HEMATOPORPHYRIN, AN ARTIFICIAL PROTEOLYTIC ENZYME.

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Introduction.

In studying the problem of enzyme action one is brought to the realization that very little is known with respect to the nature of this action. One enzyme molecule changes a very large number of molecules of the substrate without the apparent utilization of much, if any energy, whereas without the enzyme much energy is required to produce the same result. When a protein undergoes an enzymatic digestion very little energy is absorbed or given up to the surrounding medium during the reaction, whereas to hydrolyze the same protein prolonged heating at a high temperature is necessary. How are these substances able to accomplish so much without the apparent utilization of much energy? What is the source of their energy supply? An attempt to answer these questions is the objective of this thesis.

It was suggested to the writer by Professor A.P. Mathews that the digestive or hydrolytic action of hematoporphyrin on the blood protein, fibrinogen, in the presence of light was an enzymatic reaction and if this were true, then the study of the mechanism of this reaction might throw light on the nature of enzymatic action in general.

In this case the enzyme might receive its supply of energy from the light radiations, pass this energy on to the protein, being itself inactivated thereby, but at once reactivated by the absorption of light.

The modern photochemical theory accepts the Stark-Einstein equivalence law, $E = h\nu$, for the explanation of all primary photochemical processes. This law states that a quantum of absorbed energy, E, is equal to $h\nu$, where h is Planck's constant and ν is the frequency of the absorbed radiation. Upon the absorption of energy the molecule becomes activated, either by raising the valence electron to a higher quantum orbit or by increasing the kinetic energy of the molecule. The activated molecule may impart its energy either by collision, or it may in some other manner, i.e., by radiation or temporary union, transfer its additional energy to another molecule which thereby becomes unstable and chemically more reactive.

Thus it is possible for a molecule of the enzyme, hematoporphyrin, to become activated by light energy, and thereby be in a position to act chemically on the protein substrate, rupturing its weakest bonds with the formation of proteolytic products.

The action of hematoporphyrin on several protein substrates was thoroughly investigated to discover if an

actual hydrolysis of the proteins took place and to elucidate the mechanism involved. The results obtained bore out the theory that hematoporphyrin acts as a hydrolytic, proteolytic enzyme in the presence of light energy, but only if oxygen be present.

Historical

The phenomenon of the photodynamic action of dyes on living organisms was observed by O. Raab in 1900. While studying the pharmacological action of acridine on paramecia, he found that the organisms died more rapidly in the presence of the dye in the light than in the dark. His discovery has led to a great number of investigations on the action of dyes and light on animal organisms. In a recent review on photodynamic action by Blum (1932) it is stated that " the photodynamic phenomena may be ultimately described as oxidations by molecular oxygen". He has accumulated considerable evidence to show that the presence of molecular oxygen is often required for photodynamic action. The destruction of bacteria by rose bengal, methylene blue, and phenosafranine, does not occur in the absence of oxygen, according to Jodlbauer and Tappeiner (1905). Hemolysis by eosine, methylene blue, and other dyes which occurs in light, under ordinary conditions, does not take place in a vacuum or in the presence of hydrogen or pure carbon monoxide, as was demonstrated by Hasselbalch (1904), Schmidt and Norman (1922), and Blum and McBride (1932). Other investigators have shown that the presence of oxygen is necessary in the killing

and cytolysis of paramecia and of other protozoa, spirilla, and diatoms, by photosensitizers.

This photodynamic action may be inhibited by a number of substances. Sacharoff and Sachs (1905)have found that the reducing substance, sodium sulfite, will prevent the hemolysis of red blood corpuscles by light and sensitizer. Busck (1906) was able to show that red blood cells could be protected from photodynamic action by the presence of serum. Noack(1920) has shown that sodium sulfite will inhibit other photodynamic actions. Schmidt and Norman (1922) found that the presence of tyrosine and tryptophane protect red blood cells from hemolysis by eosine and sunlight. Rask and Howell's (1928) experiments on the perfused terrapin's heart, found that serum albumin had a protective action against the damaging effect of light and hematoporphyrin, while euglobulin, pseudoglobulin, eggalbumin, and peptone afforded no pretection. Awoki (1925) showed that white mice could be protected against the fatal effect of light and hemataporphyrin by injections of serum, while Lippay (1930) found that the stimulation of frog's striated muscles by light and sensitizers was inhibated by horse serum.

In regard to the action of photodynamic substances on inanimate matter it has been shown by Jodlbauer and Tappeiner (1905) that certain enzymes and toxins were destroyed

by dyes in the presence of light and oxygen.

Hasselbalch (1909) showed that hemoglobin could be oxidized to methemoglobin by fluorescein dyes, but only in the presence of oxygen.

Noack (1920) pointed out that certain plant pigments could be oxidized by light and photodynamic substances.

In regard to the action of photodynamic substances on inanimate matter, it has been shown by Howell (1921) that the blood protein, fibrinogen, is changed when exposed to light in the presence of hematoporphyrin and eosine. The protein no longer clots upon the addition of thrombin nor coagulates when heated to 56°C.

Hill and Holden (1926) found that small amounts of hematoporphyrin rapidly denatured globin in the presence of sunlight.

Harris (1926) has shown that proteins such as egg albumin, hemoglobin, serum albumin, casein and ammino acids, as tyrosine and tryptophane, undergo a photo-oxidation when exposed to ultra violet light. Upon addition of minute traces of hematoporphyrin and certain fluorescent dyes the photo-oxidations are greatly accelerated. The photo-oxidation was shown by the uptake of oxygen in a Barcroft apparatus.

Gaffron (1926) repeated Harris' work and showed that oxygen was taken up and carbon dioxide and ammonia liberated, when serum and ammino acids were exposed to light in the presence of hematoporphyrin and dyes. It is interesting to note that when Gaffron used the non-fluorescent copper and silver salts of hematoporphyrin they were inactive as sensitizers for egg albumin, but the fluorescent zinc salt was active.

Carter (1928) has made a study on the photo-oxidations of various organic compounds by a number of substances, hematoporphyrin, uroporphyrin, eosine, and methylene blue. He claims that no exception has been discovered to the generalization that the aliphatic compounds are inactive to photo-oxidation in the presence of a photodynamic substance. A number of compounds were tried including glucose, hexose-diphosphate, sodium oleate, aspartic acid and ethylamine, but they showed no oxygen up-take in the presence of dyes and light energy. However, when he studied benzene derivatives it was found that an introduction of an hydroxyl or amino group into the ring enabled photo-oxidation to take place. All other groups studied such as the carboxyl, nitro, nitrile, halogens, and sulfuric acid groups were without effect.

The purine groups, hypo-xanthine, xanthine, uric acid and guanine showed photo-oxidation.

In summing up the work of these investigators, two outstanding facts need to be considered, namely, (1) that fluorescence seems to be the essential physical property of dyes that show photodynamic action; (2) that oxygen plays a part in the photodynamic process.

In studying the digestive action of hemato-porphyrin and other substances on various protein substrates in the presence of light, the writer has taken into consideration these two important facts.

Preparation of the Artificial Proteolytic Enzyme.

Hemin Preparation;

a - Raw alpha-hemin.

Alpha-hemin was prepared according to the method of Schalfejeff Modification of Nencki and Zaleski. Three liters of glacial acetic acid were placed in a four liter flask and enough sodium chloride added to saturate the acid. The contents of the flask were heated to 95°C. and 500 c.c. of freshly defibrinated ox blood were added slowly from a separatory funnel, being careful not to let any blood adhere to the sides of the flask. The contents of the flask were given a slow whirling motion while adding the blood. The temperature of the mixture was brought again to 95°C. and 500 c.c. more blood were added. The temperature was kept at 95°C. until crystals of hemin appeared. The flask was allowed to stand over night to enable the hemin crystals to settle to the bottom. The supernatent liquid was syphoned off, the hemin crystals collected on a suction filter, washed with 1% hydrochloric acid, and placed in a calcium chloride dessicator until dry.

b - Pure hemin.

Pure hemin was prepared according to the method of Schalfejeff modified by Willstatter. Six gram portions of

raw hemin were dissolved in 18 c.c. of pyridine and 30 c.c. of chloroform. The solution was filtered and the residue was washed with a mixture of 1 c.c. of pyridine and 15 c.c. of chloroform. The united filtrates were poured into a solution of acetic acid, previously heated to 110°C. containing 6 c.c. of a saturated sodium chloride solution and 5 c.c. of a 25% hydrochloric acid solution. The mixture was allowed to stand over night before filtering off the crystals of hemin. The crystals were washed with a 1% hydrochloric acid solution, followed by a 50% solution of alcohol, and finally dried over calcium chloride.

c - The conversion of pure hemin into hematoporphyrin.

The pure crystalline hemin was converted into hematoporphyrin according to the procedure of Willstatter and Fischer (1913). Twenty grams of crystalline hemin were placed in a flask containing 500 grams of glacial acetic acid, previously saturated with hydrobromic acid at zero degrees until it had attained a specific gravity of 1.41 at that temperature. The mixture was shaken for 15 minutes and then allowed to stand for 12 hours at room temperature until the hemin was completely dissolved. The solution was then poured into 2.8 liters of water and filtered through a thin layer of talc.

The iron was removed from the solution of hematoporphyrin by the method of Hill (1924). After allowing the above filtrate to stand for three hours, 600 c.c. of saturated solution of sodium acetate containing 10% sodium potassium tartrate were added. The hematoporphyrin is precipitated in a flocculent form. The solution was filtered, the iron appearing in the filtrate as a soluble salt. The precipitate was washed with 500 c.c. of distilled water. The washed precipitate was re-dissolved in N/20 hydrochloric acid and reprecipitated with a minimum amount of Rochelle salts. This method of separating the iron was found by Hill to be preferable to the precipitation of iron and hematoporphyrin together with sodium acetate and the separation of porphyrin from the ferric hydroxide by dissolving in sodium hydroxide.

To prepare the hydrochloride salt from the crude hematoporphyrin the method of Nencki and Zalaski, as described by Howell, was used. The crude hematoporphyrin was re-dissolved in the smallest quantity of 2% NaOH on the steam bath, an equal volume of water was added and the liquid filtered while hot. The hematoporphyrin was reprecipitated by adding a minimum quantity of 33% acetic acid and filtered, the precipitate being washed free from the acetic acid with

distilled water. The precipitate was dissolved in 1% hydrochloric acid over a steam bath and filtered again. The filtrate was placed in a crystallizing dish in a dessicator under a high vacuum and allowed to crystallize. The mother liquid was poured-off and the crystals were again redissolved in 1% hydrochloric acid over a steam bath, filtered, and recrystallized in vacuo once more. This second lot of crystals were air dried in the dark after separation from the mother liquid.

In preparing a solution of 0.35% hematoporphyrin for experimental work, 175 milligrams of the crystalline hydrochloride salt were dissolved by addition of 5 c.c. of a 1% NaOH solution. 1% sulfuric acid was added until the porphyrin precipitated out of solution, then just enoughof the alkali was added to redissolve the precipitate. The solution was poured into a 50 c.c. volumetric flask and made up to volume with distilled water. This solution of hematoporphyrin was found to be very slightly acid to litmus.

Preparation of Fibrinogen

The fibrinogen solution was prepared according to the method of J.Mc Lean (1920) with a slight modification.

Citrated beef blood was centrifuged at high speed to remove the corpuscles and platelets. To one volume of the plasma one-fourth volume of saturated ammonium sulfate was added. The precipitated fibrinogen was removed from the solution by centrifuging for about five minutes, the supernatent liquid was poured-off and the fibrinogen carefully washed by layering over it a solution of one-fifth saturated ammonium sulfate. The washed fibrinogen was then dissolved in a 2% sodium chloride solution equal to the volume of the original plasma. This solution was filtered and precipitated a second time by half saturating the solution with sodium chloride. The precipitated fibrinogen was dissolved again in a 1% sodium chloride solution and filtered. Three precipitations generally yielded a clear solution of fibrinogen which coagulated upon the addition of thrombin, and did not clot either spontaneously or on the addition of calcium chloride.

Action of the Artificial Enzyme on Fibrinogen.

The first question asked was whether fibrinogen was altered so that it would no longer clot on the addition of thrombin, and whether, if it did not clot, it had undergone hydrolysis, and if so, what hydrolytic products were formed.

The general experimental procedure to answer the above questions was carried out as follows: 50 c.c. of a 0.3% solution of fibrinogen were placed in a 400 c.c. round bottom quartz flask. 1 c.c. of a 0.35% hematoporphyrin solution was added to the fibrinogen. This flask was stoppered and placed under a 500 Watt incandescent tungsten filament lamp or a mercury arc lamp. A control flask containing 50 c.c. of the 0.1% fibrinogen solution was placed beside the first flask. Another control solution identical with the solution in the first flask, was placed in a black bottle so as not to allow the penetration of light into the solution.

All containers were immersed in a running water bath at a temperature of 15°C. and exposed to the radiation of either a 500 Watt incandescent lamp or a mercury arc lamp, for a period of five hours. At the end of the light exposure 3 c.c. portions of each solution were

pipetted into small 1 x 10 cm. test tubes, 0.3 c.c. of fresh thrombin solution added to each tube, and the tubes immersed in a water bath at 38°C. This was to discover whether unchanged fibrinogen was present.

The thrombin solution used was activated serum and was prepared as follows: 1 c.c. of 2.5% calcium chloride was added to 10 c.c. of freshly citrated plasma, placed in a water bath at 38°C. and allowed to clot. 0.3 c.c. of a 0.5% cephalin solution were then added to 5 c.c. of the serum squeezed out of the clotted plasma. The cephalin activated the prothrombin to form thrombin. This cephalinized serum was the thrombin solution used.

The results in table 1 and 2 confirm Howell (1921), that fibrinogen exposed to light and hematoporphyrin loses its clotting power and ability to heat coagulate at 56°C.

The fibrinogen has evidently been changed by the light and hematoporphyrin, so that it no longer coagulates on heating nor clots on the addition of thrombin.

The next question was whether the protein had been hydrolyzed. The solution of fibrinogen and hematoporphyrin previously exposed to light was examined for the usual hydrolytic cleavage products of the proteins and the following results were obtained:

(1) The solution still gave the biuret test.

TABLE 1

Effect of hematoporphyrin on the clotting of fibrinogen by thrombin.

Type of solution.			Clotting time.
Fibrinogen	hematoporphyrin	light	No clot
Fibrinogen	hematoporphyrin	without light	3 1/2 min.
Fibrinogen	without hematoporphyrin	light	4 1/2 min.

Source of radiation - Mercury arc lamp.

Distance of lamp from solution - 25 centimeters.

Volume of protein solution - 50 c.c.

Concentration of hematoporphyrin - 0.007%

Concentration of protein solution - 0.1%

Time of exposure - 5 hours.

Temperature - 15°C.

TABLE 2

Effect of hematoporphyrin on the heat coagulation of fibrinogen.

Type of solution.		Coagulation Temperature.
Fibrinogen	hematoporphyrin	light ..... No coagulation at 99°C.
Fibrinogen	hematoporphyrin	without light..... 56°C.
Fibrinogen	without hematoporphyrin	light ..... 56°C.

Source of radiation - Mercury arc lamp.

Distance of lamp from solution - 20 centimeters.

Volume of protein solution - 50 c.c.

Concentration of hematoporphyrin - 0.007%

Concentration of protein solution - 0.1%

Time of exposure - 5 hours.

Temperature - 15°C.

(2) It contained a non-heat coaguable protein precipitated by ammonium sulfate, and similar to a proto-proteose.

Experiments were then tried with a shorter exposure than ten hours, and it was found that there appeared as an intermediate product a heat coaguable albumin which coagulates at about 78 - 80°C., just about the temperature of heat coagulation of serum albumin. This protein is apparently identical with one produced from fibrinogen by snake venom as recorded by Mathews (1928) and Billing (1930), and which was isolated from fibrin after clotting by Brunstetter (1927) and Miss. R. Hirose (1932). It is hydrolyzed to an albumose by further exposure to light and hematoporphyrin, and in this respect also it resembles serum albumin as will be shown presently.

These experiments showed that the light and hematoporphyrin acted on the fibrinogen to produce a hydrolytic cleavage, similar to the hydrolysis produced by a proteolytic enzyme. In fact, the hematoporphyrin might be called an artificial proteolytic enzyme. In ordinary light, such as that from the 500 Watt incandescent tungsten filament lamp, and also in ultra violet light from a quartz mercury arc, the hematoporphyrin is itself

ultimately destroyed. And in this respect also it resembles many proteolytic enzymes. But the destruction brought its enzymatic activity to an end.

Still another resemblance of hematoporphyrin to a proteolytic enzyme was discovered. This was the observation that ultra violet alone, in the absence of hematoporphyrin, produces the same series of changes, so far as the writer has been able to tell, in the fibrinogen as does hematoporphyrin and light; but the change goes on at a very much slower rate. So that hematoporphyrin acts the part of a catalytic agent, the presence of which greatly accelerates the reaction.

This action of ultra violet light alone as compared with ultra violet and hematoporphyrin is shown in tables 3 and 4.

Having thus established the important facts that a true hydrolytic cleavage of the fibrinogen was produced, the mechanism of the reaction was analyzed hoping thereby to throw light on the mechanisms of all hydrolytic cleavages and dehydration syntheses, reactions of such fundamental importance in living things.

The first question asked was whether the light energy was efficient in the absence of oxygen. It was

Table 4

Effect of ultra violet light alone, and ultra violet light with hematoporphyrin, on the clotting of fibrinogen by thrombin.

Hours Exposed	Fibrinogen Hematoporphyrin Ultra Violet	Fibrinogen Hematoporphyrin Without Light	Fibrinogen Ultra Violet
0.	clot	clot	clot
0.5	no clot	clot	clot
1.0	no clot	clot	clot
2.5	no clot	clot	clot
5.5	no clot	clot	partial clot
7.5	no clot	clot	no clot

Source of radiation-- Mercury arc lamp.

Distance of lamp from solution---------- 25 cm.

Volume of protein solution---------- 50 C.C.

Concentration of protein---------- 0.1 %

Concentration of hematoporphyrin---------- 0.007 %

Temperature---------- 15° C.

Table 4

Effect of ultra light alone, and ultra violet light with hematoporphyrin, on the heat coagulation of fibrinogen.

Hours Exposed	Fibrinogen Hematoporphyrin Ultra Violet	Fibrinogen Hematoporphyrin Without Light	Fibrinogen Ultra Violet
0.	+	+	+
0.5	+	+	+
1.0	+	+	+
1.5	—	+	+
2.5	—	+	+
5.5	—	+	cloudy ppt.
7.5	—	+	—

Source of radiation--Mercury arc lamp.

Distance of lamp from solution---------- 25 cm.

Volume of protein solution---------- 50 c.c.

Concentration of protein---------- 0.1 %

Concentration of hematoporphyrin --------- 0.007%

Temperature ---------- 15° c.

found that besides light and hematoporphyrin, oxygen must be present. This observation was of very great interest for it at once brought the hydrolytic cleavages and dehydration syntheses into the realm of cell respiration. Many observers from the time of Claude Bernard had pointed out the dependence of the synthetic powers of the cell in its respiration. That this hydrolysis also involves oxygen was, therefore, a discovery of the very greatest interest.

To study the role of oxygen in this reaction of hematoprophyrin and light on fibrinogen, experiments were carried out with the aid of a hydrogen atmosphere. 50 c.c. of a fresh 0.1% solution of fibrinogen were thoroughly saturated with hydrogen. The gas, electrolytic hydrogen, which was contained in a tank under pressure, was bubbled strongly through the fibrinogen solution causing it to froth greatly, thereby increasing the surface of the liquid and more completely eliminating any oxygen present. After fifteen minutes of this vigorous bubbling of the hydrogen through the fibrinogen solution the quartz flask container was quickly stoppered. 1 c.c. of 0.35% hematoporphyrin solution was carefully added to the fibrinogen solution and hydrogen was again bubbled through the solution for five minutes to insure the elimination of dissolved oxygen.

Another flask of 50 c.c. of 0.1% fibrinogen plus 1 c.c. of 0.35% hematoporphyrin containing atmospheric air was placed in the water bath beside the flask containing the hydrogen. The technique of exposure to the 500 Watt lamp was carried out according to the previous experiments. A control solution of fibrinogen and hematoporphyrin was placed in a dark bottle.

After five hours of exposure to the light 3 c.c. quantities of each solution were pipetted into small (1 x 10cm.) test tubes and 0.3 c.c. of fresh thrombin solution were added to each tube. The results of the thrombin clotting and the heat coagulation of the fibrinogen in the presence of hematoporphyrin, light, oxygen and hydrogen are shown in tables 5 and 6.

These experiments which always gave the same result on repetition, show very clearly that hematoporphyrin and light alone do not hydrolyze or alter fibrinogen so as to cause it to lose its clotting power or change its temperature of heat coagulation. Oxygen is necessary.

The fluorescence of the hematoporphyrin is not reduced, so far as it could be determined, in the hydrogen atmosphere as compared with the air atmosphere. The fluorescence does not, therefore, depend on oxygen. And further, it is clear that the hydrolysis cannot be a direct result of the fluorescence. In fact the result

TABLE 5

Effect of oxygen on the clotting of fibrinogen in the presence of hematoporphyrin and light.

Type of Solution	Clotting Time.
Fibrinogen — Hematoporphyrin — H_2 — light	⟶ 5 minutes
Fibrinogen — Hematoporphyrin — O_2 — without light	⟶ 3 minutes
Fibrinogen — Hematoporphyrin — O_2 — light	⟶ No clot.

Source of radiation - 500 Watt incandescent tungsten filament lamp.

Distance of lamp from solution. - 15 centimeters.

Volume of protein solution - 50 c.c.

Concentration of hematoporphyrin 0.007%

Concentration of protein 0.1%

Time of exposure - 5 hours.

Temperature - 20°C.

TABLE 6

Effect of oxygen on the heat coagulation of fibrinogen in the presence of hematoporphyrin and light.

Type of Solution.				Coagulation Temperature.
Fibrinogen —	Hematoporphyrin —	light —	H_2	$\longrightarrow$ 56°C.
Fibrinogen —	Hematoporphyrin —	without light —	O_2	$\longrightarrow$ 56°C.
Fibrinogen —	Hematoporphyrin —	light —	O_2	$\longrightarrow$ No coagulation at 99°C.

Source of radiation - 500 Watt incandescent tungsten filament lamp.

Distance of lamp from solution - 15 centimeters.

Volume of protein solution - 50 c.c.

Concentration of hematoporphyrin - 0.007%

Concentration of protein - 0.1%

Time of exposure - 5 hours.

Temperature - 20°C.

suggests that the fluorescence is an incidental matter, not of the importance many observers have supposed.

Another interesting observation was that the hematoporphyrin although fluorescing in the hydrogen atmosphere, was not destroyed by the light. There was no diminution in the tint of the solution after prolonged exposure to light in the presence of hydrogen. Whereas, the red color was entirely lost in the control solutions exposed to light in an ordinary atmosphere of air. This shows that the destruction of the hematoporphyrin like its proteolytic powers, when illuminated, depends on oxygen.

A - Other Photo-active Substances - Artificial Proteases.

There are other chemical substances besides hematoporphyrin which in the presence of light and oxygen act on fibrinogen and prevent its ability to clot in the presence of thrombin.

"Baumberger, Bigotti, and Bardwell (1929), have found that methylene blue and light inhibited the coagulation of a mixture of fibrinogen, calcium and prothrombin, but only in the presence of oxygen."

The writer has been able to produce the same results with other dyes besides methylene blue. The following tables show the action of eosin, fluorescein, safranin, thionine, methyl orange, and methylene blue on fibrinogen under varying conditions. The time of exposure to light was ten hours and the concentration of the dyes was 2 c.c. of 0.35% per 125 c.c. of 0.1% fibrinogen. The normal clotting time before exposure was about 3 minutes.

In tables 8, 9, and 10 it is noticed that these dyes, however, do not prevent heat coagulation of the protein upon boiling. It is observed in table 12 that methylene blue, acts on the fibrinogen in the presence of

Effect of different dyes on the clotting by thrombin and the heat coagulation of fibrinogen, in the presence of light and oxygen.

Table 7

Eosin

Type of Solution	Thrombin Clotting	Heat Coagulation
Fibrinogen – Eosin – H_2 – light	→ clot in 5'	56° C.
Fibrinogen – Eosin – O_2 – darkness	→ clot in 3'	56° C.
Fibrinogen – Eosin – O_2 – light	→ no clot	no coagula- at 56° or 99°C.

Table 8

Fluorscein.

Fibrinogen – Fluorscein – H_2 – light	→ clot in 6'	56° C.
Fibrinogen – Fluorscein – O_2 – darkness	→ clot in 3'	56° C.
Fibrinogen – Fluorscein – O_2 – light	→ no clot	no coagula- at 56°C., only on boiling.

Table 9

Safranin.

Type of Solution	Thrombin Clotting	Heat Coagulation
Fibrinogen - Safranin - H_2 - light →	clot in 5'	56°C.
Fibrinogen - Safranin - O_2 - darkness →	clot in 3'	56°C.
Fibrinogen - Safranin - O_2 - light →	no clot	none at 56°C. (Only on Boiling)

Table 10

Thionine.

Fibrinogen - Thionine - O_2 - light →	no clot	none at 56°C.
Fibrinogen - Thionine - O_2 - darkness →	clot	(Only at 90°C) 56°C.

Table 11

Methyl Orange

Fibrinogen - Methyl Orange - H_2 - light →	clot	56°C.
Fibrinogen - Methyl Orange - O_2 - darkness →	clot	56°C.
Fibrinogen - Methyl Orange - O_2 - light →	clot	56°C.

Table 12

Methylene Blue.

Type of Solution	Thrombin Clotting	Heat Coagulation
Fibrinogen - Methylene Blue - H_2 - light →	clot in 6'	56°C.
Fibrinogen - Methylene Blue - O_2 - darkness →	clot in 3'	56°C.
Fibrinogen - Methylene Blue - O_2 - light →	no clot	none at 56°C. (Only at 76°C)

Source of radiation--500 Watt incandescent tungsten filament lamp.

Distance of lamp from solution ---------- 15 cm.

Volume of solution ---------- 50 c.c.

Concentration of dyes ---------- 0.007 %

Concentration of protein ---------- 0.1%

Temperature ---------- 20°C.

Time of exposure ---------- 10 hours.

light, to produce a protein that coagulates at 76°C., which is the coagulation temperature of serum albumin. It has been suggested by Professor Mathews that this protein may be serum albumin.

The mechanism of the reaction is postponed until the facts of the hydrolysis of other proteins have been presented.

Preparation of Serum Albumin.

Serum albumin was prepared in a crystalline state according to the methods of E.G. Young (1922) and Adair and Robinson (1930).

Fresh horse serum was obtained by whipping out the fibrin at the time the blood was taken from the animal. The defibrinated blood was placed in a refrigerator for several hours to enable the corpuscles to settle out. The serum was poured-off the corpuscles and centrifuged untill it appeared clear. If this procedure is carried out within a few hours after the blood was taken from the animal the centrifuged serum will appear clear and amber-yellow in color.

The serum globulin was removed by adding an equal volume of saturated ammonium sulfate solution to the centrifuged serum. The precipitate was filtered off after four or five hours of standing. The filtration was carried out in a refrigerator. The clear yellow-orange filtrate was adjusted to room temperature and slowly acidified by meams of a 10% acetic acid. The acid was added from a burette, drop by drop, while the solution was being gently agitated by a mechanical stirrer. The acid is added to the point of the first trace of turbidity. This point corresponds to a pH of 6.0, and is usually attained upon

adding 1.26 c.c. of acid per 100 c.c. of serum. The solution should be examined for the presence of serum albumin crystals which are mono clinic in form. After allowing the solution to be constantly stirred for two hours, the acidity is again increased by adding one half as much acetic acid as previously added, about 0.63 c.c. per 100 c.c. of serum. After another two hours a third portion of acid is added, equal to the second. This should establish a pH of 4.9 to 5.0, below which it is not advisable to go. The solution is left at room temperature to crystallize over night. The yield of crystals may be further increased by the addition of more ammonium sulfate to the solution, a few c.c. at a time (1 c.c. per 100 c.c.) until precipitation ceases.

The crystals are separated from the mother liquid by centrifuging and then mixed with an equal volume of a fluid prepared by adding 60 c.c. of molar sodium acetate solution and 40 c.c. of molar acetic acid to 100 c.c. of a saturated solution of ammonium sulfate and again centrifuged. The crystals may be washed several times with this fluid without much loss.

In recrystallizing the serum albumin the quantities of reagents used will refer to a preparation of about 2 grams of washed crystals obtained from 100 c.c. of serum.

The volume of water used to dissolve these crystals is about 50 c.c. After filtration to remove the insoluble residue, 8 c.c. of molar sodium acetate is added, followed by a volume of saturated ammonium sulfate equal to the sum of the volumes of water and sodium acetate (about 58 c.c.). Lastly, the acidity is adjusted by the addition of 10.6c.c. of a mixture composed of equal volumes of molar acetic acid and saturated ammonium sulfate (1.33 c.c. of this mixture per c.c. of sodium acetate). The only reagent which needs to be added slowly is the mixture of acetic acid and ammonium sulfate, which may be mixed with the albumin solution in a period of about 15 minutes. The solution should be stirred constantly during the recrystallization. The yield may be increased by leaving the preparation over night at room temperature.

After three crystallizations, the crystalline material is usually white in appearance, having been freed from the yellow pigment which is always present in crude serum albumin. The crystals are dissolved in water, 50 c.c. for each 2 grams of crystals, and filtered again. This solution is immediately dialyzed against a buffer solution of pH 7.4 prepared by adding equal quantities of $.2/_{15}$ molar potassium mono-basic phosphate and $.8/_{15}$ molar sodium di-basic phosphate in order to convert the ammonium albuminates into sodium and potassium albuminates. The dialyzing

apparatus consisted of a large inverted 30 cm. funnel with cellaphane stretched accross the bottom and placed in a large evaporating dish containing the buffer solution. The dialyzing was continued over night in the refrigerator. The next three days the serum albumin was dialyzed against distilled water until the sulfates were reduced to nearly that of tap water. Toluene was added to the albumin solution during the dialysis.

Serum albumin prepared in this manner will keep for several weeks in the refrigerator without undergoing bacterial decomposition. The crystalline material will keep for an indefinite period in the mother liquid if placed in a refrigerator.

Action of the Artificial Enzyme on Serum Albumin

Qualitative Observations.

It was found that when serum albumin was exposed five hours to the light in the presence of hematoporphyrin and oxygen, a digestion of the protein occured. This digestion was evident when the filtrate of the heat coagulated serum albumin was analyzed for proteolytic products.

The serum albumin after exposure was heat coagulated in a boiling water bath for fifteen minutes and then filtered. Upon addition of an equal volume of 20% trichloroacetic acid to the filtrate a white flocculent precipitate settled out. The trichloroacetic acid was poured-off and a biuret test was performed on the precipitate, which resulted in the formation of a dark violet color. Neither of the filtrates from the coagulated control protein solutions, that without hematoporphyrin and in the light and that with hematoporphyrin and in the dark, gave a precipitate with trichloroacetic acid. This shows that an albumose was formed only in the protein solution exposed to light and in the presence of hematoporphyrin and oxygen.

Two volumes of 5% phosphotungstic acid plus one volume of filtrate from the heat coagulated, digested serum albumin also gave a precipitate which was obviously greater in quantity than the small precipitate formed under similar conditions in the filtrates of the heat coagulated, undigest-

ed serum albumin.

Half saturation of the filtrate from the coagulated digested serum albumin with ammonium sulfate resulted in the formation of a precipitate. Filtering-off the precipitate and adding solid ammonium sulfate to the filtrate until fully saturated did not produce further precipitation of a proteolytic product, which showed that there were no secondary proteoses present.

From the results of these qualitative tests, it is evident that the proteolytic product of the digested serum albumin is a primary proteose, for it is thrown out of solution by half saturation with ammonium sulfate, gives a deep violet biuret, and is also precipitated by trichloroacetic acid.

It is interesting to note that there is a difference between the physical aspects of the exposed serum albumin and the control solutions. The solutions of serum albumin and hematoporphyrin posses a red color before exposure to light, but after several hours of radiation to an incandescent light, daylight, or ultra violet light, the red color gradually changes to brown. This brown colored solution no longer gave the characteristic absorption spectrum bands of alkaline hematoporphyrin. Evidently, the hematoporphyrin in combining with the serum albumin in the

presence of light and oxygen has undergone a chemical change, for the control solutions of albumin and hematoporphyrin placed in the dark still retain the characteristic spectrum of hematoporphyrin.

Another interesting physical change of the radiated serum albumin and hematoporphyrin is observed during the heat coagulation process. The coagulum of the protein exposed to light in the presence of hematoporphyrin sinks to the bottom of the test tube and appears to be in a finely divided state, while the coagulated portion of the control protein solutions, both that without hematoporphyrin and in the light, and that with hematoporphyrin and in the dark, rises to the surface of the liquid in the test tubes and appears to be one intact coagulum. The coagulum had either a brown or red color showing that the unchanged and changed hematoporphyrin were attached to or entangled with the coagulated protein.

Quantitative Observations.

In order to study quantitatively the action of hematoporphyrin on serum albumin in the presence of light experiments were carried out under varying conditions. In one series of experiments the temperature of the protein solutions was varied while the concentration of the hematoporphyrin and the length of exposure to light were maintained constant. Another series of experiments were performed by varying the time of exposure to the light and keeping the concentration of hematoporphyrin and the temperature constant. A third group of experiments were carried out in which the concentration of hematoporphyrin was varied while the temperature and time of exposure were the constant factors. And lastly, a fourth series of experiments were performed to show the role of oxygen in this digestive action of hematoporphyrin on proteins in the presence of light energy.

Before considering the experimental data it is necessary to give a brief description of the methods used in determining quantitatively the amount of nitrogen in the filtrates of the heat coagulated serum albumins. The concentration of the protein solution ranged from 0.5% to 0.6% and contained 1% sodium chloride to facilitate heat coagulation. 1.25 c.c. of 0.35% hematoporphyrin were added to

125 c.c. of protein solution contained in a 400 c.c. round bottom quartz flask. The flask was stoppered and immersed in a constant temperature water bath. 125 c.c. of a control solution of only serum albumin were placed in another 400 c.c. quartz flask beside the one containing the hematoporphyrin. A second control solution of serum albumin and hematoporphyrin identical with the first solution was placed in a dark bottle and put beside the other flasks. A 500 Watt incandescent lamp was adjusted at a distance of 15 cms. from the first two flasks.

After exposure to the light, 50 c.c. portions from each container were pipetted into large 24 x 180 mm. test tubes. 0.4 c.c. of a 1% acetic acid solution were added to the test tubes containing the exposed serum albumin and hematoporphyrin, and 0.5 c.c. added to the control tubes in order to adjust all of the solutions to the pH of 5.2 before heat coagulation. The test tubes were placed in a boiling water bath and kept at that temperature for fifteen minutes. After heat coagulation the serum albumin was immediately filtered through a double 10 cm. filter paper into a 100 c.c. flask. The residue was washed on the filter paper with 10 c.c. of ammonia-free water and the washings added to the first filtrate.

The whole filtrate was then digested and analyzed according to the Van Slyke gasometric micro-kjeldahl method. 1 c.c. of a two to one sulfuric acid-phosphoric acid mixture was added to each flask and the contents digested over a sand bath until white fumes appeared. The flasks were allowed to cool and 1 gram of ammonia-free potassium persulfate plus 1 c.c. of ammonia-free distilled water were added to each flask, and the contents again digested until the digest became colorless. 10 c.c. of ammonia-free distilled water were added to each digest and the flask was twirled until the solidified melt had gone into solution. 5 drops of 1% alizarine were added to each flask and 40% sodium hydroxide was slowly added until the indicator turned pink. At the same time the flask was immersed in a beaker of cold water to prevent a rise in temperature of the contents. 10% sulfuric acid was then added slowly until the indicator turned yellow again. The contents of the flask were poured into a graduate cylinder and sufficient ammonia-free water added to make the volume to 25 c.c. The operation of the Van Slyke apparatus was performed in the following manner: Before making a series of analyses, the apparatus was checked thoroughly to be certain that no leaks existed. 10 c.c. of distilled water were drawn into the extraction chamber.

The external capillary orifice was sealed with a drop of mercury and the mercury level within the extraction chamber lowered to the 50 c.c. mark. The extraction chamber was shaken for three minutes, the water level within the chamber raised to the 2 c.c. graduation and the pressure on the manometer tube read. Several shakings were made until the pressure readings on the manometer tube checked closely, thereby being certain that no leaks were present in the apparatus.

The water in the extraction chamber was replaced with 10 c.c. of the digest to be analyzed. The external chamber was washed with 2 c.c. of distilled water, making a total volume of 12 c.c. in the extraction chamber. The dissolved air was pumped out of the digest by lowering the mercury level within the extraction chamber to the 50 c.c. mark and shaking for two minutes. The extracted gas was ejected and 1 c.c. of alkaline sodium hypobromite was drawn into the extraction chamber. The external capillary orifice was sealed with mercury and the ammonia gas formed by the action of the alkali on the acid digest was pumped out of solution. The ammonia gas reacted with the sodium hypobromite liberating nitrogen gas, according to the equation:

$$2NH_3 + 3NaBrO = N_2 + 3NaBr + 3H_2O$$

After shaking the extraction chamber for three minutes, the liquid level within the chamber was raised to the 2 c.c. mark and the pressure, P_1, was read on the manometer scale. The extracted nitrogen gas was ejected, and the second pressure, P_2, was noted with the liquid level at the 2 c.c. graduation. The temperature of the water bath surrpunding the extraction chamber was recorded and the quantity of nitrogen in the digest determined as follows: The difference between the two pressures, P_1 -- P_2, is multiplied by a factor, and the product is expressed in milligrams. The value of the factor varies with the temperature and may be obtained from a set of tables prepared by Van Slyke. Approximately 1/300 mg. corresponds to 1 mm. on the manometer scale.

4.

Effect of temperature on hydrolysis of serum albumin by hematoporphyrin and light in the presence of oxygen.

The object of this series of experiments is to determine the influence of temperature on the hydrolytic action of hematoporphyrin on serum albumin in the presence of light and oxygen. Although temperature has no effect on the primary photo-chemical reactions, it is possible that it may influence secondary chemical reactions. The temperature of the water bath was varied from 15°C. to 45°C. As bacterial decomposition is apt to take place

at high temperatures, the solutions of the 0.8% serum albumin were protected from the action of bacteria by the addition of 10 c.c. of toluene to each flask. The concentration of hematoporphyrin was 1.25 c.c. per 125 c.c. of protein solution and the time of exposure to the radiation of the 500 Watt filament lamp was ten hours.

The values in table 13 represent to amounts of nitrogen in the filtrates from the first 50 c.c. quantity of heat coagulated serum albumin. Table 14 represents the nitrogen values of the second 50 c.c. quantity of heat coagulated serum albumin. Both quantities were taken from the same exposed sample of protein. Two analyses were run on each filtrate and the average result taken, thereby making a total of sixty analyses for the two tables.

Page (48) contains a graph representing an average of all of the values in table 13 and 14. Temperature in the absicca is plotted against the milligrams of nitrogen in the filtrates on the ordinate.

Table 15 shows the increase in the filtrate nitrogen as a percentage of the total nitrogen in the 50c.c. of serum albumin at different temperatures. The total nitrogen in 50 c.c. of the protein was 44.25 mgs. The average of the filtrate nitrogen of table 13 and 14 were used in determining these percentages.

Effect of temperature on the hydrolysis of serum albumin by hematoporphyrin and light in the presence of oxygen.

TABLE 13

Milligrams of nitrogen in the filtrate from 50 c.c. of the heat coagulated protein solution.

Temperature	Serum albumin Hematoporphyrin Light	Serum albumin Hematoporphyrin without light	Serum albumin Light
15°	1.12	1.02	0.91
24°	1.82	0.92	0.90
27°	1.88	0.96	0.94
36°	2.09	0.97	0.94
45°	2.34	0.92	0.94

Source of radiation - 500 Watt incandescent tungsten filament lamp.

Distance of lamp from quartz flasks - 15 centimeters.

Concentration of hematoporphyrin -- 0.0035%

Concentration of protein solution - 0.8%

Time of exposure ---------- 10 hours.

Heat coagulated in boiling water bath for 15 minutes.

Effect of temperature on the hydrolysis of serum albumin by hematoporphyrin and light in the presence of oxygen.

TABLE 14

Milligrams of nitrogen in the filtrate from 50 c.c. of the heat coagulated protein solution.

Temperature	Serum albumin Hematoporphyrin Light	Serum albumin Hematoporphyrin without light	Serum albumin Light
15°C.	1.14	1.03	0.92
24°C.	1.80	0.92	0.92
27°C.	1.75	1.04	1.00
36°C.	2.13	0.94	0.98
45°C.	2.34	0.94	0.86

Source of radiation - 500 Watt incandescent tungsten filament lamp.

Distance of lamp from quartz flasks -15 centimeters.

Concentration of hematoporphyrin - 0.0035%

Concentration of protein solution - 0.8%

Time of exposure - 10 hours

Heat coagulated in boiling water bath for 15 minutes.

TABLE 15

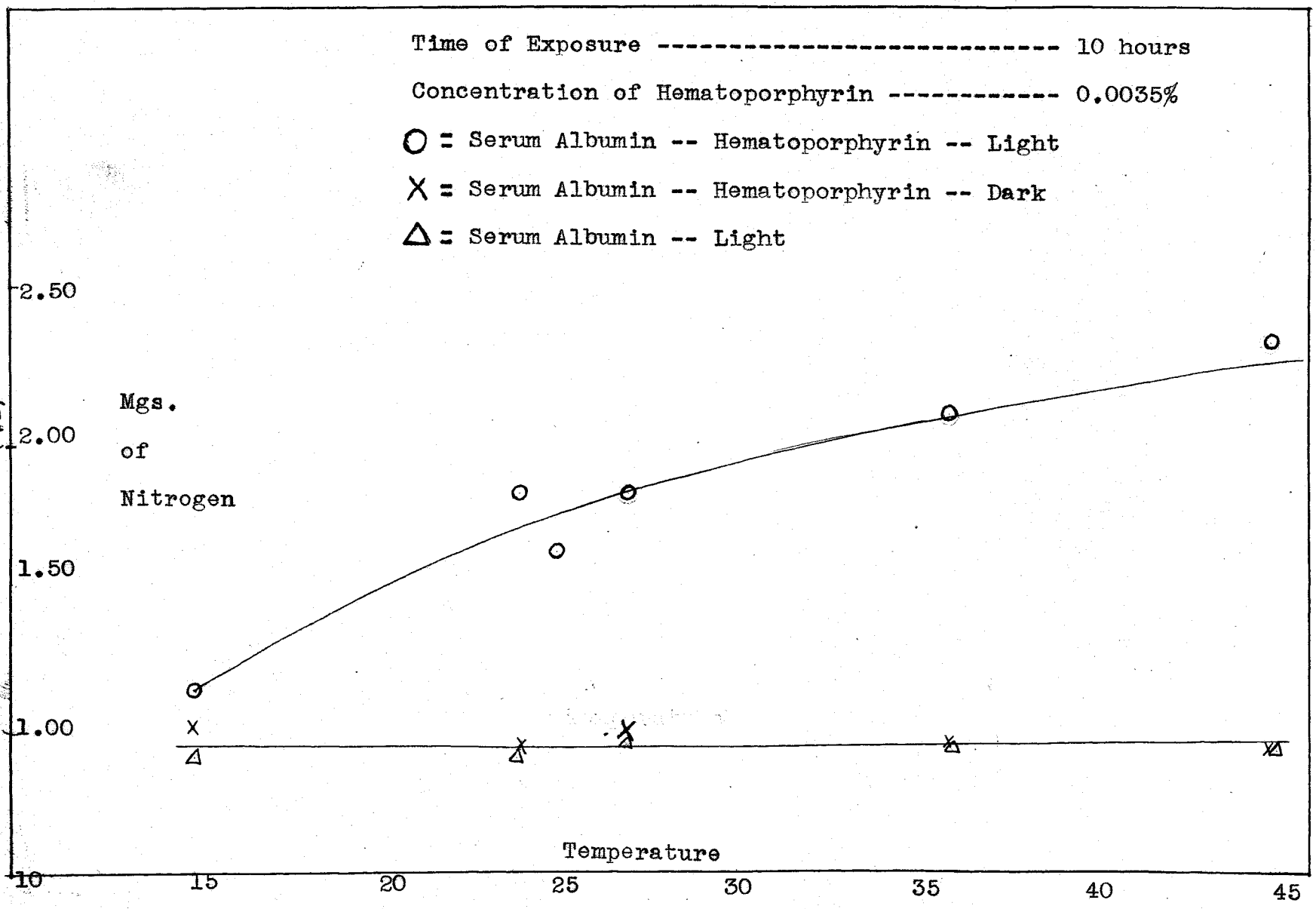
Filtrate nitrogen as a percentage of the total nitrogen.

Temperature	Serum albumin Hematoporphyrin Light	Serum albumin Hematoporphyrin in darkness	Serum albumin Light
15°C.	2.54%	2.30%	2.07%
24°C.	4.09%	2.07%	2.03%
27°C.	4.11%	2.25%	2.18%
36°C.	4.77%	2.15%	2.17%
45°C.	5.28%	2.10%	2.03%

The average of the filtrate nitrogen of Table 13 and Table 14 was used in determining these percentages.

Effect of Temperature on the Hydrolysis of Serum Albumin

by Hematoporphyrin, Light and Oxygen



From the results of these tables it is very evident that with an increase in the temperature there is a corresponding increase in the rate of hydrolysis of the serum albumin by the hematoporphyrin and light in the presence of oxygen. The possible explanation for this increase in the digestion rate is that secondary chemical reactions are taking place. The secondary chemical reactions receive energy from the increase in the temperature of the surrounding medium. It may be the expression of the dissociation of the water with increase of temperature since water enters into the reaction of hydrolysis.

B - Effect of the time of exposure to light on the hydrolysis of serum albumin by hematoporphyrin in the presence of oxygen.

The concentration of the hematoporphyrin was 1.25 c.c. per 125 c.c. of serum albumin and the temperature 25°C.

According to table 16 the quantity of serum albumin digested increased with the time of exposure to light andhematoporphyrin. The curve on page (51) would probably slope off parallel to the abscissa, although no observations were made, for the hematoporphyrin undergoes a destruction, no longer gives the characteristic absorption

Effect of time of exposure to light on the hydrolysis of Serum albumin in the presence of hematoporphyrin and oxygen.

TABLE 16

Milligrams of nitrogen in the filtrate from 50 c.c. of the heat coagulated protein solution.

Hours	Serum albumin Hematoporphyrin Light	Serum albumin Hematoporphyrin without light	Serum albumin Light
0	0.917	0.932	0.960
2	1.16	0.998	0.908
5	1.47	0.954	0.971
10	1.61	0.917	0.901

Source of radiation - 500 Watt incancescent tungsten filament lamp.

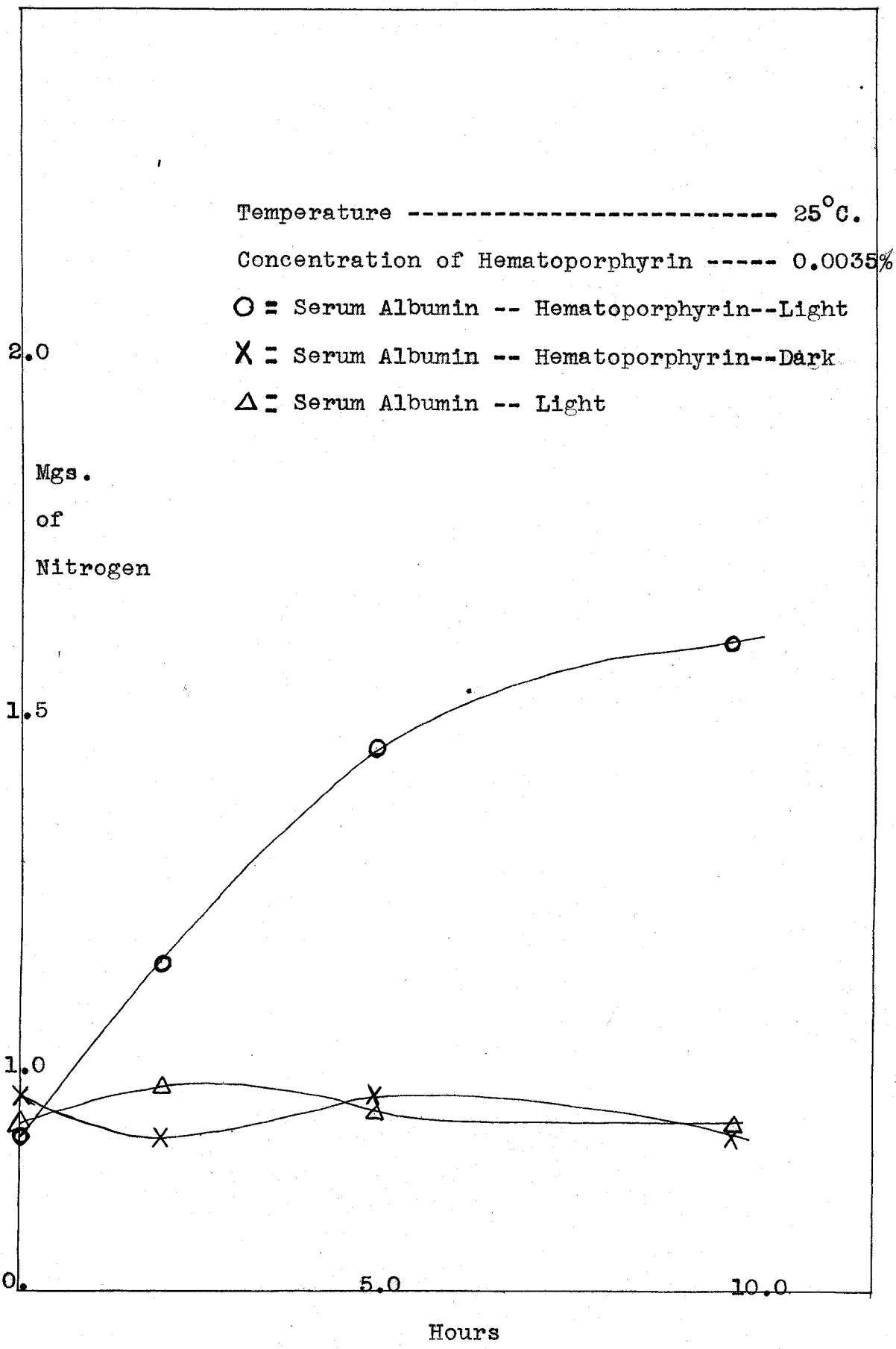
Distance of lamp from quartz flask - 15 centimeters.

Concentration of hematoporphyrin - 0.0035%

Concentration of protein solution - 0.8%

Temperature ---------- 25°C.

Effect of Time of Exposure to Light



spectrum, and experiment shows that it then loses its power to digest the serum albumin.

C - Effect of the concentration of hematoporphyrin on the hydrolysis of serum albumin in the presence of light and oxygen.

The temperature was kept at 25°C. and the time of radiation was for a period of ten hours.

Table 17 indicates that with an increase in the concentration of hematoporphyrin there is also an increase in the rate of hydrolysis of the serum albumin. The optimum concentration of hematoporphyrin is approximately 1 c.c. of 0.35% per 100 c.c. of protein solution, after which a greater increase in concentration has little effect on the rate of hydrolysis.

D - Effect of oxygen on the hydrolysis of serum albumin by hematoporphyrin and light.

It has been shown that hematoporphyrin and light have no action on fibrinogen so far as can be determined by any change in the temperature of heat coagulation or clotting by thrombin if the oxygen be removed from the solutions. Similar experiments have been carried out on solutions of serum albumin exposed to hematoporphyrin

Effect of the concentration of hematoporphyrin on the hydrolysis of serum albumin in the presence of light and oxygen.

TABLE 17

Milligrams of nitrogen in the filtrate from 50 c.c. of the heat coagulated protein solution.

No. of c.c. of 0.35% Hematoporphyrin	Serum albumin Hematoporphyrin Light	Serum albumin Hematoporphyrin without light	Serum albumin Light
0.01 c.c.	0.86	0.95	0.90
0.5 c.c.	1.46	-----	----
1.0 c.c.	1.82	0.92	0.95
5.0 c.c.	2.04	----	----

Source of radiation - 500 Watt incandescent tungsten filament lamp.

Distance of lamp from quartz flask - 15 centimeters.

Concentration of protein solution - 0.8%

Time of exposure ---------- 10 hours.

Temperature ---------- 25°C.

and light with the exclusion of oxygen. As in the case of the fibrinogen solutions, the atmospheric air of the serum albumin was replaced with hydrogen.gas. Tables 18 and 19 show the results obtained.

From these results it is obvious that oxygen is involved in the action of hematoporphyrin and light on serum albumin, for in the absence of oxygen hematoporphyrin and light alone cause no increase in the non-heat coaguable protein.

The general result is to show that serum albumin is also hydrolyzed by the combined action of hematoporphyrin, light and oxygen as was fibrinogen but the hydrolysis is at a very much slower rate. This is shown by the fact that in fibrinogen the whole of the nitrogen is in the form of protein products no longer heat coaguable after 10 hours exposure, whereas, in serum albumin only about five percent of the nitrogen has been split-off in the same time. Serum globulin, the third blood protein is even more resistent to the action of the artificial enzyme than is serum albumin.

Effect of oxygen on the hydrolysis of serum albumin by hematoporphyrin.

TABLE 18

Milligrams of nitrogen in the filtrate from 50 c.c. of the heat coagulated protein solution.

Serum albumin -	hematoporphyrin -	light -	$O_2 \rightarrow$	1.58
Serum albumin -	hematoporphyrin -	light -	$H_2 \rightarrow$	0.85
Serum albumin -	hematoporphyrin -	without light -	$O_2 \rightarrow$	0.78

Source of radiation -500 Watt incandescent tungsten filament lamp.

Distance of lamp from flask - 15 centimeters.

Time of exposure - 10 hours.

Temperature of water bath - 15°C.

Concentration of protein solution - 0.55%

Concentration of hematoporphyrin - 0.0035%

Effect of oxygen on the hydrolysis of serum albumin by hematoporphyrin.

TABLE 19

Milligrams of nitrogen in the filtrate from 50 c.c. of the heat coagulated protein solution.

Serum albumin	hematoporphyrin	light	$O_2 \rightarrow$	1.28
Serum albumin	hematoporphyrin	light	$H_2 \rightarrow$	0.63
Serum albumin	without hematoporphyrin	light	$H_2 \rightarrow$	0.65

Source of radiation - 500 Watt incandescent tungsten filament lamp.

Distance of lamp from quartz flask - 15 centimeters.

Time of exposure - 20 hours.

Temperature of water bath - 15°C.

Concentration of protein solution - 0.51%

Concentration of hematoporphyrin - 0.0035%

Action of the Artificial Enzyme on Serum Globulin

Preparation of the Serum Globulin

Serum globulin was prepared from fresh horse serum which had been highly centrifuged to remove all corpuscles and platelets. Upon the addition of an equal volume of saturated ammonium sulfate the globulin was thrown out of solution. After standing for several hours to allow complete precipitation the globulin was filtered off. It was redissolved in distilled water and reprecipitated again and filtered. A third precipitation was done and the solution of the globulin was dialyzed against running water to free it from ammonium sulfate. After two days of dialyzing the globulin was diluted with distilled water so that the protein content was about 0.4%. Sufficient sodium chloride was added to give the solution a 1% salt concentration.

Experimental

100 c.c. of serum globulin and 2 c.c. of 0.35% hematoporphyrin were placed in a 400 c.c. quartz flask. A control solution of serum globulin alone was placed in another quartz flask of the same capacity. Both flasks

were immersed in a running water bath of 21°C. temperature. The source of radiation was a mercury arc lamp provided with a plate glass screen to filter out the ultra violet. The lamp was placed at a distance of six inches from the two quartz flasks and the protein solutions were exposed twenty five and forty hours.

Procedure of analysis.

After exposure to the light, 20 c.c. portions from each flask were placed in large test tubes, 24 x 180mm., 0.2 c.c. of 1% acetic acid was added and the tubes placed in a boiling water bath for fifteen minutes. After heat coagulation the contents were filtered and the filtrate analyzed for nitrogen.

The filtrate was digested according to the Koch and Mc Meekin (1924) micro-kjeldahl method. 1 c.c. of a one to one sulfuric acid was added to the filtrate contained in the 24 x 180 mm. test tube. The tube was heated over a micro burner until the contents fumed. After cooling for a few minutes 30% hydrogen peroxide was added drop by drop until the digest was clear upon further heating. The digest was diluted with about 70 c.c. of distilled water,

poured into a 100 c.c. volumetric flask, and 15 c.c. of modified Nessler solution added. The unknown solution was immediately compared with a standard Nessler solution containing 0.5 mgs. of nitrogen.

The results from table 20 and 21 show an apparent increase in the filtrate nitrogen of the flasks containing hematoporphyrin, but the increase is within the limits of error of the determination and not sufficient to be an indication of a positive hydrolysis by the artificial enzyme.

What the nature and origin is of the nitrogen thus appearing in the filtrate is unknown. It may be due to small traces of ammonium salt left in the solution after dialysis, or to some soluble protein split from the protein on heat coagulation. Further work will be done to clear up this point. At any rate, illumination, does not increase this soluble nitrogen.

Action of hematoporphyrin, light and oxygen on serum globulin.

TABLE 20

Milligrams of nitrogen in the filtrate from 50 c.c. of the heat coagulated protein solution.

l_A Serum globulin – hematoporphyrin – light – O_2 ⟶ 0.75

l_B Serum globulin – hematoporphyrin – light – O_2 ⟶ 0.70

ll_A Serum globulin – without hematoporphyrin – light – O_2 ⟶ 0.67

ll_B Serum globulin – without hematoporphyrin – light – O_2 ⟶ 0.65

Source of radiation - Mercury arc lamp, provided with a screen for filtering out the ultra violet.

Distance of lamp from solution - 15 centimeters.

Temperature - 20°C.

Time of exposure - 25 hours.

Concentration of protein solution - 0.42%

Concentration of hematoporphyrin - 0.0035%

Action of hematoporphyrin, light, and oxygen on serum globulin.

TABLE 21

Milligrams of nitrogen in the filtrate from 50 c.c. of the heat coagulated protein solution.

1_A	Serum globulin	hematoporphyrin	light – O_2 →	1.92
1_B	Serum globulin	hematoporphyrin	light – O_2 →	2.00
11_A	Serum globulin	without hematoporphyrin	light – O_2 →	1.87
11_B	Serum globulin	without hematoporphyrin	light – O_2 →	1.82

Source of radiation - Mercury arc lamp, provided with a screen for filtering out the ultra violet.

Distance of lamp from solution - 15 centimeters.

Temperature - 20°C.

Time of exposure - 40 hours.

Concentration of protein solution - 1.0%

Concentration of hematoporphyrin - 0.0035%

Action of the Artificial Enzyme on Edestin.

A few experiments of a qualitative kind were carried out with edestin. After exposure to the unscreened light of a Cooper-Hewitt mercury arc lamp for five hours, in a quartz flask with added hematoporphyrin, the filtrate from the coagulated protein gave no biuret test. No apparant change had occured in the protein. The solution was not examined for other protein products.

While these results are too incomplete for a definite statement that there is no hydrolysis at all of the edestin, they never-the-less show that if it occurs, it is at a much slower rate, than either fibrinogen or serum albumin, for each of these under similar circumstances, yield biuret positive products not coagulated by heat.

Discussion and Conclusions.

The Mechanism of the Reaction.

The hydrolysis of a protein evidently requires that the protein molecule shall receive energy from some source or other. For the hydrolysis occurs with appreciable speed in the absence of catalytic agents, only at high temperatures. In this case the source of the energy taken up by the protein may very well be the kinetic energy of certain water molecules which are moving at greater than mean velocities of the given temperature, for only a few of the molecules split apart at any instant of time under these conditions.

In the experiments we have performed it has been shown that both hematoporphyrin and oxygen are necessary, and that there must be an additional source of energy in light, presumably the light absorbed by the hematoporphyrin. In the absence of light at such low temperatures as we have employed, 20° - 45°C., no appreciable hydrolysis of the protein occurs.

Our first idea was that hematoporphyrin which is a salt, the colored constituents being in the cation, unite with the protein molecule which at the pH we employed was electro negative. So that the first step consisted in the

formation of a hematoporphyrin-protein salt. It may be said in favor of this theory, that on boiling and coagulating the protein the whole, or a large part of the hematoporphyrin, remains attached to the coagulated protein.

Assuming then that this combination occured we supposed that the hematoporphyrin received the light energy and being activated thereby, radiated a part of it at a lower frequency as a fluorescent light in the red part of the spectrum, and the remainder of the energy was passed over to the attached protein, activating it. Being thus activated it became capable of uniting with any active water molecules, and was thereby hydrolyzed at some amino-acid junctions. The effect of temperature which increased the rate very materially might be supposed to increase the number of active water molecules and thus play its part in the velocity of the reaction. It is certain that the radiated energy (fluorescent radiation) is not necessary for the hydrolysis, first because it occurs in the red end of the spectrum and such frequencies do not affect the protein and second because it occurs in the absence of oxygen and produces no hydrolysis.

This view was found to be incomplete when I discovered that oxygen as well as hematoporphyrin had to be present. And further that hematoporphyrin is not

necessary, although oxygen is necessary for hydrolysis if we use ultra violet light. It was found that ultra violet light plus oxygen hydrolysed the protein, although at a relatively slow rate. Hematoporphyrin in this hydrolysis appears to play a role not unlike that of chlorophyll in the synthesis of formaldehyde. Ultra violet light alone will convert formaldehyde to a carbohydrate, but in the presence of chlorophyll ordinary light will do the same.

Here ultra violet light hydrolyses in the absence of hematoporphyrin, but ordinary light will accomplish the same result if hematoporphyrin be present. The hematoporphyrin makes available the energy of longer wave lengths.

We have, then, three or four elements recognized in the reaction (1) molecular oxygen; (2) hematoporphyrin; (3) water; and (4) radiant energy.

The next fact which must be considered is that the hematoporphyrin is itself destroyed in the presence of light and oxygen; but is not destroyed as rapidly, if it is at all, by light in the absence of oxygen. This fact indicates that the oxygen oxidizes hematoporphyrin when the latter has been excited by absorbing light.

Our theory of what happens is hence as follows: Hematoporphyrin unites with the protein to form a hemato-

porphyrin protein salt compound. The hematoporphyrin absorbs light both in the ultra violet and in the visible spectrum. This absorption increases its energy content, it does not, however, increase it sufficiently to lead to the destruction of the molecule, nor to the hydrolysis of the protein. At any rate if any hydrolysis occur it is at a slow rate. Most if not all the absorbed energy is radiated as fluorescent or Raman light.

But when oxygen is present with its large store of energy, it is able to unite with the excited hematoporphyrin molecule, and to oxidize it, passing its energy over to it. This has a double result: it so increases the energy content of the hematoporphyrin that the latter becomes unstable and decomposes; and at the same time a portion of its energy is passed through its union with the protein to the latter and leads to its excitation also. This excited or anakinetic protein molecule then unites with the water and is hydrolyzed.

The whole process would be a true catalytic process were it not that the hematoporphyrin was destroyed. For in that case the molecule of hematoporphyrin might function many times as a carrier of energy from the oxygen to the protein.

It is suggested that with this limitation, hematoporphyrin may be called a proteolytic enzyme.

Furthermore, it is an enzyme of considerable specificity since it is a proteinase acting with any speed only on a few proteins and preeminently on fibrinogen. In fact, the results of its action appears to be closely similar to that of thrombin, and even more similar to the fibrinoginase in Crotalus adamanteus venom as Professor Mathews has pointed out.

Acknowledgement

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