FURTHER STUDIES ON EOSIN HEMOLYSIS.

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It is a well known fact that if a dilute solution of a photosensitive substance such as eosin is added to a suspension of washed red blood cells and the mixture is exposed to sunlight, hemolysis of the red cells promptly takes place, while no action is observed when the mixture is kept in the dark. Busck (1) and later Sellards (2) found that the addition of certain substances such as blood serum and egg white to solutions of photobiologic sensitizers results in decreasing or completely inhibiting the toxic action, but no protection is afforded to cells by the addition of glucose, starch, or gelatin. Recognizing that there are fundamental differences in the chemical make-up of those substances which afford protection and those which do not, Schmidt and Norman (3) carried out experiments to determine the relation between the amino-acid content of the protein molecule and protective action. They found that eosin hemolysis can be prevented by the addition of tyrosine, tryptophane, and proteins which contain these amino-acids. Certain other organic compounds which contain the hydroxyphenyl ring also afford protection. They pointed out that the inability of gelatin to protect red blood cells against eosin hemolysis is due to the absence of the above essential amino-acids. As a tentative explanation of this phenomenon, they consider that the protection afforded by certain substances against the photodynamic effect of eosin may possibly be due to the absorption of the active rays by the protective substance.

Since the publication of these experiments, we noted a striking similarity between the substances which protect red blood cells against hemolysis by eosin, and the substances which were found by Gortner and Holm (4) to react with the Folin and Denis (5) phosphotungstic-phosphomolybdic reagent to give a characteristic blue color. These

substances were found by Gortner and Holm to include tyrosine, tryptophane, uric acid, α-methyl indole, and ferrous iron. Abderhalden (6) states that the list also includes oxyproline and oxytryptophane. The property which is possessed in common by all of these substances is that they are easily oxidizable.

Experimental work was accordingly undertaken to determine whether inorganic reducing substances in addition to tyrosine, tryptophane, and proteins which contain these amino-acids in the molecule can afford protection to red cells against eosin hemolysis, and our results appear to answer this question in the affirmative. As in the previous work, 0.5 cc. of a 5 per cent saline suspension of red blood cells (ox or sheep) was placed in each of a number of small test-tubes, and to each, 1 cc. of a 1:10,000 dilution of eosin (Grübler's) in salt solution was added. The substances to be tested for protective action were likewise made up in normal saline solution in the concentrations, as given in Table I, and the reaction was adjusted to approximate neutrality. The tubes were placed in direct sunlight for 30 minutes and after exposure they were immediately placed in the ice chest. The tubes were inspected at the end of several hours to determine the amount of lysis which had taken place. Control tubes which were kept in the dark eliminated factors other than that of photodynamic action. The experimental results are given in Table I. They indicate that inorganic reducing substances afford marked protective action to red blood cells against eosin hemolysis. The list of inorganic reducing substances which may be used in experiments of this type is limited, since many of the best reducing agents such as ferrous chloride, ferrous sulfate, and ferrous ammonium sulfate, yield solutions of high acidity, and when these are added to red cells the latter are agglutinated. Oxyproline and oxytryptophane were not available for experimental work. It is doubtful whether the former substance can protect red cells against the toxic action of eosin, since gelatin, which contains 14 per cent (7) of this substance, lacks protective ability. Marked protection was shown by each of the two preparations of histidine. Both gave a trace of blue color when tested by the Folin and Denis reagent, indicating the possible presence of tyrosine. Valine, serine, proline, creatinine, and cinnamic acid afford no protection, while marked protection is afforded by skatole and tryptophane.

Since the action of the protective substance appears to be that of a reducing agent, it seemingly follows that eosin hemolysis is a phenomenon involving oxidation. Hemolysis may be wholly prevented by placing the eosin-red cell mixture in a highly evacuated glass tube or by saturating the cells with illuminating gas or hydrogen. These observations are in agreement with the statement of Sellards, that an atmosphere of hydrogen is as effective as total darkness in preventing

TABLE I.

The Effect of the Addition of Certain Substances on the Hemolysis of Red Cells by Eosin.

Substance added.	Concentration.*	Result.
Sodium chloride (control)		Complete hemolysis.
Valine	M/10	" "
Serine	M/10	" "
Proline	M/10	" "
Cinnamic acid	M/10	" "
Creatinine	M/30	" "
Tryptophane	M/30	No hemolysis.
Skatole	Saturated solution.	" "
Sodium sulfite	M/30, M/90	" "
Sodium thiosulfate	M/30	" "
Ferrous lactate	M/30	" "
Potassium ferrocyanide	M/30	" " Some hemolysis over night.
Histidine 1	M/20	No hemolysis.
Histidine 2	M/20	" "

* Sufficient NaCl was added to each of these solutions to make them isotonic.

the toxic action of eosin in sunlight. It must be admitted that in certain instances the reducing agent may react with the fluorescent substance and in this way partially inhibit its toxic action. Thus, when sodium sulfite is added to a solution of eosin and the mixture is exposed to sunlight, the latter substance is rapidly reduced to fluorescein.

The action of sunlight in accelerating oxidative reactions is a well known phenomenon. Bilirubin when exposed to sunlight is oxidized to biliverdin (8), many of the vegetable oils are oxidized by light, and the bleaching of the triphenylmethane dyes (9) is a phenomenon in

which the dye itself appears to catalyze the oxidation which is accelerated by the sun's rays. We have noted that the bleaching of eosin solutions when exposed to sunlight may be markedly inhibited by the addition of tryptophane, while alanine, glycocoll, and phenylalanine afford little or no protection.

Three possible reactions may conceivably take place in the oxidation of the protective protein or amino-acid. The first is oxidative deamination which yields an α-ketonic acid. For tyrosine the reaction is represented by the equation:

$$C_6H_4OH \cdot CH_2 \cdot CHNH_2 \cdot COOH + O = C_6H_4OH \cdot CH_2 \cdot CO \cdot COOH + NH_3$$

This is a universal reaction and represents a step in the normal catabolism of the amino-acids. The protective action against eosin hemolysis afforded by tyrosine and tryptophane cannot be due to this reaction, since it is not at all specific for these two amino-acids. Moreover, ammonia, which itself possesses hemolytic properties, is set free in the reaction. It is possible that the latter substance may be a factor which is concerned in eosin hemolysis. Experimental work, however, does not appear to support this hypothesis. A series of test-tubes containing 100 mg. each of glycocoll, alanine, and tryptophane, dissolved in 5 cc. of normal salt solution and 1 cc. of 1:10,000 eosin was exposed to sunlight for 1 hour. At the end of this time the eosin was decolorized in the tubes containing glycocoll and alanine. The ammonia was taken up with permutit and subsequently set free by the addition of NaOH and the solutions were Nesslerized. A trace of ammonia was found in each instance while none was evident in the control tubes which had been kept in the dark. After 4 hours exposure, the tryptophane solution gave an ammonia content which corresponds to approximately 0.1 cc. of a 0.03 normal solution, about half of the amount necessary to hemolyze completely the dosage of red cells. A series of test-tubes, each containing 1 cc. of a 0.1 normal solution respectively of glycocoll, alanine, phenylalanine and tryptophane, and 1 cc. of 1:10,000 eosin solution was exposed to sunlight for 1 hour, and after exposure 0.5 cc. of a 5 per cent saline suspension of washed sheep cells was added to each tube, the mixtures were shaken and placed in the ice chest. Only a trace of hemolysis was shown by the tubes after standing over night. In this connection it might be

mentioned that the ammonia, which is formed as a result of the oxidation of the amino-acid, is probably not free but is combined with the ketonic acid.

Neuberg (10) studied the mechanism of the reaction which takes place when solutions of certain amino-acids to which uranium salts have been added are exposed to sunlight. In addition to oxidative deamination, he found that CO_2 was split off from the carboxyl group yielding an aldehyde, the reaction being similar to that which takes place when an amino-acid is oxidized with H_2O_2. When serine is exposed to sunlight or is oxidized with H_2O_2, glycol aldehyde is formed according to the reaction:

$$\begin{array}{l} \text{COOH} \\ | \\ \text{CHNH}_2 \\ | \\ \text{CH}_2\text{OH} \end{array} + \text{O} = \begin{array}{l} \text{CHO} \\ | \\ \text{CH}_2\text{OH} \end{array} + \text{CO}_2 + \text{NH}_3$$

This reaction like the one discussed previously is universal, and fails to explain the specific reducing action of tyrosine and tryptophane.

It is a well recognized fact that benzene and a large number of aromatic substances of varied types undergo substitution of the hydrogen atoms in the nucleus to a more or less marked extent, and *in vitro* this reaction can be brought about by ozone, H_2O_2, and by photochemical action (11). We have attempted to test the possible application of this reaction to the subject of eosin hemolysis. Suspensions of washed red cells were treated respectively with H_2O_2 (saline solution of the neutralized product), H_2O_2 plus a small amount of catalase, and H_2O_2 plus platinum black. In each instance the oxyhemoglobin was converted into methemoglobin, but hemolysis did not take place. On saturating red cells with ozone a similar result was obtained. Tubes containing red cells to which platinum black and colloidal palladium were added, showed no hemolysis after exposure for a half hour to sunlight. Evidently hemolysis cannot be brought about with the aid of H_2O_2. The fact that substances which contain either the hydroxyphenyl ring which apparently facilitates the introduction into the nucleus of other OH groups, or the indole ring which is easily oxidized to indoxyl and then to indigo blue (Abderhalden (12) has noted that tryptophane and adrenalin are sensitive to light), can afford protection

to red blood cells against the toxic action of eosin, leads us to believe that we are dealing with a special type of oxidation which is markedly accelerated by fluorescent substances. The highly specific action of tyrosine and tryptophane as reducing agents likewise indicates that these amino-acids which are contained in the protein molecule are attacked and undergo oxidation as a result of the photodynamic action of eosin. It is not possible at this time to state how far this oxidation proceeds or the mechansim whereby lysis takes place. It appears logical to assume that the oxidation concerns itself with the proteins of the stroma and this results in the necrosis of the cell (13).

Bovie (14) has demonstrated that coagulation of proteins can be brought about by exposure to ultra-violet light. This reaction is presumably one of denaturation (15) and does not involve oxidation. To eliminate the possibility of denaturation being a factor in eosin hemolysis, the following experiment was carried out: a test-tube containing 2 cc. of horse serum and 0.1 cc. of 1:1,000 eosin was exposed to sunlight for a period of 4 hours and subsequently incubated at 37°C. There was no visible coagulation.

SUMMARY.

Additional experimental work on the subject of eosin hemolysis has been carried out. This indicates that red cells may be protected against the toxic action of eosin in sunlight by the presence of inorganic reducing agents. It is pointed out that a marked parallelism exists between the substances which react with the Folin and Denis reagent and the compounds which afford protection to red cells against the photodynamic action of eosin. The property which is possessed in common by all of the substances is that they are easily oxidized, and their ability to protect red cells lies in their power of reduction. The toxic action of eosin probably involves the oxidation of tyrosine and tryptophane which are contained in the protein molecules of the stroma.

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