogen. However, the experiments described in this paper show that the polyphenol oxidase catalyzed aerobic oxidation of catechol is not inhibited by ascorbic acid. It is possible that there is a slow reaction between free polyphenol oxidase and ascorbic acid which does not occur when the enzyme is protected with oxygen or catechol.

Test of Inhibition by Ascorbic Acid.—The report[6] that ascorbic acid is an inhibitor for polyphenol oxidase described an experiment that used Sreerangachar's method[9] (the amount of ascorbic acid oxidized in 5 min.) and potato as a source for the enzyme. This experiment was checked with an identical method on 3 enzyme preparations (A, B and C) prepared from potatoes by the procedure described by Baruah and Swain.[6] With only one of these preparations (A) was it possible to find any inhibition by ascorbic acid. Furthermore, the inhibition of the preparation (A) could not be repeated quantitatively and it foamed badly during the activity determination. It is quite possible that the ascorbic acid in some way affects surface denaturation of the enzyme. When the consumption of oxygen in solution was measured by the rotating platinum electrode,[10] which involves no bubbling, the activity was found to be independent of the ascorbic acid concentration. The results for this enzyme preparation (A) are summarized in Table I. Sreerangachar's method was also used to test the effect of ascorbic acid on polyphenol oxidase from mushrooms. These results, summarized in Table II together with results from the other two potato enzyme preparations (B) and (C), show no effect of ascorbic acid on activity. The inhibition found with preparation A could not be duplicated by adding enough egg albumin to preparations B and C to make them foam.

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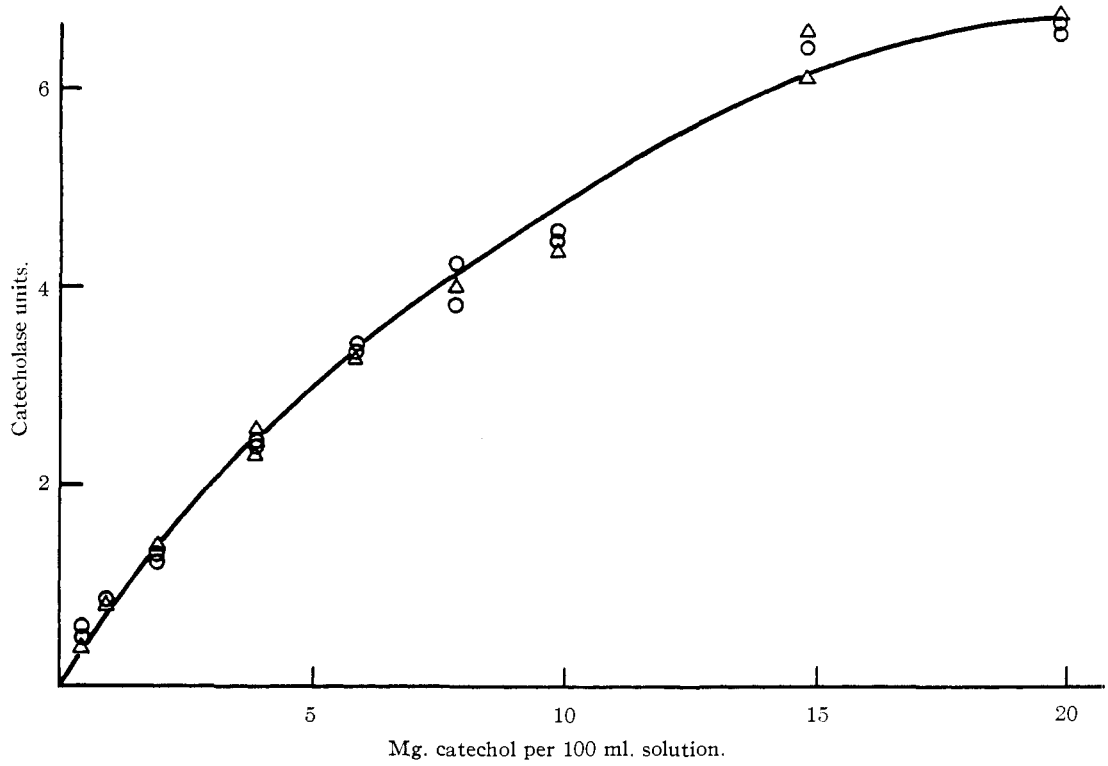

Fig. 1.—Catecholase units per ml. of mushroom juice at various amounts of catechol. The triangles refer to solutions containing 25 mg. of ascorbic acid per liter and the circles refer to solutions containing 100 mg. ascorbic acid per liter.

Initial Rates of Oxidation at Different Concentrations of Ascorbic Acid.—It is possible that Sreerangachar's method for determining enzyme activity may be a partial measure of the reaction–inactivation of the enzyme.[4] If the time were 20 min. instead of 5 min. the method would give an approximate measure of k_0E_0/k_I where k_0 is the rate constant for the oxidation and k_I is the rate constant for reaction–inactivation.[4]

To make sure that k_0 is independent of ascorbic acid concentration and not merely that the ratio k_0/k_I is independent, measurements were made during the first minute of the reaction with a polarized rotating platinum electrode.

The results shown in Table III for mushroom juice and prune enzyme together with those in Table I for potato enzyme indicate that in all of these enzyme sources there is no inhibition by ascorbic acid.

Determination of a Values at Different Concentrations of Ascorbic Acid.—In order to test whether k_I, the rate constant for reaction–inactivation,[4] is dependent upon ascorbic acid concentration, a study was made of the ultimate amount of ascorbic acid oxidized at various concentrations of ascorbic acid and for enzymes from different sources. The ultimate amount of ascorbic acid oxidized is commonly designated by the symbol a and is equal to k_0E_0/k_I.[4] Commercial mushroom enzyme[11] and apple enzyme were chosen for this study because both of these preparations showed a great deal of reaction–inactivation and these two sources of enzyme were used in our previous studies[4] of reaction–inactivation.

(11) Obtained from Mann Fine Chemicals, Inc.

The results of these experiments are presented in Table IV.

The relative constancy of these values indicates that not only k_0 but also k_I is independent of ascorbic acid concentration. This suggests that re action–inactivation is not the result of a reaction between semiquinone or o-quinone and enzyme, because the steady-state concentrations of these

TABLE I

ACTIVITY OF POTATO ENZYME PREPARATION A BY TWO METHODS

Sreerangachar's method[a] Ascorbic acid, mg./l. Initial	Used	Polarized platinum electrode Ascorbic acid, mg./l.	Catecholase units per ml. of enzyme
161	99	50	6.2
215	120	100	6.0
398	59	500	6.2
806	22	1000	6.8

[a] 0.10 ml. of potato enzyme was used in these experiments.

TABLE II

EFFECT OF VARIATION OF ASCORBIC ACID CONCENTRATION ON MUSHROOM ENZYME BY SREERANGACHAR'S METHOD

Ascorbic acid (mg./l.) Mushroom[a] Initial	Oxidized in 5 min.	Potato B[b] Initial	Oxidized in 5 min.	Potato C[c] Initial	Oxidized in 5 min.
109	>109	116	>116	136	96
209	117	235	232	253	106
485	119	503	238	403	104
990	112	998	233	937	109

[a] 0.20 ml. of a solution of 2 mg. of enzyme per 10 ml. of solution was used in these experiments. This enzyme was obtained from Worthington Biochemical Corp. [b] 0.10 ml. of potato enzyme was used. [c] 0.25 ml. of a 1 to 10 dilution of enzyme C was used in these experiments.

TABLE III

INITIAL RATES OF REACTION IN CATECHOLASE UNITS PER ML. OF ENZYME AT VARIOUS CONCENTRATIONS OF ASCORBIC ACID

Mushroom juice		Prune enzyme	
Ascorbic acid, mg./l.	Rate	Ascorbic acid, mg./l.	Rate
39	2.3	50	4.0
64	2.6	100	4.4
114	2.6	500	4.8
264	2.6	1000	4.4
514	2.5		
1014	2.3		

would be reduced by increasing the ascorbic acid concentration. However, ascorbic acid would not necessarily reduce the concentration of the short-lived enzyme–semiquinone complex that has been proposed to explain reaction–inactivation.[4]

Test for Competitive Inhibition.—In the above experiments there is the small possibility that the inhibition is competitive and the substrate concentration was always too high to detect inhibition. In order to test this possibility the activity of the enzyme was measured as a function of catechol concentration at two different concentrations of ascorbic acid. The results shown in Fig. 1 do not show any competitive inhibition by ascorbic acid even at quite low concentrations of catechol.

TABLE IV

"*a*" VALUE AT VARIOUS ASCORBIC ACID CONCENTRATIONS[a]

Initial ascorbic acid, mg./l.	Mushroom[b] enzyme	Apple[c] enzyme
200	>200	95
300	250	
400	240	95
500	250	
600	230	96
700	280	
800	240	
900	240	
1000	240	

[a] In mg. of ascorbic acid. [b] 1.76 mg. of Mann amino enzyme/100 ml. reaction mixture. [c] 0.40 ml. of enzyme/100 ml. reaction mixture.

Experimental

The potato enzyme preparations were prepared by the method of Baruah and Swain.[6] The prune enzyme was prepared by the same method as the potato enzyme except that frozen depitted prunes were used. The preparation of the apple enzyme[4] and mushroom juice[12] have been described previously. The *a* values were determined by a method described previously[12] also. Water redistilled through all-glass equipment was used in all experiments.

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Effects of Some 6-(Substituted)-purines on Regeneration of Hydra

BY CHARLES G. SKINNER, WILLIAM SHIVE, RICHARD G. HAM[1], DAVID C. FITZGERALD, JR., AND ROBERT E. EAKIN

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Adenine has been found to inhibit regeneration of hydra whose tentacles and hypostome have been removed. Sixteen new 6-(substituted)-aminopurines and six new 6-(substituted)-thiopurines have been prepared and characterized. The activity in inhibiting regeneration of "decapitated" hydra has been determined for these new substances and for thirteen other compounds whose syntheses have been reported previously. Certain of these adenine derivatives were found to have more than one thousand fold the activity of adenine in inhibiting regeneration; kinetin has an effect 20 times that of adenine.

The effects of 6-(2-furfuryl)-aminopurine (kinetin) on cell division in tobacco wound callus tissue[2,3] as well as the effects of a series of 6-(substituted)-aminopurines on development in mosses[4,5] have been reported. These results, together with the unpublished observations of several investigators, suggest a widespread importance of the 6-(substituted)-aminopurines to processes of cell division and development, at least within the plant kingdom.

Methods for quantitative study of the regeneration of hydra in a chemically defined environment recently have been developed in this Laboratory, and are being published elsewhere.[6] Since the process of regeneration in hydra involves obligatory cell division[7] and since the process is under investigation as a possible model for development and differentiation in higher animals, a study of the effects of some 6-(substituted)-aminopurines on regeneration of hydra seemed desirable and was undertaken.

The preparation of most of the 6-(substituted)-aminopurines (Table I) was effected by heating an excess of the appropriate amine with 6-methylthiopurine in a sealed tube, by a procedure similar to that of Elion, Burgi and Hitchings.[8] This method was suitable for the preparation of 6-dimethylamino- and 6-diethylaminopurine but, with other dialkylamines, the reaction mixture gave oils which did not crystallize; however, a procedure similar to that used by Daly and Christensen[9] in

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