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Reactive Sulfhydryl and Disulfide Groups of Protyrosinase and Tyrosinase.* (20471)

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The methods of conversion of protyrosinase, the inactive form of tyrosinase, to tyrosinase have been the object of extensive studies(1). In general, it may be said that amphipathic anions, carbamates, and temperatures between 60° and 70°C for 5 minutes will bring about this change. The object of this and subsequent studies is an attempt to ascertain what physical or chemical changes occur in such a conversion.

Materials and methods. Protyrosinase was obtained from diapause grasshopper eggs. These eggs were triturated with a mortar and pestle and diluted with 0.9% NaCl to a volume of 10 cc per g of eggs. This was centrifuged and the lipoidal layer removed. The remaining supernatant was recentrifuged, decanted and 1 g of KH_2PO_4 per 250 cc of supernatant added. After standing 2 hours at 0°C, this was centrifuged and the supernatant decanted. Enough $(NH_4)_2SO_4$ was added to make the supernatant 19.3% saturated (12% solution). Two hours later the precipitate was centrifuged away and the supernatant brought to 31.1% saturation with $(NH_4)_2SO_4$ (18% solution). After 2 hours at 0°C, this was centrifuged, the supernatant discarded and the precipitate resuspended in a volume of 0.9% NaCl equal to one-half that of the solution centrifuged. After one hour this was centrifuged, the precipitate discarded and the supernatant brought to 31.1% saturation with $(NH_4)_2SO_4$. After 2 hours, this was centrifuged and the precipitate resuspended in a volume of NaCl equal to that used in the previous step. This was dialyzed against 3 changes of NaCl for at least 24 hours. The resulting preparation showed no tyrosinase activity and contained on an average 0.5 mg of nondialyzable material per cc, which in turn had in it 0.21% copper and 14% nitrogen.

A modification of the methods employed by Mirsky and Anson(2) was used to determine the reactive sulfhydryl and disulfide groups. The limitations of this method have been adequately discussed by these authors. In the determination of the sulfhydryl groups, one cc of proenzyme or enzyme plus 2 cc of 0.9% NaCl plus 0.1 cc of phosphate buffer (pH 7.5) plus 0.1 cc of 0.1 M $K_3Fe(CN)_6$ were allowed to stand at 35°C for one hour. At the end of this period, the protein was precipitated with 0.1 cc of 0.05 N H_2SO_4 and 3.3 cc of a saturated solution of $(NH_4)_2SO_4$, filtered out and one cc of Folin and Malmros(3) ferric sulfate solution added to 3 cc of the supernatant. This was allowed to

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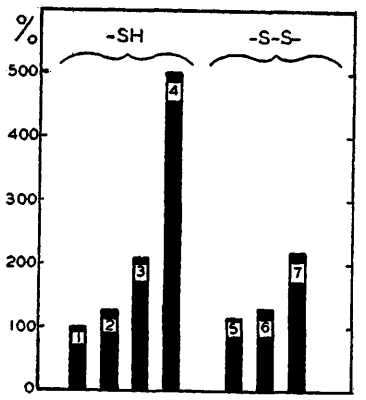

FIG. 1. Percentage amounts of sulfhydryl groups (columns 1 to 4) and disulfide groups (columns 5 to 7). Column 1, 5: the native proenzyme; column 2: the enzyme produced by Aerosol OT; column 3: the enzyme produced by exposure to 65°C for 10 min.; column 4, 7: the enzyme denatured by exposure to 100°C for 10 min.; column 6: the enzyme produced by sodium dodecyl sulfate (dialyzed away after the activation of the proenzyme).

stand 30 minutes at 35 C and the color intensity determined in a photoelectric colorimeter designed and constructed similarly to that described by Evelyn and Cipriani(4). A red filter (Corning No. 241) was used. The disulfides were determined by reduction with thioglycolate and then testing for the increase in sulfhydryl groups. Three to 5 cc of the proenzyme or enzyme solution were used with 2.5 cc of neutralized thioglycolate (app 0.7 M) and 0.5 cc of phosphate buffer (pH 7.5). The protein was precipitated with $(NH_4)_2SO_4$ and washed 4 times. A control underwent similar treatment with the exception that a NaCl solution was added in place of the thioglycolate. The precipitated protein was tested for sulfhydryl groups.

The colorimeter was standardized with known quantities of $Na_4Fe(CN)_6$. With the possible exception of very low concentrations of the ferrocyanide, the ammeter readings were linear with increasing concentrations between 0 and 8 x 10^{-7} mols of ferrocyanide added and were accurate to within 2%.

Results. The native proenzyme reduced 1 x 10^{-7} mols of ferricyanide per cc of proenzyme. This is expressed as 100% in column 1, Fig. 1. When the enzyme was prepared using Aerosol OT, a slight increase in reactive sulfhydryls occurred (Col. 2, Fig. 1), but analysis of the data of 10 different determinations showed this to be nonsignificant. Activation of the proenzyme at 65°C for 10 minutes, resulted in a significant increase in —SH groups (Col. 3, Fig. 1). This is undoubtedly due to the enzyme that is concomitantly denatured. In the data of Bodine and Allen(5), about 28% of the enzyme is destroyed by exposure to 65°C for 10 minutes. The increase in —SH noted here is approximately 27% of the amount of —SH liberated upon complete denaturation by heat (Col. 4, Fig. 1). This increase upon heat denaturation is in agreement with the findings on other proteins(6). The amount of reactive disulfide present is slightly greater than the —SH as measured by these methods (Col. 5, Fig. 1) and no significant increase occurs upon activation (Col. 6, Fig. 1). The change from proenzyme to enzyme was brought about in this case by sodium dodecyl sulfate which was then dialyzed away to facilitate precipitations. Heat denaturation doubles the number of reactive —S—S— (Col. 7, Fig. 1). It is then evident that no significant increase in —SH nor —S—S— groups is occasioned by the change from protyrosinase to tyrosinase.

It next seemed of interest to ascertain whether either the oxidation of the —SH by ferricyanide or the reduction of —S—S— with thioglycolate influences the activity of the enzyme. Both substances delayed the oxidation of the substrate by reaction with the intermediary products rather than by a primary effect on the enzyme. This is evident in Fig. 2, which shows the effects of the addition of small amounts of $K_3Fe(CN)_6$ to the reaction mixture of tyrosinase and substrate. The ferricyanide delays the initiation of the oxygen uptake and also the appearance of the red color in the reaction mixture. When the time to reach 100 μl oxygen uptake is plotted against the concentration of ferricyanide, the points, save that at zero concentration, fall on a straight line (inset). Extrapolation of this straight line gives evidence that some of the ferricyanide has been reduced to ferrocyanide by reaction with the proenzyme. It is interesting to predict that if 1 x 10^{-6} moles of ferricyanide delayed the initiation of oxygen uptake or color formation 30 minutes, then 1 x

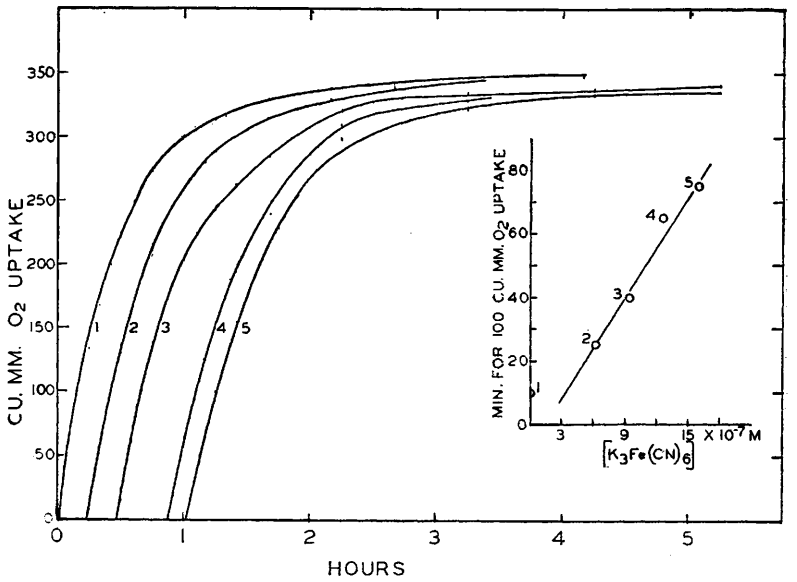

FIG. 2. Effect of potassium ferricyanide on activity of tyrosinase on tyramine-HCl. Abscissa, time in hr; ordinate, mm³ of oxygen. Concentrations of ferricyanide used may be read from the corresponding numbers on the inset. Inset: time to reach an oxygen uptake of 100 μl (ordinate) plotted against the moles of $K_3Fe(CN)_6$ added to the reaction mixture (abscissa).

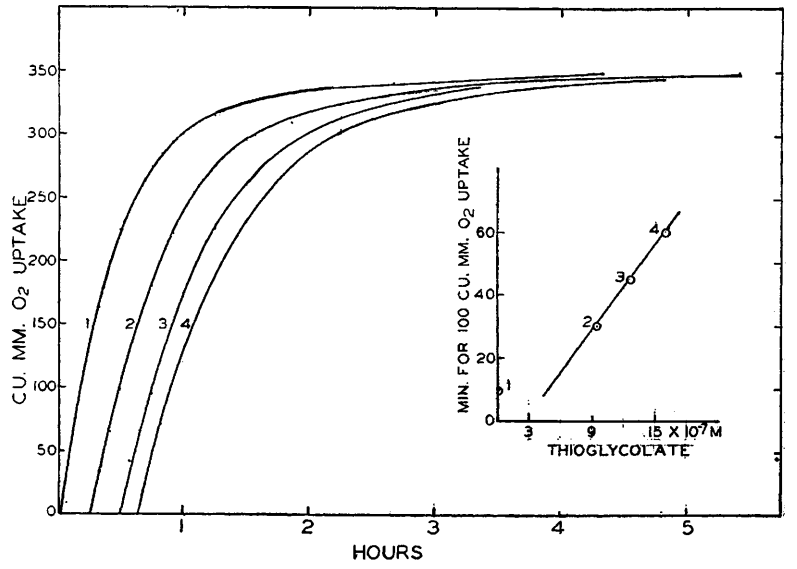

FIG. 3. A plot similar to Fig. 2, but for thioglycolate.

10^{-3} moles would delay the reaction for more than 20 days. Sodium ferrocyanide had no effect on the enzyme activity. In Fig. 3, similar data are shown using thioglycolate. Note on the inset that the straight line extrapolated indicates a greater number of —S—S— groups, this being in agreement with the colorimetric determinations.

Summary and conclusions. It seems evident that no change in sulfhydryl nor disulfide groups occurs upon activation of protyrosinase and further that the sulfhydryl and disulfide groups do not seem essential for enzyme activity. Any apparent inhibition caused by oxidation of the sulfhydryl groups or the reduction of the disulfide groups is due

to a reaction of the oxidant or reductant with the intermediary products of the oxidation of tyramine-HCl. If the production of tyrosinase is occasioned by the denaturation of an inhibitor, an increase in —SH groups seems probable; this, however, is not the case.

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Inhibition of Utilization of Aspartic Acid for Growth of *Leuconostoc mesenteroides* P-60. (20472)

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γ-L-Glutamylhydrazide was an effective inhibitor of growth of 2 strains of streptococci(1) and of *L. arabinosus*(2) under conditions in which small amounts of glutamine produced a marked acceleration of growth. In both instances it was found that β-aspartylhydrazide was a weaker inhibitor than the glutamyl derivative. α-DL-Methylglutamic acid was also found to be an inhibitor of the utilization of D- and L-glutamic and α-ketoglutaric acids by *L. arabinosus*(2,3), while α-DL-methylaspartic acid and α-DL-methylasparagine were ineffective. It was of interest to determine whether the aspartic acid analogues could inhibit the utilization of aspartic acid under suitable circumstances.

Materials and methods, compounds used. L-Aspartic acid was employed in all experiments. γ-L-glutamylhydrazide (GGH), β-DL-aspartylhydrazide (BAH), α-DL-methylglutamic acid (AMG), α-DL-methylaspartic acid (AMA), and α-DL-methylasparagine (AMAS) were kindly given to us by Dr. Karl Pfister of Merck and Co., Inc. The procedure of Hac and Snell(4) was employed for microbiological assay of L-aspartic acid with *L. mesenteroides* P-60, and growth was determined turbidimetrically and the results expressed in optical density units (O.D.). Solutions of all of the substances tested for inhibition were sterilized by Seitz filtration. The final volume of the assay medium was 1.5 ml per tube. The basal medium and the test samples were added to the assay tubes with the rapid automatic dispenser of Cannon.

Experimental results. In Fig. 1A is seen a typical standard curve obtained after 75 hours of incubation at 37°C, showing the graded response of the bacteria to increasing amounts of L-aspartic acid. An experiment was performed simultaneously in which incubations were made with 60 γ of L-aspartic acid per tube in the presence of 1-5 μM of the ana-

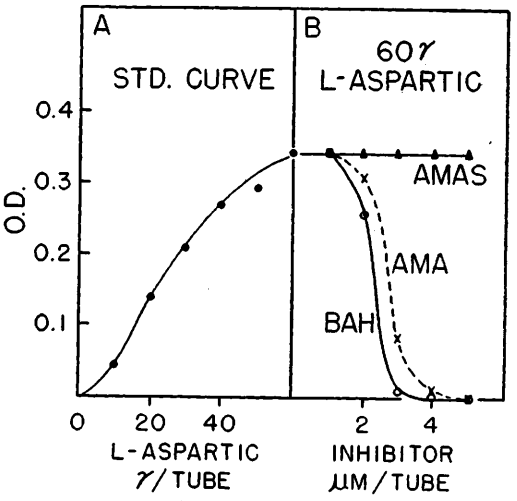

FIG. 1. Influence of varying amounts of aspartic acid analogues on growth on 60 γ of L-aspartic acid. BAH, β-DL-aspartylhydrazide; AMA, α-DL-methylaspartic acid; AMAS, α-DL-methylasparagine.