A STUDY OF SULFHYDRYL GROUPS IN CELL DEVELOPMENT

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ABSTRACT.—The distribution of –SH groups in the cellular components of the cleidoic grasshopper eggs and the increment of these chemical groups during embryogenesis has been determined using amperometric methods. The greatest concentration of the –SH groups was found in the microsome portion of the egg; this is in accord with other work which indicates that these subcellular particles are sites of biorganic synthesis. A noted increase of –SH groups was observed when various reagents such as sodium borohydride, guanidine hydrochloride and Dupinol P.C. were used to break disulfide and hydrogen bonding.

Although the existence of the sulfhydryl group, sometimes called the mercaptan or thiol group, has been known for considerable time, only recently has the universal importance of the group in compounds come to be recognized. Since the discovery of this active group, its universal distribution in animal tissue has been clearly established. It is known that a large number of enzymes necessary for protein synthesis and metabolism depend on the presence of this –SH group for activity as proved by Barron and Seki (1952). The sulfhydryl group can be reacted by oxidation to form the disulfide group (–S–S–), or possibly by hydrogen banding as postulated by Mazia (1956).

In recent years extensive research has been done to discover the role of the –SH group in cell production, enzyme activity, clotting of blood, protein denaturation and many other physiological activities. It has been known for some time that the –SH group is present in compounds known as the papinases or sulfhydryl enzymes. These enzymes are activated by certain reducing agents and inactivated by aeration or other mild oxidation treatment. Whether these activating agents remove metal inhibitors or simply reduce the –S–S– to –SH is apparently not settled. It is customary to speak of free sulfhydryl groups and bound groups. Whether these bound groups are hydrogen bonded or disulfide forms is not settled and there is considerable evidence that possibly both exist at the same time.

The purpose of this investigation has been a quantitative distribution study of the sulfhydryl groups in cellular components using the cleidoic eggs of the grasshopper, *Melanoplus differentialis* as living material. Bodine (1929), (1932), Bodine et al. (1940), and Norman (1954), have shown that the sulfhydryl content increases after the egg is laid until it reaches the diapause stage.

An attempt has been made to study the increase in the sulfhydryl content by treatment of the so-called bound groups of the egg with the reagent sodium borohydride to reduce the –S–S– linkage. Other reagents, such as guanidine hydrochloride, Dupinol P.C. (a detergent) and urea have been studied. These are not strong enough to break the disulfide linkage but apparently exer-

cise a physical swelling effect to free the sulfhydryl groups bound by hydrogen bonding according to the postulations of Mazia (1956) and Boell (1935) working independently.

Materials and Methods

Grasshopper embryos of known age and temperature history were used. Samples containing 100 embryos per determination were homogenized in the cold for ten minutes, then transferred to an ordinary 250 ml. beaker for titration. A titration medium consisting of ethanol, ammonium hydroxide, ammonium nitrate and Versene was used. The resulting pH of this medium was determined with a Beckman pH meter to be 8.54. Embryos were titrated with silver nitrate, 5×10^{-4} M.

Samples of embryos and yolk were placed in a refrigerated centrifuge and centrifuged at speeds of 62,500 rpm to separate the nuclei, 200,000 rpm to separate mitochondria or large particles, and 370,000 rpm to separate the microsomes or small granules. Samples to give the quantity equivalent of 100 embryos and yolk were taken for determination and titration with silver nitrate. Another sample of 100 embryos and yolks were treated for 30 minutes with 15 mg of sodium borohydride and similar titrations were run after centrifugation. Other samples of like size were treated with 80% guanidine hydrochloride, with saturated urea solution, and saturated Dupinol P.C. for 30 minutes, then titrated after centrifugation.

Amperometric Titration Method.— In this particular study the amperometric method for determination of -SH groups, first described by Kolthoff and Harris (1946) and later modified by Benesch and Benesch (1950) for living material was used. This method is sensitive, highly specific, and not interfered with by any of the common amino acids, purines, pyrimidines, eczymase or ascorbic acid. A rotating platinum electrode, perfected by Wald and Brown (1952) for this type of apparatus was used. This method of titration is simple, as no E.M.F. need be applied to the cell which consists of an indicating and reference electrode. The current which flows through the cell during titration is read on a microammeter.

The rotating platinum electrode used was about 6 mm long and sealed in a 6 mm diameter soft glass tube. This electrode was rotated at 600 rpm by a synchronous motor. Electrical contact was made with the platinum electrode by placing mercury in the tube over it and dipping a wire into the mercury. A reference electrode was used which had a potential of -0.23 volts against the saturated calomel electrode. Electrical connection between the reference and rotating electrode was made by means of a salt bridge.

Silver Nitrate Method. — In titrating a mercaptan with silver nitrate, using a saturated calomel electrode as the reference electrode, the current was zero until the end point was reached. After this there was an excess of silver ions in solution. The diffusion current of the silver ion was measured with a microammeter, the diffusion current being proportional to the concentration of the silver ions in solution. When the current readings during titrations were plotted against vol-

ume of reagent added, two lines were obtained, which intersected at the end point. A 5 x 10^{-4} solution of silver nitrate prepared from a stock solution was used as the titrating agent. The titrating agent was delivered by means of a microburette. A commercial piece of apparatus, a Sargent "Ampot", using either the rotating platinum electrode or dropping mercury electrode, was used.

One tenth ml of 0.01 N ethylene diamine tetraacetic acid, (Versene) was added to the titrating mixture. This acted as a powerful chelating agent and prevented the metal-catalyzed oxidation of soluble non-protein mercaptans in an alkaline titration medium as described by Benesch and Benesch (1950).

A sample to be determined was diluted to 40 ml with 22% aqueous ethanol. This solution was made up to 0.25 M in ammonium hydroxide and 0.05 M ammonium nitrate. The sample is titrated with the 5 x 10^{-4} M silver nitrate solution at the rotating electrode, and the -SH groups were removed by combination with the silver ions. The further addition of silver ions caused a flow of current which was proportional to the concentration of silver ions in solutions. Two or three readings of the microammeter were made before the end-point was reached. As long as the silver nitrate was not in excess, the current was zero. After the end-point was reached, the deflection of the ammeter corresponded to the diffusion currents of the excess silver ions. When the ammeter indicated that the end-point was passed, two or three small increments of silver nitrate were added and the current was read after each addition.

Results and Discussion

The results obtained by titration of untreated embryos showed that 52.08% of the sulfhydryl groups were in the microsome fraction, 27.07% in the mitochondrial fraction, 16.70% in the cell nuclei, and 4.14% in the clear cytoplasm. The results that were obtained by treating the embryos with various reagents, followed by centrifugation and titration, demonstrated an increase in amount of titratable sulfhydryl in every instance. The greatest percentage of increase was with sodium borohydride which gave an increase of 37.50% and represented breaking of disulfide bonding. The results that were obtained by using reagents for breaking of hydrogen bonding demonstrated that guanidine hydrochloride with a 25.00% increase was the most effective and Dupinol P.C. with a 12.50% increase and saturated Urea with 7.25% increase in amount of titratable sulfhydryl were less effective.

The developing embryo in the grasshopper egg showed high cellular activity. This indicates that an increase of sulfhydryl groups is associated with periods of active growth. The abrupt rise of titer of -SH groups during development, occurs at the same time as morphogenetic growth and differentiation. This increase in sulfhydryl content parallels metabolic, mitotic and morphogenetic activity. The fact that the greater portion of the free sulfhydryl groups is located in the

microsome fraction supports the contention of Hammet (1929), Rapkine (1931), and Barron and Seki (1952) that the microsomes are concerned with protein, fat, and to some extent carbohydrate synthesis in the cell.

The increase in amount of titratable sulfhydryl after treatment with a strong reagent like sodium borohydride and the relatively weak or mechanical reagents such as guanidine hydrochloride, saturated urea, and Dupinol P.C. would indicate that in addition to occurring as free groups, the titratable sulfhydryls also occur as bound groups in at least two different types of linkages, the disulfide and hydrogen bonding.

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