with plasma. In the absence of added calcium no precipitate formed and no changes in ultracentrifugal properties of β-lipoproteins occurred. Rice starch sulfate precipitates human β-lipoproteins in absence of added calcium, but for maximal precipitation of several animal β-lipoproteins calcium was required.

Quantitative evaluation of analytical ultracentrifuge patterns is fraught with difficulties which combine to cause a sizable error in determinations, particularly when lipoprotein concentrations are either very low or very high. Thus, the difference from quantitative precipitation values for human sera seen in Table I are well within the experimental error of the method.

Summary. Ultracentrifuge analysis confirms that polyanion-complex precipitation method for human serum β-lipoproteins is adequate and does not denature the lipoproteins when either rice starch sulfate or mepesulfate are used as precipitating agent. The method is applicable to isolation of β-lipoproteins from several but not all species of animals.

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Received April 29, 1960. P.S.E.B.M., 1960, v104.

Determination of Ceruloplasmin Oxidase in Serum. (25928)

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Ceruloplasmin is a copper oxidase which can be determined photometrically by rate of oxidation of p-phenylenediamine(1,2,3,4). Oxidation of p-phenylenediamine (PPD) by serum is catalyzed by serum ceruloplasmin and by copper and iron present in serum as well as in water and reagents used in the test. To determine oxidation due to ceruloplasmin alone, it is necessary either to correct for or inhibit catalytic oxidation due to metals. Both approaches have been used. In the method of Ravin(3) a serum blank is determined in which ceruloplasmin is inhibited by azide. In methods of Humoller *et al.*(2) and Broman (4), catalytic oxidation by metals is inhibited by complexing them with ethylenediaminetetraacetate (EDTA). This paper presents (a) a modification of Ravin's method which points out an error in original method, (b) a study of various factors which must be controlled in the procedure, (c) normal values obtained with this procedure and (d) results of study of validity of methods employing EDTA to inhibit metal-catalyzed oxidation of PPD.

Methods. Reagents. 1. Acetate buffer, 0.1 M, pH 6.0. Add 10 ml 0.1 M acetic acid (0.57 ml glacial acid plus water to 100 ml) to 200 ml 0.1 M sodium acetate (1.36 g $CH_3COONa \cdot 3H_2O$/100 ml). pH should be 5.95-6.00. 2. Sodium azide, 0.1% in acetate buffer. pH must be 5.95-6.00. Store in refrigerator. 3. p - Phenylenediamine · 2HCl, 0.25% in acetate buffer. Recrystallize commercial salt as follows: Dissolve in water, add Darco charcoal, warm in water bath at 60°C with occasional mixing and filter. Add acetone to filtrate until turbidity appears, refrigerate several hours, filter off PPD · 2HCl and dry the crystals in the dark in vacuum

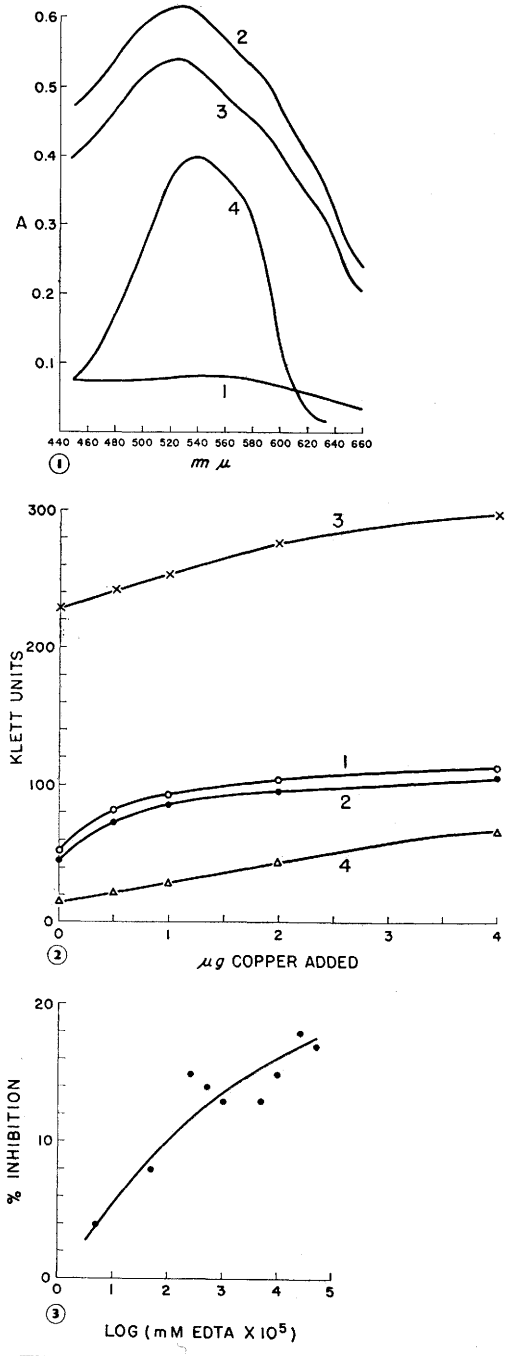

FIG. 1. Absorption curves in test for ceruloplasmin. Curve 1—blank *vs* water at 30 min. Curve 2—normal serum *vs* water at 30 min. Curve 3—normal serum *vs* blank. Curve 4 — Pontacyl violet 6R, 0.0159 mg/ml of 0.5% acetic acid, read *vs* water. Perkin-Elmer recording spectrophotometer, model 4000-A.

FIG. 2. Effect of azide on substrate oxidation by added copper. Curve 1—reagent blank. Curve 2—reagent blank + azide. Curve 3—serum. Curve 4—serum + azide.

FIG. 3. Inhibition of oxidase activity by EDTA.

desiccator over anhydrous $CaCl_2$. Store in brown bottle. To prepare 0.25% reagent, dissolve 12.5 mg in 3.0 ml acetate buffer and, using narrow range pH paper, adjust pH to 6.0 by adding 1 N NaOH dropwise from 0.2 ml serologic pipet (approximately 0.1 ml required). Add acetate buffer to final volume of 5 ml. This reagent can be used to about 2 hours after preparation if kept in the dark. *Procedure with Beckman model DU spectrophotometer, equipped with thermospacers.* Compartment temperature kept at 37°C. To "blank" cuvet (1 cm light path) add 1 ml azide reagent, 1 ml buffer and 1 ml of PPD. To "test" cuvet add 2 ml buffer and 1 ml of PPD. Place cuvets in compartment and allow 5 minutes for temperature equilibration. Add 0.1 ml serum to each tube from a TC pipet. Read absorbance of "test" against "blank" at 530 mμ at exactly 10 min. and again at 40 min. after addition of serum. The unit of ceruloplasmin activity is arbitrarily defined as increase of 0.001 in absorbance in 30 min. under our conditions. Calculation: ceruloplasmin units = (A_{530} at 40 min − A_{530} at 10 min.) x 1000. *Procedure with Klett filter photometer.* The "test" and "blank" are set up in double quantities in matched test tubes. Tubes are incubated in water bath at 37°C and covered by heavy black cloth to exclude light except when tubes are removed at 10 and 40 minutes for reading with Klett 54 filter. An artificial standard of Pontacyl violet 6R (Du Pont) is employed. A solution containing 15.9 mg of dye/liter of 0.5% acetic acid is equivalent to 400 units (units same as absorbance units defined above). Calculation:

$$\text{Ceruloplasmin units} = \frac{\text{Klett reading at 40 min. minus Klett reading at 10 min.}}{\text{Klett reading of dye standard against water}} \times 400.$$

Investigation of method. Artificial standard. Because of the complicated nature of oxidation of PPD, a standard cannot be set up with this substrate. A dye could not be found which had an absorption curve identical to

that of PPD oxidized by ceruloplasmin (Fig. 1). The closest was that of Pontacyl violet 6R (Fig. 1). The artificial standard is for use with Klett 54 filter with Klett photometer and was not checked with any other filter photometer. *Inhibition of ceruloplasmin by azide.* Experiments in which serum tests were run with varying concentrations of sodium azide and read against reagent blanks without serum, revealed that complete inhibition of enzyme activity occurred in the range of 0.008 to 0.016%. Final concentration of sodium azide employed for the blank is 0.032%.

Destruction of ceruloplasmin at low pH. Erratic and spuriously low results were frequently obtained by original technic of Ravin (3) in which serum is mixed with unbuffered, unneutralized PPD reagent prior to addition of acetate buffer. Variable time of contact with this acid reagent (final pH 2.8) resulted in variable destruction of oxidase activity. In one typical experiment, periods of contact of serum with acid PPD reagent of 1 and 10 minutes prior to addition of buffer, gave ceruloplasmin results 55 and 89% lower, respectively, than result obtained by mixing serum with buffer prior to addition of PPD. This apparently constitutes a technical error in Ravin's method which is avoided in the method presented by buffering PPD.

Results. Ascorbic acid concentration and the lag phase. Evidence indicates that PPD oxidized by ceruloplasmin is again reduced by ascorbic acid present in serum until all ascorbic acid is used up(5). This produces a lag phase in the test which can reach significant proportions. Addition of ascorbic acid to serum to a concentration of 20 mg/100 ml produced a lag of about 5 minutes, during which no increase in absorbance occurred. This level of ascorbic acid is considerably higher than physiological levels. Study of approximately 100 sera by the DU method in which absorbance readings were made at 1-minute intervals for the first 10 minutes, followed by readings at 10-minute intervals up to 60 minutes, revealed that up to 10 minutes from zero time was required for rate to increase to a stable maximum. For this reason the rate in our method is determined after 10 minutes from zero time.

Effect of light. Catalytic oxidation of PPD was increased by exposure to visible light. The test, therefore, must be carried out in the dark.

Variation in pH. Rates of enzyme action were determined in 2 sera by the DU method over pH range of 5 to 7.1 (acetate buffer). Rates were calculated from 10 to 30 minute interval because of a curious phenomenon. At pH 6.0 and higher, the rates, after the lag phase, were constant from 10 to 60 minutes. At pH 5.8 and lower, however, a decrease in rate invariably occurred during 30 to 40 minute period. In most instances this did not appear to be curvilinear, but rather a sudden change to a lower but constant rate. Oxidase activity had a fairly sharp maximum at pH 6.0 with about 15% decrease at pH 5.5 and 25% at 6.5. The buffer has sufficient capacity to keep the increase in pH upon addition of even aged serum to within 0.05 pH unit of buffer pH. Optimal pH has been reported previously to be 5.5(6), between 6 and 7(1), between 5.5 and 6.0(2) and 6.0(7).

Effect of temperature. Activities of 2 sera were studied at 22, 32, 37 and 45°C by the DU method. Arrhenius plots of log of activity against reciprocals of absolute temperature gave straight lines with slopes of −3700. This gives an activation energy μ of 17,000 calories/mole and a Q_{10} between 30 and 40°C of 2.45.

Precision. Reproducibility of the method (95% limits) calculated from 4 sets of 6 replicates on normal sera was ± 4.5% (Klett method).

Normal values. Determinations (DU method) were made on 20 normal adult males and 20 normal adult females. There was no significant sex difference (t test, $P>0.05$) so values were pooled. The 95% normal limits determined by the "normal equivalent deviate method"(8), without log transformation of data, were 280 to 570 units (mean 425 units).

Stability of serum. Stability of 6 normal sera was studied at 25°C, at 4°C and in the frozen state. At 25°, 4 sera deteriorated up to

15% within 24 hours. The other 2 showed no significant decrease in 2 days. No change was observed at 4° within 2 days or in the frozen state within 2 weeks. Stability at 4° and in the frozen state has been reported previously(3,9,10).

The effect of ethylenediaminetetraacetate (EDTA). Experiments were performed by "Klett photometer method." Fig. 2 shows the effect of copper added as cupric sulfate to reagent blanks without azide (curve 1) and with azide (curve 2) and to serum tests without azide (curve 3) and with azide (curve 4). Points are averages of 4 experiments, ordinates are increase in Klett readings made against water from 10 to 30 minutes after start of reaction, and abscissae are μg of copper added to 6.2 ml of test solution. Azide had a measurable, but practically insignificant, effect on copper-catalyzed oxidation of substrate. In serum tests azide inhibited enzymatic oxidation but had no significant effect on copper catalysis. The shape of reagent blank curves is different from that of serum test curves and, without added copper, oxidation rate in reagent blanks is much higher than in tests with serum plus azide. These effects are probably due to binding of free copper by serum proteins other than ceruloplasmin(11,12). Addition of EDTA to final concentration of 0.2 mM decreased Klett units in all reagents blanks of Fig. 2 to less than 6 and in all tests containing serum and azide to less than 18. Iron added as ferric chloride to reagent blanks showed one-twentieth or less the catalytic activity of cupric ions. Azide had no effect on ferric ion activity and, furthermore, as previously reported(4), EDTA was without effect. "Blanks" without added copper or iron showed significant oxidation of substrate (Fig. 2). Analysis after wet ashing(13) indicated that copper content of a reagent blank including azide was less than 0.017 μg/ml. Normally, serum contains about 100 μg of copper/100 ml up to 10 μg of which is loosely-bound, non-ceruloplasmin copper(14,15,16). Use of such a serum in the test for ceruloplasmin adds only 0.0033 μg of copper/ml of test solution.

The effect of EDTA (disodium salt) on test for ceruloplasmin is shown in Fig. 3. Points are averages of 4 experiments. EDTA was added to both "blanks" and "tests" and "tests" were read against "blanks." The % inhibition was calculated from increases in Klett readings from 10 to 40 minutes after start of reaction. Inhibition appeared to approach a maximum of about 20%. The effect of EDTA was preponderantly on rate of color development in the "test," not the "blank," indicating an actual inhibition of enzymatic activity. To obtain an apparent inhibition, there would have to be either lower rates in the "test," higher rates in the "blank" or both. Addition of EDTA produced lower "blanks."

Discussion. Humoller *et al.*(2) used EDTA in final concentration of 0.17 mM (log (mM x 10^5) = 4.23) and read the test against a water blank. They obtained lower results with EDTA than without it but assumed that this was due solely to chelation of free metals present. Broman(4) employed EDTA in the same manner. He stated that EDTA was used in a final concentration of 4×10^{-5} M and presented data indicating no inhibition of purified ceruloplasmin at this concentration. Recalculation from Broman's description of his method, however, indicates that final concentration actually was 4.47×10^{-4} M, at which concentration his data indicated inhibition.

Our experiments led to the following conclusions: (a) reading color produced by serum oxidase activity on PPD against a serum blank containing azide is a valid measure of enzymatic oxidase activity of serum; (b) EDTA successfully inhibits catalytic oxidation of substrate by copper, presumably the predominant non-enzymatic catalyst present, but also produces significant inhibition of enzymatic oxidase activity of serum. It is not known whether EDTA forms a complex with ceruloplasmin through its copper, whether it successfully removes copper from ceruloplasmin, thus leading to inactivation, or whether inhibition is a nonspecific polyvalent ion effect(7). It has been shown that copper can be dissociated from ceruloplasmin under certain conditions(5,18,19). It is also not clear

why only about 20% inhibition of serum oxidase activity could be obtained with EDTA. Presence of 2 ceruloplasmins has been demonstrated in serum(4) and it may be that one is much more resistant to EDTA than the other.

Summary. A method for determination of ceruloplasmin in serum is presented in which rate of oxidation of p-phenylenediamine is measured spectrophotometrically or photometrically. Correction for catalytic oxidation by free metals present is made by reading the test against a serum blank in which enzymatic activity is inhibited by azide. The effects of pH and temperature on the reaction have been investigated and normal values obtained by our method. Ethylenediaminetetraacetate significantly inhibits enzymatic oxidase activity of serum.

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Received March 15, 1960. P.S.E.B.M., 1960, v104.

Uptake of Glycine-1-C^{14} by Connective Tissue. III. Effects of Adrenal Hormones and Aging.* (25929)

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Changes in connective tissue constituents in response to administration of adrenal cortical hormones have been described many times. Histological studies have afforded evidence for their involvement in metabolism of skin connective tissue and granulation tissue of a number of species of animals(1,2,3,4). Metabolic and chemical studies, including those employing tracers, support the conclusion that glucocorticoids inhibit formation of connective tissue components, especially those of the ground substance(5,6,7). However, the extent to which these hormones influence metabolism of the collagenous components of connective tissue is uncertain. Perrone(8) was unable to demonstrate an effect of cortisone upon incorporation of labeled glycine into the collagen of rat skin. Robertson and Sanborn(9), however, found that cortisone reduced (and adrenalectomy increased) collagen concentration in carrageenin granulomas in guinea pigs. Like the carrageenin granu-

* Supported by research grants from Nat. Inst. Health, U.S.P.H.S., Bethesda, Md. For paper II in this series, see Frieden, E. H., *Endocrinol.*, 1956, v59, 69.