load in adrenal-demedullated anesthetized rats pre-treated with large doses of deoxyglucose. This suggests that other pathways of glucose disposal must be utilized which compensate for inhibition of oxidation. These avenues of glucose disposal will be explored. Inhibition by DHE of the hyperglycemia which results from injection of deoxyglucose into intact fasted rats suggests that this glucose analogue is capable of stimulating release of epinephrine from the adrenal medulla. The lack of hyperglycemia following deoxyglucose injection in adrenal demedullated rats lends support to this hypothesis.

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Mechanism of Inactivation of Serum Oxidase (Ceruloplasmin) Activity by Ultraviolet Radiation. (24729)

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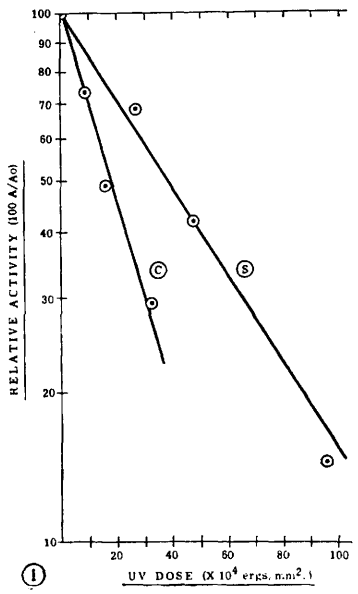
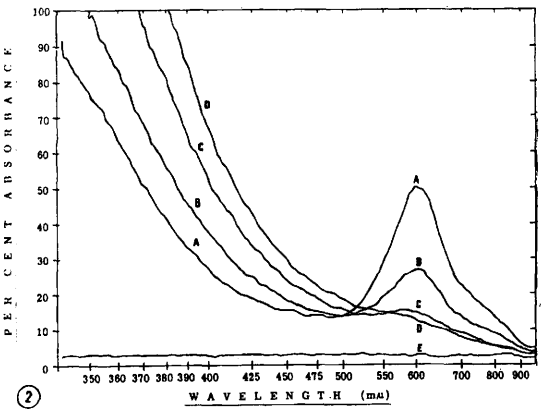
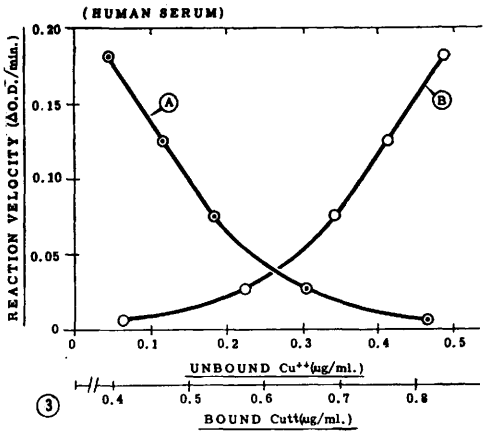
This paper is concerned with the effect of ultraviolet radiation on oxidase activity of ceruloplasmin, the copper containing protein in blood. Ceruloplasmin has a molecular weight of approximately 151,000 and 8 atoms of copper/molecule(2,3). It contains over 90% of copper of blood and is thought to be the copper storage and transport protein. That it also has oxidase activity is interesting. The exact enzymatic role of this copper protein is unknown at present although there have been reports that it can oxidize serotonin(4,5). Data are presented to show that as oxidase activity of serum or ceruloplasmin is destroyed by UV radiation, bound copper in the enzyme is changed resulting in increased amount of "unbound" copper.

Materials and methods. Total copper(6) and direct copper(7) determinations were made by the method of Gubler *et al.* A stainless steel water bath was constructed to hold Petri dish 5 cm in diameter. Ice water at 0°C and not varying more than 0.1° was continuously circulated through water bath. The solution to be irradiated with ultraviolet light was placed in the Petri dish. After temperature equilibration was established, the sample was exposed to UV radiation for definite time interval. A Mineralight model V-41 ultraviolet lamp (Ultraviolet Products, San Gabriel, Calif.) was used as ultraviolet (UV) source. The UV source was mounted above the solution so that it could be moved in vertical or horizontal direction. The lamp was placed 2.5 cm above the solution. At this height the intensity of radiation striking the solution was $[1.63 \pm 0.09] \times 10^4$ ergs/minute/mm^2 as determined by uranyloxalate actinometric method(8). Value of quantum yield used in the calculations was 0.60(9). Serum volumes were kept at 6 ml permitting thin uniform layers to be irradiated (3 mm). With purified ceruloplasmin, volumes of 1 ml and 3 ml were used. Evaporation was not noted either with ceruloplasmin or serum. After various samples were irradiated, they were permitted to warm up to 25° C. Aliquots were then analyzed for oxidase activity, total copper, and direct reading copper. Copper free glassware and reagents were used throughout. Serum samples were obtained from staff personnel. Limited amounts of ceruloplasmin were kindly donated by Upjohn Co., Ortho Research Fdn., and Red Cross Blood Program. *Assay.* When N,N-dimethyl-p-phenylenediamine dihydrochloride (DPP) reacts with serum or ceruloplasmin, the substrate is oxidized to a red colored com-







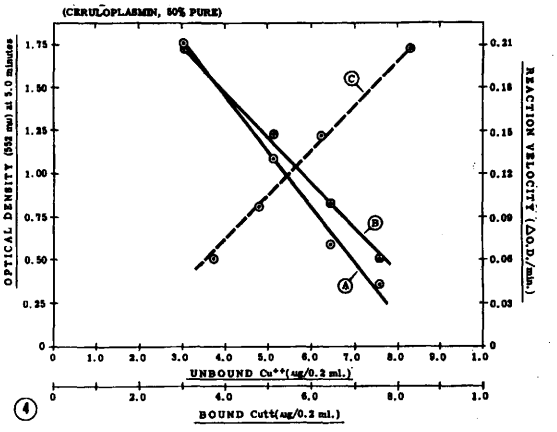


FIG. 1. Effect of UV light (2537 Å) upon ceruloplasmin oxidative activity and human serum oxidase at 0°C. Ordinates: Relative activity ($A\% = 100\ A/A_0$); abscissa: UV dose expressed as ergs/mm². Serum sample (S) contained 87 mg/ml protein and 0.83 μg Cu^{++}/ml in the bound state; the ceruloplasmin sample (C) contained 25.8 mg/ml protein and 41.2 μg Cu^{++}/ml in the bound state.

FIG. 2. Absorbance curves in 340-900 mμ region for 4 ceruloplasmin samples (A-D) exposed to 0, 32.6×10^4, 97.6×10^4, and 196×10^4 ergs/mm² of UV radiation (2537 Å), respectively, and one sample of a $CuSO_4$ solution containing 30 μg Cu^{++}/ml. Before irradiation, ceruloplasmin samples each contained 17.5 μg Cu^{++}/ml in the bound state and 12.1 μg Cu^{++}/ml in the unbound state as well as 10.6 mg/ml protein.

FIG. 3. Relationship between serum oxidase activity and unbound copper (curve A) and bound copper (curve B) in 5 serum samples exposed to different doses of UV radiation.

FIG. 4. Relationship between oxidase activity of human ceruloplasmin (50% pure) and unbound copper (curves A and B) and bound copper (curve C) in 4 samples irradiated with UV light for different lengths of time.

pound(10). Ceruloplasmin and serum oxidase activity was measured by noting change in optical density with time at 552 mμ in a model DU Beckman Spectrophotometer, the cuvette chamber of which was kept at 25 ± 0.1°C by means of thermospacers. Enzymatic activity was expressed either as initial reaction velocity (ΔOD/min or ΔOD/min/μg

Cu^{++}) or as optical density after a given interval of time, such as 5 min. Optical density of serum at 552 mμ was minimal. The blank cuvette contained 1.5 ml distilled water plus 1.5 ml of serum while in the experimental cuvette 1.5 ml of 0.35% DPP solution was added to 1.5 ml serum. The timer was started just as substrate was added. Final pH was 6.2. Under these conditions, the enzyme was assayed at optimum substrate concentration, at optimum pH and at constant temperature (11). Buffer ions which interfere with the reaction(12) were not present. In experiments using purified ceruloplasmin, 1.3 ml of UV irradiated serum (oxidase activity destroyed) plus .2 ml ceruloplasmin were substituted for 1.5 ml serum(13). Absorbance curves of irradiated ceruloplasmin samples were run in model DK-2 Beckman Recording Spectrophotometer.

Results. The effects of UV irradiation on human serum and purified ceruloplasmin kept at 0°C are presented in Fig. 1. The logarithm of relative activity (ordinate) is plotted against corresponding dose of UV irradiation (abscissa). The logarithm of relative activity was proportional to incident energy within experimental error. The results may be represented mathematically by equation 1: $A = A_0 e^{-kD}$(1), where A is oxidase activity after a UV dose D (ergs/mm^2), A_0 is activity of control or nonirradiated sample, and k is a constant. The ratio A/A_0 is the relative activity.

If the irradiation process is continued for sufficiently long period it is possible to inactivate completely the oxidase activity. Of course the thinner the sample layer the shorter the irradiation period necessary to accomplish inactivation.

In experiments in which ceruloplasmin samples were irradiated with UV light, the characteristic blue color changed to lighter shades and the change in intensity of color appeared proportional to amount of radiation absorbed by the solution. The sample receiving enough UV radiation to reduce oxidase activity to less than 5% of normal was light greenish-blue resembling the color of an aqueous solution of cuprous chloride. Absorbance curves in the region 340-900 mμ of 4 ceruloplasmin samples irradiated for 0, 20, 60 and 120 min., respectively, and one sample of cupric sulfate (E) containing 30 μg/ml of Cu^{++} are shown in Fig. 2. Maximum absorbance peak for ceruloplasmin occurred at 600 mμ. Sample A, the non-irradiated control, had the highest absorbance. Sample D, which received a UV dose of 196 x 10^4 ergs/mm^2, showed practically no peak at 600 mμ. Samples B and C fell between A and D in the expected sequence. After passing through their respective maxima, absorbance curves crossed each other in the 500-550 mμ region resulting in complete reversal in the order noted at 600 mμ. This may be due, in part, to greater denaturation of protein material in samples exposed to higher doses of radiation which then could cause a considerable amount of non-specific absorption to occur at the lower wavelengths.

In Fig. 3 data from a typical experiment in which five 6 ml samples of human serum were each exposed to different amounts of UV radiation, are presented to show relationship between resulting fall in enzymatic activity and change in direct reading copper hereafter referred to as "unbound" copper (curve A) and bound copper (curve B) content of samples determined as the difference between total copper and direct reading copper. On curve A, the sample represented by highest enzymatic activity and lowest "unbound" copper is the control which received no irradiation. The other 4 samples, represented by points indicating an increase in "unbound" copper values, received 24.4 x 10^4, 48.9 x 10^4, 97.8 x 10^4, and 196 x 10^4 ergs/mm^2 of UV radiation (2537 Å), respectively. Increase in direct reading copper was inversely proportional to fall in activity in the range in which enzyme activity varied from normal to approximately 30% of normal. Below this point, the change in "unbound" copper was greater than the corresponding fall in oxidase activity. When oxidase activity was plotted against bound copper (curve B), a mirror image of curve A was obtained. The same type of relationship was found when purified human ceruloplasmin was employed in place of serum. The data from such an experiment are shown in Fig. 4. Enzymatic activity was expressed in 2 ways: (1) in curve A, optical density read

at 552 mμ 5 min. after the reaction began was plotted against "unbound" copper content, and (2) initial reaction velocity was used as measure of enzyme activity in curve B. The linear relationship between reaction velocity and bound copper is shown by curve C. The difference between the shape of curves presented in Fig. 3 and those in Fig. 4 is that in the case of the experiment with partially purified ceruloplasmin the relationships were linear over the complete range studied.

The effect of —SH inhibitors on serum oxidase was also tested. Mercuric chloride, p-chloromecuribenzoate (sodium salt), o-iodobenzoic acid, and iodoacetic acid (sodium salt), were used in final concentrations of 5×10^{-4}M and 5×10^{-3}M. Inhibitors were incubated with serum for 30 minutes before the assay began. All showed no significant effect on activity except mercuric chloride. At lower concentrations, an increase in optical density was noted without an increase in rate (O.D. *vs.* time) and this was due to formation of a cloudy reaction upon addition of DPP. At higher concentration, a flocculent precipitate occurred when the substrate was added and the reaction mixture turned a deep red color.

In a limited number of experiments, an attempt was made to reactivate oxidase activity which had been initially destroyed by UV radiation. This was done by incubating the inactivated enzyme with either ascorbic acid plus copper ions or cysteine. All results to date have been negative. In addition, it was not possible to protect adequately the oxidase activity from the effects of UV radiation with cysteine.

Discussion. Apparently the mechanism of inactivation of serum oxidase (ceruloplasmin) is not due to oxidation of –SH groups, but to a change in state of copper in the protein molecule from bound to "unbound." Removal of copper ions by chemical methods also results in loss of enzyme activity(10,14). A similar phenomenon has been shown by Schade and Oyama in the case of siderophilin(15). Its iron binding capacity was inactivated after exposure to UV irradiation.

Recently Heyndrickx(16) has shown that when human serum is exposed to UV irradiation there is an increase in number of –SH groups as measured by the amperometric technic. This as well as the increase in viscosity that is seen when serum is irradiated with UV light, can be taken as indication of unfolding or increase in asymmetry of the protein by splitting of certain bonds or less stable linkages in the molecule. In the case of ceruloplasmin, the decrease in its bound copper content after irradiation could also be explained by assuming rupture of linkages between copper atoms and specific groups of the protein. Apparently ceruloplasmin is a conjugated protein, with copper as an essential part of the molecule, held in a coordination complex or in some similar arrangement. At the present time very little is known in regard to the nature of the chemical bond or linkage between copper and the apoprotein of ceruloplasmin as well as this type of linkage in other copper containing enzymes(17). Although Corwin (18) has given an excellent theoretical review of some of the possible complex configurations in which the copper atom can exist, experimental data are still lacking to show the exact nature of the linkage in copper proteins.

Summary. Oxidase activity of serum and purified ceruloplasmin is inactivated by ultraviolet irradiation at 2537 Å. Loss of enzymatic activity was correlated with a change in state of bound copper of ceruloplasmin to "unbound" copper. When relative enzymatic activity is plotted against radiation dose, the relationship was exponential ($A = A_0e^{-kD}$).

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Use of Pressure Cell to Prepare Cell Walls from Mycobacteria.* (24730)

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In a recent report(1) separation of cell wall from internal protoplasm of mycobacteria based on the principle used by Salton and Horne(2) was described. Although yields of cell walls obtained were small, they permitted limited biologic analyses which demonstrated the immunologic significance of this fraction. The demand for larger quantities of cell wall material was met by utilizing a commercial pressure cell in place of the Mickle apparatus (1,2) to achieve selective disruption of organisms. In the resulting cell wall fractions, however, considerable amounts of denatured protoplasmic substances adhered to inner surface of walls and could not be washed off. Further investigation revealed that an increase in temperature at the orifice of the needle valve during bleeding of very highly compressed bacterial suspension apparently caused partial denaturation of protoplasmic components. Since conventional cooling systems were not adequate, an experimental instrument was devised which allowed sufficient cooling of the orifice and needle by exposing them to compressed CO_2 released at a separate needle valve. This paper describes application of such a pressure cell for preparation of cell walls of mycobacteria.

Description and operation of pressure cell. The instrument (Fig. 1) consists essentially of a steel cylinder and piston (A) and 2 needle valve systems (B and C). The outlet at bottom of cylinder is connected by steel tubing to the body of the high pressure needle valve (B) used for bleeding the compressed bacterial suspension. This connection included a high pressure stainless steel nipple, gland nuts, and compression sleeves purchased from American Instrument Co., Silver Spring, Md. The 3-way cross-type stainless steel valve (B) with compression packing was ordered from the same company. Its upper opening (D) is connected by steel tubing to the expansion chamber of the needle valve C (brass body and steel needle) used to bleed compressed CO_2 to cool the needle and orifice of valve B. The lower opening (E) is connected by plastic tubing with suction flask from which glass tubing leads to closed hood equipped with exhaust fan and incinerator. The piston ring, a Parker O Ring No. 2-218, made of compound 49-031, was obtained from Parker Seal Co., Clevland, O. *Operation.* Piston and cylinder were cooled in ice water and the cylinder was then filled with the cold mycobacterial suspension (maximum quantity about 100 ml).

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