stances produced by irradiation of this medium. About two-thirds of the total effect can be attributed to H_2O_2 or its reaction products, while the rest results from an action on the medium not due to H_2O_2. On the other hand, the frequency of genetic changes is not affected by the medium in which the organisms are irradiated nor does irradiated medium or medium to which H_2O_2 was added induce such changes. The failure to find an effect upon the genetic mechanism is interpreted as probably the result of the failure of the substances produced in the medium to reach the micronuclei which are situated some microns below the cell surface. The corollary to this is that the nongenetic changes are due to alterations in the superficial layers of the cell.

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Spectrophotometric Studies on Mixtures of Purified Diphtherial Toxin and Ferriprotoporphyrin IX.*† (19680)

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Pappenheimer(1-3) has proposed that diphtherial toxin is the protein moiety of diphtherial cytochrome b. Direct evidence for this concept would be available if it could be demonstrated that purified diphtherial toxin interacts with iron-porphyrins in a limiting molar ratio of 1:4 to form complexes spectrophotometrically and enzymatically related to cytochrome b. In other porphyrin-protein systems, specific interactions occur *in vitro* under a variety of experimental conditions. In the case of hemoglobin synthesis, the heme-globin linkage is not affected by considerable alteration of the protohemin‡ structure(4). Thus, mesohemoglobin and hematoglobin have been prepared by Hill and Holden(5) and synthetic hemoglobins from rhodohemin, diacetyldeuterohemin and pheohemin-b by Warburg and Negelein(6). All of these compounds combined reversibly with oxygen. Experiments on the recombination of the protein moiety of horseradish peroxidase with various hemins have been summarized by Maehly(7). The formation of methemalbumin has been shown by Rosenfeld and Surgenor(8) to occur over a wide pH range and to be unaffected by small changes in ionic

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‡ The nomenclature and terminology of Lemberg and Legge(4) is used in this paper when designating porphyrin derivatives and complexes.

strength and the specific nature of the buffer ions.§ Serum albumin also binds bilirubin and protoporphyrin(9).

Rawlinson and Hale(10,11) have shown that the major iron-porphyrin present in *cells* of *C. diphtheriae* is ferriprotoporphyrin IX, which is also considered to be the prosthetic group of mammalian cytochrome b(4). The possibility exists, therefore, that diphtherial toxin would interact with hemin (ferriprotoporphyrin IX chloride) if, indeed, diphtherial toxin were the protein moiety of diphtherial cytochrome b. Accordingly, the present report concerns results of spectrophotometric examinations of purified diphtherial toxin-hemin mixtures and the failure to obtain evidence for the formation of specific complexes.

Experimental. Materials. The highly purified *diphtherial toxin,* previously described (12), contained between 2250 and 2420 flocculating units/mg protein nitrogen. The preparations were at least 85% electrophoretically homogeneous in pH 7.5-7.6 phosphate buffer of ionic strength 0.2. The protein molarity was calculated assuming a mean molecular weight of 72,000(13) and a nitrogen-to-protein factor of 6.25. *Human serum albumin, Fraction V,* was prepared according to the method of Cohn and his associates(14), and was at least 95% electrophoretically homogeneous in pH 8.6 barbiturate buffer of ionic strength 0.1. The molarity of solutions was calculated, using a molecular weight of 69,000 and a nitrogen-to-protein factor of 6.25. *Crystallized hemin* (ferriprotoporphyrin IX chloride, Eastman Kodak) was recrystallized 3 times from pyridine-chloroform by the method of Fischer(15). Solutions were prepared by dissolving an accurately weighed sample of the 3 times recrystallized hemin in 10 ml of 0.1 N NaOH and immediately diluting to 1 liter with distilled water. The solutions were used within a 4-hour period and then discarded(8). *Spectrophotometric measurements* were made in the Beckman model DU spectrophotometer with Corex cells of 1 cm optical path.

Effect of molar ratio of hemin to toxin. Molar ratios of hemin to toxin of 0.5:1, 1:1, 2:1, 4:1, and 8:1 were prepared by adding calculated volumes of hemin solution, previously adjusted to ionic strength 0.15 with sodium chloride, to aliquots of an 8.1 x 10^{-6} M toxin solution in pH 7.4 phosphate buffer of ionic strength 0.15. The absorption spectra were measured at 380-640 mμ after initial mixing, after incubation for one-half hour at 38°C, after further incubation for one hour at 38°C, and after standing overnight at 0°C. The spectra were compared with those of freshly prepared hemin solutions in the absence of toxin and of a toxin solution in the absence of hemin. All samples were read against a blank consisting of the appropriate proportions of phosphate buffer and 0.15 M sodium chloride.

No evidence for the interaction of diphtherial toxin with hemin was observed in the 16-fold range of molar ratios tested. Reversal of the order of mixing of the reagents was without effect. Four to one molar ratios of hemin to diphtherial toxin at pH 7.4 and ionic strength 0.15 flocculated with antitoxin to the same titer as toxin controls. The Minimum Reacting Dose in the rabbit was also unaffected by the presence of hemin.

Effect of pH and buffer ions. Glycine, veronal, acetate and phosphate buffers were prepared in overlapping pH values from pH 2-12 at ionic strength 0.15. One volume of hemin solution, adjusted to ionic strength 0.15 with sodium chloride, was added to one volume of an 8.1 x 10^{-6} M solution of diphtherial toxin in 0.15 M sodium chloride. Two volumes of buffer were added and the solutions incubated at 38°C for one-half hour. The pH was then measured and the samples read in the spectrophotometer at 380-640 mμ. The final ionic strength of each sample was 0.15. The molar ratio of hemin to toxin was constant at 2:1. The spectra of hemin solutions in the absence of toxin under the same conditions of pH, ionic strength, incubation time, and temperature were also obtained.

The results at 3 typical pH values are shown in Fig. I. An increase in extinction in

§ The author wishes to thank Drs. Rosenfeld and Surgenor for making available a manuscript of their paper prior to its publication. The technics employed in the present study were patterned after those of Rosenfeld and Surgenor(8).

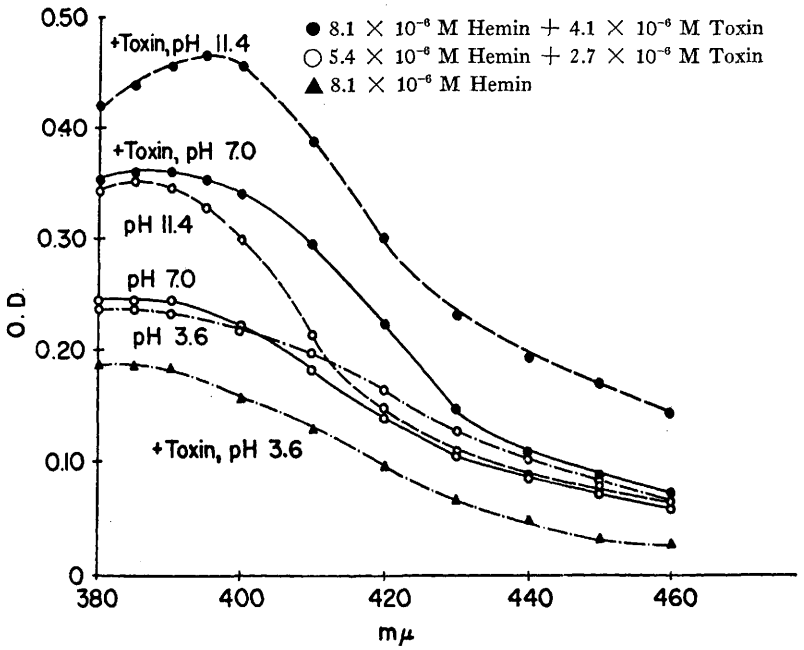

FIG. 1. Comparison of absorption spectra of hemin in presence and absence of diphtherial toxin at representative pH values in buffers of ionic strength .15: pH 3.6 veronal, pH 7 phosphate, and pH 11.4 glycine-sodium hydroxide.

the presence of diphtherial toxin was observed at all pH values where the toxin remained soluble. This non-specific dispersing effect of proteins on hemin solutions has been observed with other protein-hemin systems(8). A shift in the hemin absorption maximum in the presence of diphtherial toxin was noted only at pH values exceeding pH 10.

Precipitation occurred at pH 4.4, 4.9 and 5.6 when acetate or veronal buffers were added to toxin or to toxin-hemin mixtures. The precipitates were insoluble in water and in pH 2-10 buffers of ionic strengths 0.15 but soluble in dilute sodium hydroxide. The latter solutions, when prepared from toxin-hemin mixtures, gave absorption spectra of the type shown for pH 11.4 in Fig. 1. No spectrophotometric evidence for specific interaction could be obtained when toxin-hemin mixtures precipitated at pH 5.0 with acetate buffer were shaken at 0°C in a 10 KC sonic oscillator (Raytheon, 150 watts) for 10, 20, 30 or 60 minutes.

Effect of ionic strength. One volume of hemin solution in 0.001 N NaOH was added to one volume of a 6.16 x 10^{-6} M aqueous solution of diphtherial toxin to give a molar ratio of hemin to toxin of 4:1. Two volumes of buffer of appropriate concentration to give the desired final ionic strength were then added. The absorption spectra from 380-600 mμ were compared with hemin blanks consisting of the above system except for the substitution of water for toxin. Samples were prepared and measured in this manner at ionic strengths 0.05, 0.10, and 0.32, each at pH 2.0 (glycine buffer), pH 4.0 (acetate buffer), pH 7.1 (phosphate buffer), and pH 9.4 (glycine buffer).

No evidence for the specific interaction of diphtherial toxin was observed under any of the above conditions. The absorption spectra varied with pH as previously described but were essentially independent of ionic strength. The non-specific increase in extinction in the presence of toxin was observed at all wavelengths and at all pH values and ionic strengths.

Comparison of diphtherial toxin-hemin and serum albumin-hemin mixtures under alkaline conditions. The shift in absorption spectra observed when hemin was added to diphtherial

toxin above pH 10 prompted a comparison of the spectra of toxin-hemin and albumin-hemin mixtures under the same conditions of alkalinity in the presence and absence of a reducing agent. Qualitative examination of 2:1 molar ratios of hemin:protein in dilute sodium hydroxide showed identical absorption maxima for diphtherial toxin and serum albumin hemichromes at 392-398 mμ and a very diffuse maximum at 560-580 mμ. Reduction with sodium hydrosulfite gave peaks at 423-424 mμ, 527-528 mμ, and 557-558 mμ for both hemochromes. These results were highly indicative of a non-specific reaction between hemin and denatured protein.

A stoichiometric study was carried out by adding one volume of varying concentrations of hemin to one volume of a constant concentration of diphtherial toxin or human serum albumin. The mixture was brought to 1% Na_2CO_3 by the addition of an equal volume of 2% Na_2CO_3. Hemin samples were prepared in the same manner, substituting water for the toxin. The blank consisted of 1% Na_2CO_3. A small amount of hydrosulfite was added to each sample and the optical densities measured at intervals of 10 minutes at 424, 528, and 558 mμ until the extinction began to decrease. The maximum reading at each wavelength was used for comparison of extinctions at the various molar ratios tested. Measurements on the hemichromes were made at 395 mμ immediately after mixing the reagents and prior to the addition of hydrosulfite.

The results were qualitatively identical at each of the 3 hemochrome absorption maxima. The optical density of the reduced albumin-hemin mixtures was found to be a linear function of the molar ratio between 0.5:1 and 8:1. The reduced toxin-hemin curve was superimposable on the albumin-hemin curve up to a molar ratio of about 4:1. Beyond this point, a small but reproducible decrease of slope was observed. Neutralization of 4:1 hemin-toxin alkali hemochromes resulted in dissociation. A typical experiment at the 424 mμ hemochrome absorption maximum is shown in Fig. 2. The optical densities of the albumin-hemin mixtures have been corrected to the same concentration as the toxin-hemin mixtures and the curves for each protein are directly comparable.

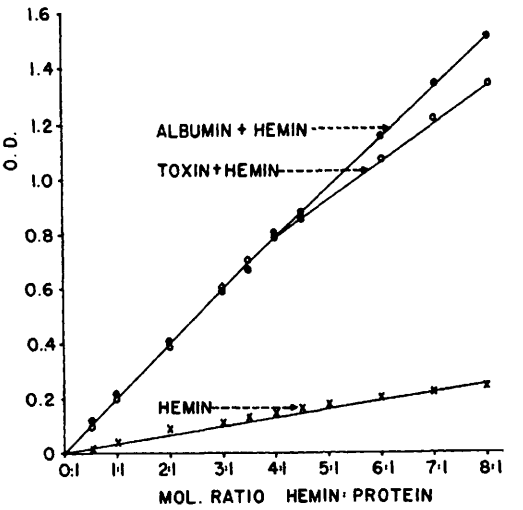

FIG. 2. Comparison of human serum albumin and diphtherial toxin alkaline hemochromes at 424 mμ.

The albumin and toxin hemichrome absorptions at 395 mμ were identical linear functions of the molar ratio of hemin to protein. The slight break in the toxin hemochrome curve at 4:1 was not observed with the oxidized mixtures.

Discussion. Diphtherial toxin does not specifically interact with hemin under the conditions investigated. Present knowledge of protein-porphyrin interactions would lead to the prediction that such an interaction would occur *in vitro* if diphtherial toxin were the protein moiety of cytochrome b. However, it cannot be assumed that the diphtherial toxin-hemin system necessarily falls within the generalizations previously cited for other protein-porphyrin interactions. The results of this study may only be interpreted as a failure to provide evidence for the Pappenheimer proposals on the primary relationship between diphtherial toxin and cytochrome b.

Summary. Diphtherial toxin-hemin mixtures prepared under a wide range of conditions of pH, ionic strength, buffer ions, molar ratio of hemin to toxin and incubation time have been examined spectrophotometrically. Combination of purified diphtherial toxin with hemin was observed only under conditions leading to non-specific alkali hemi- and hemochrome formation. The significance of these

findings to the Pappenheimer proposals relating diphtherial toxin to cytochrome b is discussed.

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Effect of Certain Plant Growth Substances on Oxidative Phosphorylation In Rat Liver Mitochondria.* (19681)

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Recent work has resulted in technics whereby mitochondria from a number of mammalian tissues can be isolated by differential centrifugation and their metabolism studied using conventional procedures(1). It appears that the mitochondria are the chief intracellular sites of oxidative phosphorylation(2). A number of compounds, among them dinitrophenol(3,4) and the barbiturates (5), have been shown to uncouple phosphorylation from oxidative processes with a concomitant increase in respiration. Bonner(6) reported that 2,4-dinitrophenol in concentrations which inhibited section growth was a powerful promoter of *Avena* respiration. The effects of plant growth substances on certain plants is reflected in increased total respiration, with or without accelerated growth and development(7). It was suggested(8) that perhaps 2,4-dichlorophenoxyacetic acid (2,4-D) and other plant growth substances might be uncoupling agents. The above considerations led us to investigate the effects of various plant growth substances on a mammalian tissue system which would test this possibility.

Experimental. General procedures and methods were similar to those previously described(5,9). Rat liver mitochondria were isolated according to the methods of Schneider and Hogeboom(10). In the experiments designed to demonstrate a stimulatory effect on respiration the phosphate-deficient system of Judah(3) was used. The mitochondria used in these experiments were washed 3 times with 0.25 M sucrose in order to remove most of the inorganic phosphate. Other experimental details are given in the figure legends.

Results. In the experiments designed to show the effect of 2,4-D on oxidative phosphorylation (Fig. 1) it was found that this compound was a potent uncoupling agent. At concentrations of 2,4-D as high as 1×10^{-3} M there was little effect on respiration in the test system and yet the P:O ratio fell to 20%

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