ENZYMES IN ONTOGENESIS (ORTHOPTERA)

XIV. THE ACTION OF PROTEINS ON CERTAIN ACTIVATORS OF PROTYROSINASE*

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INTRODUCTION

The enzyme tyrosinase can be obtained from the egg of the grasshopper, *Melanoplus differentialis*, in an inactive form, designated protyrosinase (Bodine, Allen, and Boell, 1937; Allen, Ray, and Bodine, 1938). Protyrosinase may be activated by a number of surface active compounds including a naturally occurring substance found in the lipoidal layer of a centrifuged egg brei (Bodine, Allen, and Boell, 1937; Bodine and Allen, 1938 *a* and 1938 *b*). It may also be converted to the active form (tyrosinase) by heat (Bodine and Allen, 1938 *b*), by acetone, chloroform, urea, and urethane (Bodine and Allen, 1938 *a*, 1938 *b*), and by dialysis against distilled water (Ray and Bodine, 1939). Furthermore, it has recently been shown that some constituents in the egg brei inhibit the activating power of certain of these activators (natural activator, sodium oleate, Duponol, Aerosol) presumably by forming films over the surface of the activator micelles (Bodine and Carlson, 1940). It is not unreasonable to assume that this inhibition of the activators is due to proteins which are normal constituents of the brei since proteins have been shown to form films on quartz or collodion particles placed in their solutions (Moyer and Moyer, 1940) as well as on oil droplets found in living forms (Danielli and Harvey, 1934; Danielli, 1935).

It is the purpose of the following to show that inhibition of the various activators may be accomplished by pure protein solutions and that the inhibition may be enhanced by higher temperatures. Since the effect of heat suggests a possible relation to a denaturation, exposures of these activator-protein complexes to ultraviolet light were also made previous to the application of heat.

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Material and Methods

The protyrosinase employed was similar to that previously described and designated as B₁ (Bodine *et al.*, 1939). The activators used were sodium oleate (Merck) and Aerosol (American Cyanamid). Protein solutions were made by placing 1 gm. of the protein in 99 cc. of distilled water. This was allowed to stand and then filtered. In the case of egg albumin the stock solution was 1 per cent; in the case of hemoglobin, the solution was diluted to 0.2 per cent. Casein and edestin are very sparingly soluble and hence their concentrations in final solutions were difficult to evaluate. The brei minus AC was prepared in the same manner as that already described (Bodine and Carlson, 1940).

Temperature exposures were accomplished by placing a test tube containing the protein and activator in a water bath kept at the required temperature for a period of 10 minutes after which the tube was removed and cooled to 25°C. or below as rapidly as possible. Concentrations of activators were chosen so as to fall on or below the critical or maximum point of activation for the amount of enzyme employed (see Bodine and Carlson, 1940).

The source of ultraviolet light was the entire spectrum of an air-cooled quartz mercury vapor lamp operating on 110–115 volts A.C. at 3 amperes. The solutions were placed at a distance of 15 cm. from the light source in glass dishes covered with quartz plates 2½ mm. thick (controls with glass). The dishes were placed on damp towelling or in a water bath of the desired temperature. The length of exposure in these experiments was 20 minutes.

The effects of these various substances and treatments were checked by testing the ability of the activator to convert protyrosinase to tyrosinase. The amount of active enzyme formed was determined in standard Warburg manometers at 25°C. using a known amount of tyramine-HCl as a substrate. The activity is expressed as $1/T \times 10^8$ where T is the time required to oxidize one-half of the substrate.

RESULTS

The addition of increasing amounts of protein to sodium oleate greatly decreases or inhibits the latter's activating potencies for protyrosinase with the exception of egg albumin which produces relatively slight inhibition at room temperature (Fig. 1). If, however, egg albumin is heated with sodium oleate at 80°C. for 10 minutes, a marked inhibiting action is then observed (Fig. 1). The relative effectiveness of the proteins used in producing this inhibition of the activating properties of sodium oleate is clearly indicated in Fig. 1. When B₁ (protyrosinase) is first mixed with the sodium oleate and the protein then added, no inhibition occurs except for a very slight effect in the case of casein. Upon the simultaneous addition of two proteins to the activator, previous to the B₁ addition, protyrosinase is always activated less than if the second protein were not present. Slight additive effects of the proteins seem evident but are rather difficult to evaluate.

As increasing amounts of protyrosinase are added to a constant amount of sodium oleate an increasing rate of oxidation of the substrate results (Bodine and Allen, 1938 *b*). The possible mechanism of this activation has been previously described (Bodine and Carlson, 1940) as being the adsorption or orientation of the proenzyme on oleate micelles. If one assumes that the proenzyme is protein in nature, and there seems good evidence for such an assumption, the relation between the activator and other proteins may be the same as that between protyrosinase and sodium oleate. To

FIG. 1. The effect of varying amounts of protein solutions on the activating power of a constant amount of sodium oleate. Abscissa, amounts of solution added; ordinate, per cent of rate given with sodium oleate alone. ○, 1 per cent albumin; ○·, 1 per cent albumin heated 80°C. for 10 minutes with the sodium oleate; ●, 0.2 per cent hemoglobin; □, casein; △, edestin; ▽, obverse curve of protyrosinase activation curve. The values shown on the abscissa are one-half actual value for the protyrosinase curve.

illustrate this an obverse curve of the activity of increasing amounts of proenzyme in relation to a constant amount of activator is shown in Fig. 1. This curve is the reverse of what would actually be shown if the activity of the mixture were placed on the ordinate and demonstrates that while certain proteins remove the potential activating power of sodium oleate, protyrosinase does essentially the same thing.

The effect of heat on activator-protein complexes was tested by taking a ratio of protein to activator which, as the result of experimental tests, showed moderate inhibition and heating this mixture and then rechecking its activating capacity. Hemoglobin and casein in such systems are unaffected by temperatures up to 80°C. (Fig. 2). The effect of different

temperatures (25–80°) on albumin and edestin is shown in Fig. 2. It will be noted that for each of these proteins there is little effect of temperature until a certain point is reached when there is a rapid increase in inhibition of the activator (compare with results for brei minus Ac—Fig. 4). This occurs between 55 and 70° for albumin and between 45 and 60° for edestin. It should be emphasized again that these effects are not obtained unless the activator and protein are heated together (see previous paper on heat and brei (Bodine and Carlson, 1940)).

FIG. 2. The effect of temperature on mixtures of proteins and sodium oleate. Abscissa, temperature in degrees C.; ordinate, the per cent rate in terms of the mixtures at 25°C. ●, albumin with sodium oleate; ○, edestin with sodium oleate. △, casein; □, hemoglobin. Concentrations of proteins constant for any protein throughout experiments and chosen so that rate changes could be easily followed.

Inasmuch as protyrosinase (B_1), a protein, can in itself be activated by temperature (Bodine and Allen, 1938 *b*) it becomes of some interest, in light of the above results, to determine the action of heat on it in the presence of sodium oleate. The addition of a small amount of sodium oleate to protyrosinase lowers the temperature at which heat activation becomes evident (Fig. 3). This change in the effect of temperature is in accord with the results obtained when other proteins are similarly treated with sodium oleate. It is known that the number of micelles increases with increase in temperature (Hartley, 1936). Yet the nature of the increase in degree of activation at different temperatures as well as the fact that varying amounts of sodium oleate show the same relationships seems to indicate

that the explanation of this phenomenon is not simply an increase in micelles. Moreover, mixtures of B₁ with an excess of sodium oleate that have been heated at 90° for 10 minutes show no activity when more B₁

FIG. 3. The effect of temperature with and without ultraviolet light on mixtures of protyrosinase and sodium oleate. Abscissa, temperature in degrees C.; ordinate, rate of the reaction. □, the effect of heat on protyrosinase alone; ●, the effect of heat on protyrosinase in combination with a small amount of sodium oleate; ○, the effect of heat following treatment with ultraviolet light on the same combinations. Top graph shows effect with less activation by oleate than in bottom graph.

is added, indicating that the oleate-enzyme complex maintains and is irreversible or even increased in strength after such exposure to temperature; *i.e.*, the oleate is "covered over" and no longer is capable of activating protyrosinase.

It has previously been noted that all of the agents activating protyrosinase

also act upon proteins, denaturing them (Bodine and Allen, 1938 *b*). The effect of temperature on brei proteins as well as edestin and albumin in the presence of sodium oleate suggests a possible relation to denaturation. Since ultraviolet light is known to lower the temperature of protein denaturation (Stedman and Mendel, 1926; Clark, 1935; Bovie, 1913), these

FIG. 4

FIG. 5

FIG. 4. The effect of ultraviolet light and heat on brei minus AC with sodium oleate. Abscissa, temperatures to which the mixtures were exposed; ordinate, per cent of the rate at 25°C. ●, the effect of heat alone; ○, the effect of treatment with ultraviolet light preceding exposure to heat; □, the effect of heat treatment preceding ultraviolet exposure; Δ, the effect of simultaneous exposure to ultraviolet light and heat; ⊖, the calculated summation effect of ultraviolet light and heat.

FIG. 5. The difference in the effects of ultraviolet light when used before or subsequent to heat or brei minus AC with sodium oleate. Abscissa, temperature in degrees C.; ordinate, the values for solutions treated with both ultraviolet light and heat in per cent of those treated with heat alone. ○, mixtures treated with heat and then ultraviolet light; ●, mixtures treated with ultraviolet light and then heat.

activator-protein complexes were subjected to ultraviolet light previous to, simultaneous with, and subsequent to heating. Figs. 4 and 5 show that exposure to ultraviolet alters the temperature effect on a mixture of protein and activator. It is evident in Fig. 4 that if ultraviolet light is applied before or simultaneous with heat, the potency decrease due to heat is greater. If, however, the ultraviolet light is used subsequent to heating, the effect is the same as that obtained at 25°C. The data in Fig. 4 are replotted in Fig. 5 to more clearly demonstrate this point. Combinations

of albumin or edestin with sodium oleate which are previously treated with ultraviolet are affected similarly.

When a mixture of protyrosinase and sodium oleate is irradiated with ultraviolet before subjection to temperature, the activity of the enzyme is reduced and the temperature of inactivation (complete denaturation) is lowered (Fig. 6). This effect of ultraviolet light on the proenzyme is analogous to the effect noted with albumin and edestin.

Many of the above reactions have been tested using Aerosol as the activator and some using olive oil and the results have been qualitatively

FIG. 6. The change in the heat effect on albumin or edestin with sodium oleate caused by treatment with ultraviolet. Abscissa, temperature, degrees C.; ordinate, the values for solutions treated with both ultraviolet light and heat in per cent of those treated with heat alone. ●, albumin; O, edestin.

similar. Thus, the above mentioned conclusions seem to hold irrespective of the particular lipoidal activator.

DISCUSSION

A consideration of the above results suggests a parallelism between the mechanism of activation of protyrosinase and that for the inhibition of the activator produced by the addition of proteins, such as the brei proteins, albumin, hemoglobin, edestin, and casein. Since sodium oleate has been most extensively employed as an activator the present discussion will be based almost solely upon results obtained through its use although comparable results are produced by all activators.

The possible modes of action or the relationships between activator and protein may be looked upon in one of two ways. First, the oleate (activator) may be adsorbed on the protein. Secondly, the protein may be adsorbed on the activator micelle. (Hartley, 1936, has described these micelles for paraffin chain salts. Each micelle consists of a hydrophobic core and a hydrophilic shell.) The evidence at hand from the present work seems to more or less favor the second of the above possibilities.

Du Noüy (1926) in studying the changes in surface tension of serum upon the addition of sodium oleate concludes that the oleate is on the surface of the protein. Anson (1939) in his study of protein denaturation by detergents suggests that the denatured protein is kept in solution by detergent molecules adsorbed on their surfaces. However, he makes no mention of the mode of denaturation.

It is difficult to explain a number of the results obtained in the present experiments on the assumption that the protein adsorbs the sodium oleate molecules. Results of rather crude cataphoresis experiments show that an activator hemoglobin or activator enzyme complex moved at the same rate as the protein alone and not at a rate similar to that of oleate alone. Data from cataphoresis (Moyer, 1940; Moyer and Moyer, 1940) and surface tension (Danielli and Harvey, 1934; Danielli, 1935) methods give evidence for the adsorption of proteins on fat droplets, collodion, or quartz particles in solutions, and on fat globules in animal cells. Many of the reactions studied and especially those with heat give different results if the protein and oleate are treated together or separately. Also, if activation of the enzyme is a phenomenon similar to protein adsorption, it is difficult to conceive of it as being activated by adsorbing oleate. It seems more logical, with the evidence at hand, that it be adsorbed on the oleate micelles and thus changed in structure or configuration.

The mechanism by which the protein is adsorbed on these activator micelles is at the present not well understood. Because of the diverse types of activators and proteins it is difficult to explain adequately the relationships obtained by invoking the phenomenon of coacervation as developed by Bungenberg de Jong (1936). Micelles of oleate possess a net negative charge as well as a hydrophilic shell. At the pH (6.8) at which these studies were made hemoglobin and edestin are on the positive side of their isoelectric points. They are strongly adsorbed (Fig. 1). Albumin, negatively charged at this pH does not inhibit to a great degree until it is heated. Casein, however, with an isoelectric point similar to albumin behaves much like hemoglobin. While heat affects both edestin and albumin when they

are in contact with the activator, it has no effect on hemoglobin or casein under similar conditions. It might well be that hemoglobin and casein are completely denatured on the micelles while edestin and albumin are not. Heat then continues the denaturation process making the adsorption more complete. This seems also to be the case with mixtures of sodium oleate and the proenzyme. The changes in activity occur at temperatures below the point of heat denaturation of the protein alone. This supposition is given further support by the action of ultraviolet light on these protein-activator complexes. Exposures to ultraviolet irradiation lower the temperatures at which proteins denature (Clark, 1935; Stedman and Mendel, 1926; Bovie, 1913, etc.). Bovie (1913), repeating Chick and Martin's work on denaturation of albumin with irradiated protein, obtained a similar type of curve except that it occurred 10 to 15°C. lower than the untreated albumin. In all cases where heat affects the protein activator complexes in these experiments, ultraviolet caused a lowering of the temperature at which it occurred (Figs. 3, 5, and 6).

Activation of protyrosinase, which may be accomplished by a number of factors, seems related to a change in configuration or orientation of the protein. Northrop (1939) has shown that comparatively slight changes in an inactive protein may result in the formation of an active enzyme. In the case of the trypsinogen to trypsin conversion, the change is in the splitting of a peptide bond (Northrop, 1939).

When the activation of protyrosinase is brought about by surface active compounds, it seems to be accomplished by an adsorption on the surface of micelles of these compounds. Other proteins are adsorbed also but the adsorption of protyrosinase yields a unique configuration possessing the enzymic properties measured experimentally. This change from inactive to active enzyme may well be a mild denaturation exposing certain groups *via* opening of bonds. Other chemicals and treatments known to cause similar changes in proteins are capable of changing protyrosinase to tyrosinase.

SUMMARY

1. Proteins, when added to activators (sodium oleate, Aerosol) of protyrosinase, greatly decrease the degree of activation.
2. Certain proteins adsorbed on activator micelles are markedly affected by temperature and are rendered more sensitive by ultraviolet light.
3. Ideas are expressed as to the possible nature of activating and inhibiting phenomena especially as they relate to the enzyme tyrosinase.

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