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Localization of Ceruloplasmin Biosynthesis in Human and Monkey Liver Cells and Its Copper Regulation

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Hepato-lenticular degeneration, or Wilson's disease, is a genetic error in copper metabolism in man[1]. In this disease, poisoning due to the accumulation of copper is usually accompanied by high deficiency in copper proteid, ceruloplasmin, in blood and tissues. Copper entering the organism is not bound by ceruloplasmin, transferred from a ceruloplasmin molecule onto the appropriate acceptors[2], or secreted from the cell by this protein[3,4], but is accumulated in the cells in large quantities affecting the brain, liver and other organs[1]. In this connection, it would be important to study the mechanism of ceruloplasmin deficiency in Wilson's disease.

Theoretically, ceruloplasmin deficiency may be due either to the formation of a mutant protein with a defect in its molecular structure and function, or to the disturbance in its biosynthetic regulation (and, therefore, a decreased ceruloplasmin concentration) or, finally, both. The present paper considers only the second aspect of this problem, that is, ceruloplasmin biosynthesis and its regulation.

There are data obtained from the study of isolated rat liver by perfusion method with ^{64}Cu indicating that both the label incorporation into ceruloplasmin and the biosynthesis of this protein occur in liver[5]. However, in such experimental procedure there is always a likelihood that ^{64}Cu is incorporated into an already formed ceruloplasmin molecule rather than involved in the protein biosynthesis. Therefore, in our experiments, an attempt has been made to obtain direct evidence of ceruloplasmin biosynthesis in human and monkey liver cells.

The study of the biosynthesis of human and monkey ceruloplasmin has proved, because of a close similarity of the 2 proteins[6], extremely convenient, and it has allowed us to combine experiments both in vivo and in vitro. Thanks to this fact, it has become possible to show that liver seems to be the only organ involved in ceruloplasmin biosynthesis and to demonstrate the induction and repression of ceruloplasmin biosynthesis by copper salts.

Materials and methods

Liver slice incubation technique. The experiments were conducted on male monkeys, *Macacus rhesus*, of 2–2.5 kg body weight. The animal was sacrificed by bleeding, the liver was quickly removed and washed in Ringer-Lock physiological solution by perfusion. Then the liver was placed on ice and slices $2\times3\times0.3$ mm in size were prepared. The slices were washed 3 times in physiological solution to remove loose tissues and were placed in Thunberg's tubes (80–100 mg protein per sample).

Incubation was done in the Warburg apparatus at 37 °C (80–100 c/min) for 30 min in 5 ml incubation medium. The medium composition (in M/l): mannitol, 2.5×10^{-1}; *Tris*-buffer, pH 7.6, 5×10^{-2}; $MgCl_2$, 1×10^{-2}; NaCl, 4×10^{-2}; KCl, 1×10^{-1}; KH_2PO_4, 1×10^{-2}; ADP, 2×10^{-3}; GTP, 1×10^{-3}; succinate, 5×10^{-3}.

The control were incubated in the same medium in a cold bath in a shaker (80–100 c/min) for 30 min.

Liver slices were prepared on the criostat and treated by COONS indirect method[7]. Their examination for a complete removal of ceruloplasmin and remaining blood plasma was made by fluorescent microscopic technique. All the media were prepared with deionized water.

After incubation the slices were separated from the medium by centrifugation at 3,000 g in polyethelene tubes. The precipitated slices were homogenized in a plexiglass homogenizer in 3 ml 0.1 M of sodium acetate

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buffer, pH 5.5. The homogenate was centrifuged at 15,000 *g*. Ceruloplasmin concentration was determined from the catalitic oxidation of paraphenylendiamine in the supernatant fluid of the homogenate (further: the soluble fraction, SF) and in the incubation medium[8].

Ceruloplasmin identification technique by fluorescent antibodies. Ceruloplasmin identification was made in human and *Macacus rhesus* tissues. Slices of organ tissues were taken from a practically normal male, aged 37, 2 h after clinical death due to a severe trauma. In addition, biopsy was made in several patients with liver cirrhosis, and ceruloplasmin identification was conducted in liver cells. The material from monkeys was taken by biopsy in vivo and after the animals were sacrificed.

Indirect COONS' method by fluorescent antibodies was used. Slices 6.5 μ thick were prepared in the criostat at the environmental temperature −20 °C. Rabbit serum from a rabbit immunized by human ceruloplasmin was used as an intermediant. As will be shown later, rabbit serum containing antibodies to human ceruloplasmin is capable of agglutinating monkey ceruloplasmin. To remove antibodies to other plasma proteins, anticeruloplasmin serum was preliminary depleted by adsorption with the diazotized plasma protein complex from a patient with Wilson's disease. Diazotizing was done by OLOVNIKOV's method[9] with the only difference that human liophylized serum albumin was used as the stabilizing protein. Fluorescein isothiocyanate labelled antirabbit serum from a monkey was employed as staining serum.

After fixation in pure acetone the slices were covered with intermediate serum and incubated for 30 min at 37 °C, after which they were washed in buffer saline at pH 7.2 and stained by fluorescent antirabbit serum for 30 min. The preparations were washed, dried and studied microscopically.

The following systems were used as controls of the specificity of fluorescence observed: (1) slices treated by normal rabbit serum; (2) slices treated by fluorescent antirabbit serum only; (3) slices treated by rat serum; (4) mouse liver slices treated by rabbit antiserum containing antibodies to human ceruloplasmin and stained with antirabbit serum. Microscopically observed to be specific only if the controls showed no fluorescence.

Preparation of ceruloplasmin. Ceruloplasmin was isolated from human placenta serum by Broman's method[10] with a slight modification. The yield of the protein was 70–75% of the initial catalic serum activity in paraphenylendiamine oxidation. Ceruloplasmin increase during the isolation process was 110-fold. The isolated protein was homogeneous in ultra-centrifugation studies and in starch gel electrophoresis. From spectrophotometric data (coefficient E_{610}/E_{280}) and from copper concentration in the protein ceruloplasmin was shown to be 100% pure.

Ceruloplasmin was also isolated from monkey serum (*Macacus rhesus*) by BROMAN's method with the modification described earlier[11]. Ceruloplasmin concentration in monkey serum was 2–3 times that in human serum; therefore, the isolation procedure and the yield have some advantages (yield is 95%, increase is 140-fold). Monkey ceruloplasmin was also obtained in a pure form.

Preparation of antisera against human ceruloplasmin. Antiserum was obtained by immunizing rabbits with human ceruloplasmin. Some injections were made with ceruloplasmin adsorbed in aluminium hydroxide suspension. During the immunizing process each rabbit was injected 220 mg ceruloplasmin in total. The antiserum obtained agglutinates both human and monkey ceruloplasmin in specific precipitation on agar gel by Ouchterloni's method[12].

Analytical procedures

Determination of ceruloplasmin concentration. 2 ml 0.4 *M* sodium acetate buffer (pH 5.5) and 0.5 ml of 0.5% paraphenylenediamine hydrochloride (PPD) were added to 2 ml medium in which monkey liver slices had incubated (see [13]). The mixture was incubated for 1 h at 37 °C. The reaction was inhibited by adding 0.5 ml of 0.5% solution of sodium azide, and the other manipulations were performed according to RAVIN's method[8]. The concentration of the stained product of PPD oxidation was measured by a spectrophotometer from extinction at 530 nm. To determine ceruloplasmin concentration in the supernatant after centrifugation of the homogenate from liver slices (soluble fraction, SF) 1 ml SF, 3 ml of acetate buffer pH 5.5 and 0.5 ml 0.5% PPD were taken. After incubation the reaction was inhibited by adding 0.5 ml of 0.5% solution of sodium azide. Ceruloplasmin concentration in samples was determined from the calibration graph showing the dependence between spectrophotometrically measured extinction at 530 nm stained product and human homogeneous ceruloplasmin concentration, the protein in samples being determined by LOWRY's technique[14].

Sedimentation constant, diffusion coefficient, and molecular weight measurement. The sedimentation constant was measured by UCA-5 ultracentrifuge (SKB BFEM, Moskwa). To register the mobile boundary of the

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sedimenting protein Philpot-Swensson refractometric technique and a polarization interferometer were used. The diffusion coefficient was determined by ZWETKOV's polarization diffusiometer which allows us to study protein concentrations as low as 0.06 to 0.1%[15].

Ceruloplasmin molecular weight was calculated from the sedimentation constant and the diffusion coefficient by Svedberg's equation.

Ceruloplasmin electrophoresis. Ceruloplasmin zone electrophoresis was made in starch gel by SMITHIES' method[16] followed by amino black staining and specific *o*-dianizidine staining of the resulting reaction.

Copper content determination in ceruloplasmin. Copper concentration in the solution following the treatment of the protein by high concentration of hydrochloric acid was determined by the MASHKOV-DMITRIEV method[17] based on copper interaction with diphenylcarbazon in benzol. The concentration of the stained product was spectrophotometrically measured from extinction at 540 nm. The number of copper atoms in a protein molecule was calculated (ceruloplasmin molecular weight was taken to be 160,000).

Spectrophotometric studies. All the studies were carried on with SF-4 spectrophotometer (Leningrad, USSR).

Results

Physico-chemical and antigenic properties of monkey ceruloplasmin. Our studies were designed to show that human and monkey ceruloplasmins are identical in their properties. A close similarity of the 2 proteins enables us to study ceruloplasmin biosynthesis more thoroughly and to compare the experimental data in animals with those in man. Therefore, we have determined the basic physico-chemical properties of monkey ceruloplasmin and compared them with the properties of human ceruloplasmin.

The Figure 1 shows a diagram of monkey ceruloplasmin sedimentation, indicating that this protein was obtained in the homogeneous form. Figure 2 gives a comparison of electrophoregrams for human and monkey ceruloplasmin. The analysis confirms homogeneity of both proteins indicating, at the same time, a certain difference in their charge values. Different electrophoretic mobility of these proteins has proved to be according to our data, the only property which distinguishes one from the other. As is seen from the data in Table I, human ceruloplasmin differs only slightly from monkey ceruloplasmin in quite a number of its physico-chemical properties.

The study of their antigenic properties by means of specific precipitation on agar gel by OUCHTERLONI's method has also demonstrated the identity of the 2 proteins. Agar immunodiffusion of native antiserum of rabbits immunized by human ceruloplasmin gives 2 or 3 precipitation arcs with human and monkey ceruloplasmin as well as with the sera of the same origin, while depleted rabbit antiserum gives only one arc joining the neighbouring arcs (Figure 3). Since

Table I. Physico-chemical properties of human and monkey (*Macacus Rhesus*) ceruloplasmins

Properties	Human ceruloplasmin	Monkey ceruloplasmin
Concentration in serum (mg/100 ml)	30 35[1]	60–90
Color	blue[18]	blue
$E^{610}_{1\%,\,1\,cm}$	0.68[1,19] 0.64[18]	0.66
$E^{280}_{1\%,\,1\,cm}$	14.6[19]	14.0
Copper/protein, %	0.32[18] 0.31[19] 0.34[1]	0.33
E_{610}/E_{280}	0.047[19] 0.044[20]	0.047
Copper atoms/mol. wt. of protein	7.5[18] 8.0[19]	8.0
$S_{20,\,W} \times 10^{-13}$	7.29[1] 7.1[19] 7.6[21]	7.4
$D_{20,\,W} \times 10^{-7}$ cm^2 sec^{-1}	4.4[21] 4.5[1]	4.5
Molecular weight	151,000[1 18] 160,000[22]	160,000
Oxidase activity (per 1 mg protein)	11.6 ± 0.4[23]	11.5
KM (in moles of paraphenylene diamine)	5.9×10^{-4}[24]	5.2×10^{-4}

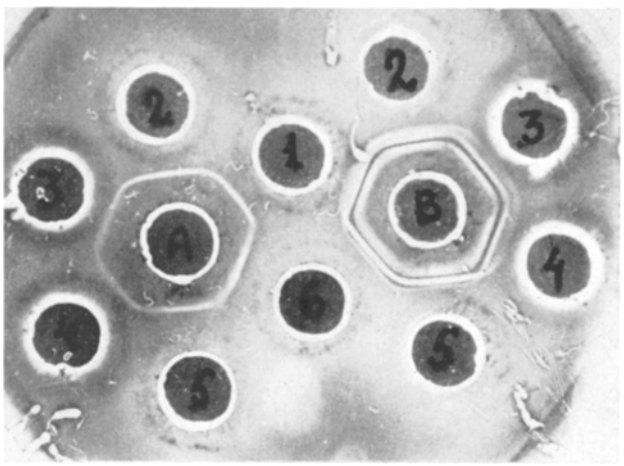

Fig. 1. Agar immunodiffusion of blood serum ceruloplasmin from a normal subject, patient with Wilson's disease and monkey. A, depleted antiserum; B, native antiserum. 1, 6, normal human ceruloplasmin; 2, normal human blood serum; 3, monkey ceruloplasmin; 4, monkey blood serum; 5, blood serum in Wilson's disease.

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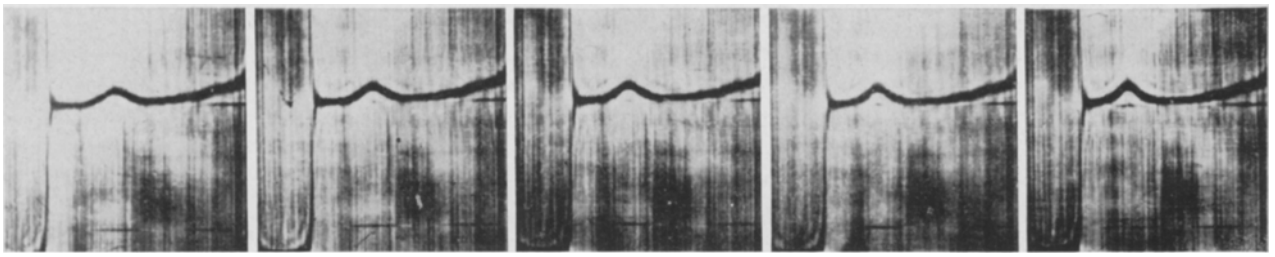

Fig. 2. Sedimentation diagram of monkey ceruloplasmin. Protein concentration 0.1%; rotor velocity 52,000 rpm; temperature 20 °C. The films were made in 18, 21, 24, 27 and 30 min at maximal rotor velocity.

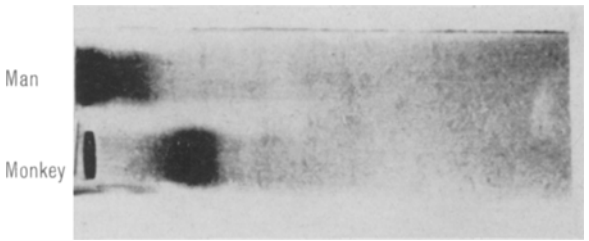

Fig. 3. Electrophoresis of human and monkey ceruloplasmin on starch gel. Experimental conditions: 0.5 mg protein; 0.1 *M* acetate buffer, pH 6.8; ionic strength 0.1; potential gradient 6 v/cm; current 15 mA. Amino black staining.

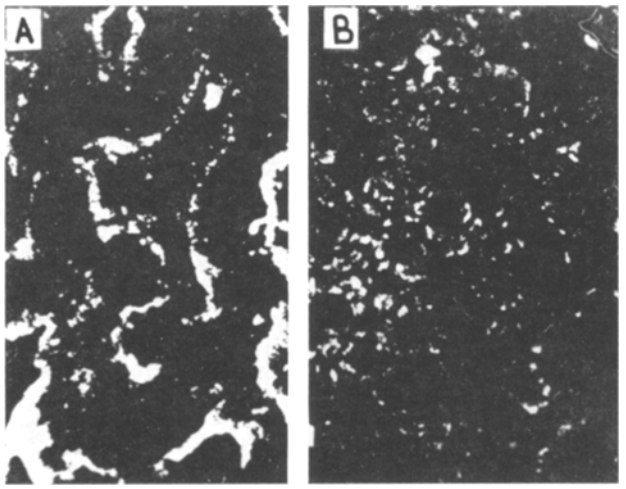

Fig. 4. Staining of monkey liver slices for ceruloplasmin by Coons' indirect method before and after perfusion of liver in saline. A, before perfusion; specific fluorescence determined along blood capillaries and inside liver cells. B, after perfusion; remaining specific fluorescence determined only inside parenchimatous cells.

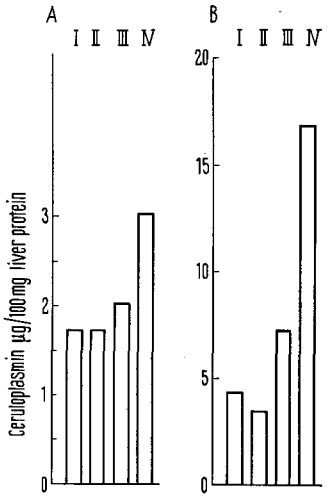

Fig. 5. Ceruloplasmin biosynthesis in monkey liver slices. Along the ordinate axis ceruloplasmin concentration in úg/100 mg protein in liver tissue. A, ceruloplasmin concentration in the incubation medium; B, ceruloplasmin concentration in SF. I, incubation in the cold (control); II, incubation at 37 °C with addition of chloramphenicol; III, incubation at 37 °C with addition of $CuSO_4$; IV, incubation at 37 °C without additions (original incubation medium).

antiserum depletion was attained with plasma proteins from a patient with Wilson's disease (whole plasma was almost entirely ceruloplasmin-defficient) and since from antiserum antibodies to other plasma, proteins, except those of ceruloplasmin, were removed, the presence of one precipitation arc between depleted rabbit antiserum and monkey ceruloplasmin indicates directly the antigenic identity of both ceruloplasmins.

Ceruloplasmin biosynthesis in monkey liver slices. Additional experiments with fluorescent antibodies were performed to see whether liver slices were free from blood (as a result of perfusion), whose contamination might be a source of ceruloplasmin. In the Figure 4 micrographs are presented of monkey liver preparation before and after perfusion. It is seen that, if before perfusion specific fluorescence is found both inside liver cells and adjacent vessels, there is no extracellular fluorescence in the perfused liver, but it is observed only intracellularly. These experiments allowed us to eliminate any presence of blood ceruloplasmin. After this we started experiments on liver slice incubation.

The Figure 5A presents ceruloplasmin concentration data in the incubation medium. It is seen that when the slices were incubated in the cold, ceruloplasmin concentration was as low as 1.6 mg/100 mg protein of liver slices, while in incubating at 37 °C in the above conditions there was 2-fold increase in oxidative activity (2.8 µg/100 mg protein) due to synthetized ceruloplasmin release from the cells. The addition of 40 µg/ml chloramphenicol to the incubation medium completely inhibited oxidase activity. Decrease release of ceruloplasmin into the medium indicates that this release

is the evidence of the actual protein biosynthesis rather than some activation of oxidase activity in the incubation medium. The addition of 4 μg/ml copper sulphate to the samples slightly increased oxidase activity in comparison to the controls.

The experiments in regard to ceruloplasmin release from the slices in the incubation medium demonstrate that in our conditions de novo ceruloplasmin remains intracellular and released into the medium in negligible quantities. This becomes evident from the data obtained previously in our laboratory[25]. It was shown that in stabilization of plasma membrane structure of liver cells (presence of ATP, Mg^{++} ions, active oxidative phosphorylation) protein release from the cells is significantly inhibited. The medium and incubation conditions used in our experiments actively contributed to respiration and oxidative phosphorylation. Therefore, it might have been expected that synthetized ceruloplasmin is largely retained by liver cells rather than released into the medium and that liver cells accumulate this protein. Experiments on ceruloplasmin determination in SF, i.e. in the soluble fraction of liver cells, have confirmed our theory.

In fact, ceruloplasmin concentration in SF of control samples (incubation in the cold) was 3.6 μg/100 mg protein, but in the experimental samples it was 16 μg/100 mg protein (Figure 5B). The addition of copper sulphate to the incubation medium (4 μg/ml) gave double inhibition of ceruloplasmin synthesis in the slices. However, ceruloplasmin concentration in these samples was nevertheless much higher than in the controls. 40 μg/ml concentration of chloramphenicol completely inhibited ceruloplasmin biosynthesis.

Though the experiments with chloramphenicol have convincingly shown that there is actual synthesis of the protein possessing oxidase activity, i.e. ceruloplasmin biosynthesis, we have undertaken some additional control testings. It was the purpose of the studies to show whether the increase of oxidase activity was due to the synthetized ceruloplasmin or to another protein possessing oxidase activity.

The specifity of the increased oxidase activity due to ceruloplasmin was successfully shown by tests with rabbit antiserum containing antibodies against ceruloplasmin. The experiments were conducted as follows. Ceruloplasmin-immunized rabbit serum was added to SF. The validity of such an approach is confirmed by the above evidence of immunological identity of human and monkey ceruloplasmins. After incubation for 3 h at room temperature, the samples were allowed to stand in the refrigerator over night, after which the precipitate was separated by centrifugation and oxidase activity was determined both in the precipitate and supernatant. As a control for the possible nonspecific liver protein precipitation by rabbit blood serum, an experiment was made in which blood serum (instead of antiserum) from a nonimmunized intact rabbit was added to SF. The Figure 6 shows the results of this experiment. It is seen that the addition of 'intact rabbit serum' to SF results in the summation of SF and rabbit serum oxidase activities. Quite a different result is observed when adding to SF specific rabbit antiserum. In this case, there is a fall out, the oxidase activity in the solution being only the activity of rabbit antiserum. This finding is evidence of the presence of a substance in SF capable of reacting specifically with rabbit antibodies to form an antigen-antibody complex which precipitates. Such a substance, capable of forming a specific complex, could be only ceruloplasmin from SF. Indeed, when analysing the precipitate its oxidase activity was found equal to that of SF.

Thus the experiments with the addition of antiserum have shown that it is SF ceruloplasmin formed in incubating liver slices rather than any other protein with oxidase activity which is responsible for the increase of oxidase activity.

Immunocytological study of ceruloplasmin in human and monkey organs. In microscopic studies of human and monkey kidney, brain, and heart, there was no localized specific fluorescence in the cells of these organs. The fluorescence was of diffuse character and slight intensity. In preparations of human and monkey spleens, a distinct specific fluorescence was observed only in the sini; intracellular fluorescence, however, was not found. On the other hand, when studying monkey liver preparations obtained in biopsy, that is, with normal circulation, oxygen supply and nutrition, specific fluorescence was observed in the form of granules of various size localized in parenchimatous cells (hepatocytes), these granules being particularly

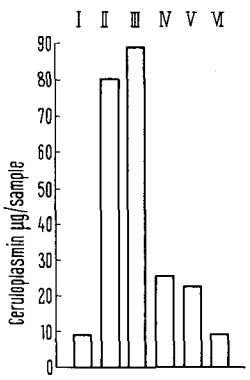

Fig. 6. The effect of specific rabbit antiserum on ceruloplasmin oxidative activity in the soluble fraction (SF) of liver cells. Along the ordinate axis ceruloplasmin concentration in SF in úg. I, soluble fraction from liver cells; II, blood serum of intact rabbit; III, SF + intact rabbit serum; IV, specific antiserum; V, SF + specific antiserum; VI, ceruloplasmin-specific antiserum complex.

[25] I. M. VASSILETZ, Biokhimiya *29*, 983 (1964).

well-shaped near the cell nucleus. Intracellular fluorescence was hardly visible (Figure 7). In the preparations from monkey liver in autopsy, that is, in oxygen deficiency and anoxia, most of the fluorescent material was localized extracellularly (Figure 8D). It is clear from the Figures 8C and 8D that in human and monkey liver fluorescence was detectable between liver cords and in the intracellular space. However, certain human and monkey parenchimatous liver cells had sites of specific fluorescence (Figures 8A and 8B). In the lumen of blood vessels of larger size (Figure 8E) stained by fluorescent antihuman serum, there was distinct specific fluorescence, no fluorescence being found after treatment with anticeruloplasmin serum. This fact indicates that the specific fluorescence observed is not related to the presence of blood or plasma in the preparations but rather is due to ceruloplasmin released from the cells into the intracellular space. In the preparations obtained in autopsy, the capacity of cell membranes to retain ceruloplasmin is most likely to be the disturbance in energy metabolism and cellular ATP balance produced by anoxia. Indeed, in liver preparations obtained in biopsy from cirrhotic patients specific fluorescence of ceruloplasmin was observed primarily inside parenchimatous cells.

In other words, the absence of fluorescence in human and monkey liver blood vessels clearly demonstrates that the specific fluorescence detected in our studies is due to intracellular localization of ceruloplasmin and its migration into the intracellular space only as a result of post mortem changes in the organ. This finding is also confirmed by the fact that specific fluorescence in perfused monkey liver is localized only intracellularly (Figure 8B). It is evident from the Figures 7, 8A and 8B that specific fluorescence can be detected in liver cell organelles surrounding the cell nucleus.

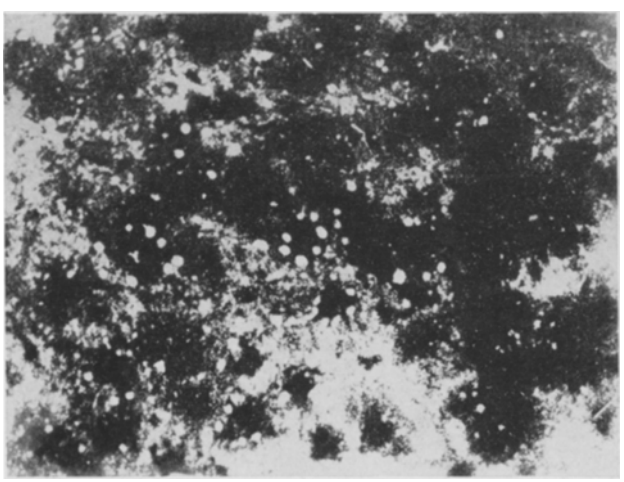

Fig. 7. Specific fluorescence in monkey liver slice (biopsy). Staining by Coons' indirect method, lens × 40, ocular × 1.7.

Copper effect on ceruloplasmin biosynthesis. Conducting the experiments on copper sulphate administration to monkeys, we were faced with 2 problems. First, an attempt was made to create an experimental model phenotypically imitating certain symptoms of Wilson's disease. Second, it was desirable to know whether ceruloplasmin biosynthesis is affected by copper level in the organism. For this purpose, a series of experiments was carried on in which one group of monkeys was injected s.c. 1–1.5 mg copper/1 kg body wt. per day (solution of copper sulphate), and the other group was injected higher doses of copper: 3.2 mg/1 kg body wt. during 3–6 days.

It appears that a single administration of copper neither noticeably affects the animal nor produces any pathological symptoms and, which is of particular interest, ceruloplasmin concentration in blood serum increases, on the average, 1.5 times. On the contrary, administration of high doses of copper results, within 2 or 3 days, in decreasing ceruloplasmin concentration in blood serum. The Table II present data of ceruloplasmin concentration in monkey blood serum after

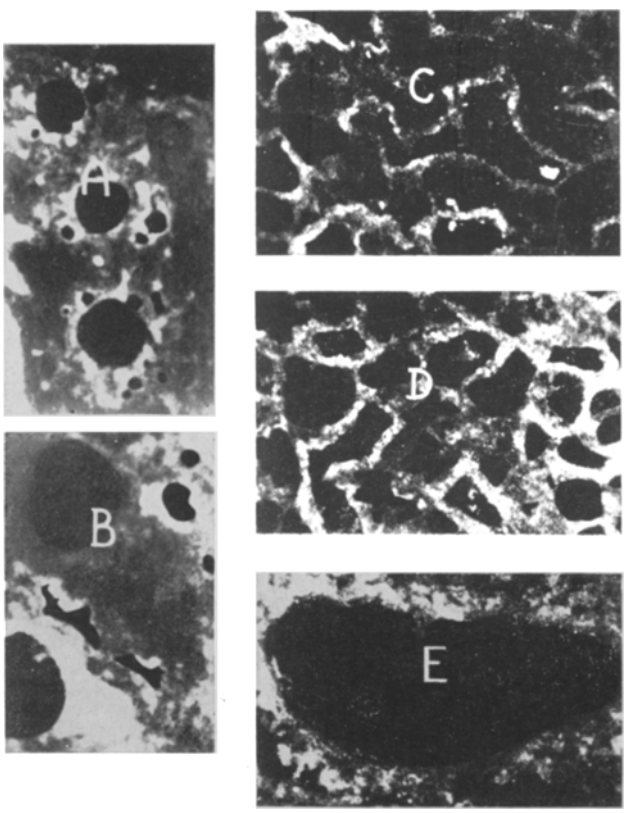

Fig. 8. Specific fluorescence in human and monkey liver parenchimatous cells (autopsy). a, specific fluorescence in monkey liver parenchimatous cells; lens × 100, ocular × 3; b, specific fluorescence in human liver parenchimatous cells, lens × 100, ocular × 3; c, specific extracellular fluorescence in monkey liver slices, lens × 40, ocular × 1.7, aqueous immersion; d, specific extracellular fluorescence in human liver slices, lens × 40, ocular × 1.7, aqueous immersion; e, absence of specific fluorescence in the lumen of monkey liver blood vessel, lens × 40, ocular × 1.7.

repeated administration of copper. It should be noted that monkey N 2 died in 4 days and monkey N 1 in 5 days after the first injection.

The monkeys, one day before death, showed symptoms characteristic of patients with Wilson's disease: shaking and involuntary movements of limbs, muscle tention in all groups of muscles, etc. Thus, both biochemical features and clinical symptomes found in monkey as a result of ceruloplasmin synthesis inhibition are phenotypically identical to changes pathognomic to Wilson's disease. It must be emphasized that, when studying liver preparations from monkeys killed by copper poisoning, no intracellular fluorescence was found.

Repeated administration of copper completely inhibited ceruloplasmin biosynthesis in monkey liver. In this case we found no difference as to ceruloplasmin concentration in the incubation medium and in SF, between the controls and experimental samples, with the addition of chloramphenicol and without.

Discussion

When incubating monkey liver slices under the conditions of active respiration and oxidative phosphorylation we observed increased catalytic activity of ceruloplasmin in PPD oxidation process ('oxidase reaction'). This increase in the activity takes place primarily intracellularly (in the soluble cell fraction, SF) and only a small part of the activity is transmitted into the medium. A few factors indicate that the higher the intensity of oxidase reaction, the higher the concentration of ceruloplasmin; in other words this increase in the intensity corresponds to the actual de novo protein production rather than to a mere increase of its specific activity. This conclusion is supported by the following findings: (1) more intensive oxidase reaction occurs only at 37 °C and it is not observed in the cold; the increase in its intensity is found only if the slices are incubated in the medium providing active oxidative phosphorylation, and it is not observed unless the slices are aerated; (2) the addition to the medium of chloramphenicol in 40 μg/ml concentration completely inhibits the increase in oxidase activity in liver cells; (3) rabbit serum containing antibodies against ceruloplasmin precipitates the whole oxidase activity involved in the soluble fraction of liver cells. Therefore, there is hardly any doubt that in the incubation of monkey liver slices ceruloplasmin biosynthesis does take place.

Furthermore, the question arises as to whether ceruloplasmin biosynthesis in man occurs in liver as well. Naturally, as to human beings, this question cannot be solved by direct experiments, but rather an indirect approach is required.

We have used COONS' fluorescent antibody technique and applied it to the study of various human and monkey tissues. The investigations of liver, kidney, brain and heart have clearly shown that it is only in liver of all organs that ceruloplasmin is found, both in monkey and man. Moreover, it is only in the parenchimatous cells, and in the organelles surrounding the cell nucleus in particular, that ceruloplasmin is localized. Various control tests eliminate the possibility of artefact, and the problem of ceruloplasmin localization in the human and monkey organism should be solved unambiguously. The following indirect conclusion from these data can be made: if ceruloplasmin is localized only in liver cells and is not found in other organs from which it could be transported to the liver, then it is only in the liver that ceruloplasmin biosynthesis seems to take place. This conclusion as to the human organism seems to be highly probable in view of the fact that monkey too shows the same localization of ceruloplasmin and that monkey liver cells have been shown to be capable of ceruloplasmin biosynthesis.

Finally, the question of the role played by copper in the regulation of ceruloplasmin biosynthesis should be discussed. During the incubation of monkey liver slices the addition of copper sulphate to the medium (4 μg/ml) markedly inhibits the biosynthesis (Figure 5). A more convincing effect was obtained by injecting copper into the animal. A single administration of copper in small doses has been found to increase ceruloplasmin concentration in blood serum, while repeated injections of large doses of copper results in decreasing ceruloplasmin concentration or in inhibiting its biosynthesis (Table II).

Therefore, it is obvious that ceruloplasmin biosynthesis is under copper control and that the regulation of the rate of this biosynthesis depends on copper metabolism and its level in the tissues. It is noteworthy that copper, which is the object of transport function of ceruloplasmin, controls in its turn the biosynthesis of the transport protein, i.e. ceruloplasmin.

In toxic inhibition of ceruloplasmin biosynthesis by copper, monkeys develop some symptoms similar to those in Wilson's disease. It is likely that these symptoms show partly the pathogenic pattern of this disease. In Wilson's disease, as a result of copper poison-

Table II. Decrease of ceruloplasmin concentration in monkey blood serum after i.v. administration of high doses of copper

Day of injection	Diurnal quantity of injected copper (mg/kg body wt. per day)	Ceruloplasmin concentration in blood serum (mg/100 ml)	
		Monkey N 1	Monke N 2
1	3.2	90	86
2	3.2	115	51
3	3.2	70	30
4	3.2	31	10
5	3.2	24	Animal died
6	3.2	15	

ing, there develops a peculiar vicious circle: on the one hand, the tissues accumulate copper which is not involved in metabolism due to ceruloplasmin deficiency; on the other, the excess of copper in liver cells further inhibits ceruloplasmin biosynthesis, impairing the state of the patient's metabolic system. A reasonable way to treat this disease appears to consist in overcoming the vicious circle.

Zusammenfassung. Aus Affenserum (*Macacus rhesus*) ist das Zeruloplasmin in Form einer homogenen Eiweisssubstanz isoliert und in ihren physikalisch-chemischen Konstanten näher bestimmt worden. Der Vergleich der Eigenschaften des Zeruloplasmins des Menschen und des Affen ergibt eine Ähnlichkeit dieser beiden Substanzen. Bei der Untersuchung mittels spezifischer Präzipitation im Agar haben sich beim Antiserum des Kaninchens Beweisgründe für eine antigene Identität beider Zeruloplasmine ergeben. An Gewebeschnitten der Affenleber inkubiert bei aktiv oxydativer Phosphorylierung ergab sich die Bindung des Zeruloplasmins in vitro. Resynthetisierte Eiweißsubstanz bildet einen spezifischen Niederschlag mit dem Antiserum des Kaninchens, welches gegen das Zeruloplasmin des Menschen immunisiert ist. Mit Hilfe lumineszierender Antikörper ist die spezifische Lumineszenz des Zeruloplasmins nur in parenchimatösen Leberzellen gefunden worden. Die Biosynthese des Zeruloplasmins ist in der Leber lokalisiert. Aus In-vitro-Versuchen an Gewebeschnitten der Leber beim Affen und entsprechenden In-vivo-Versuchen ergibt sich, dass Kupfersalze in geringen Konzentrationen die Biosynthese verstärken, in grossen Konzentrationen hingegen die Biosynthese des Zeruloplasmins hemmen.

SPECIALIA

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Kinetics of Discharge of Cadmium (II) at Dropping Mercury Electrode in KNO_3-Thioglycolic Acid System

The reduction of Cadmium (II) at dropping mercury electrode (D.M.E.), studied in KNO_3-TGA mixtures of constant ionic strength (0.5 μ), has been found to be irreversible. Kinetics of the electrode reaction has been investigated, adopting the treatment followed by KORYTA, which gave the values of transfer coefficient (α) and rate constant ($K°$) as 0.506 and 2.544×10^{-3} cm/sec (22 °C) respectively.

Polarographic behaviour of Cd^{++} at D.M.E. in presence of complexing and non-complexing electrolytes had been of considerable interest[1], but the nature of its reduction in medium comprised of thioglycolic acid (TGA), which figures prominently in the discussion of sulphur containing ligands and provides 2 possible coordination sites, viz. –COOH and –SH groups, has not yet been explored. The present investigation has, therefore, been initiated.

Experimental. All inorganic chemicals and thioglycolic acid were either reagent grade or Merck's guaranteed extra-pure, which were used without further purification. Gelatin was used as maximum suppressor, and purified and equilibrated nitrogen was used to remove oxygen from the solutions.

A Cambridge (G.P.) polarograph was used for recording c–v curves in conjunction with a thermostated H-cell (22 ± 0.05 °C) containing a saturated calomel reference electrode. All the polarograms were run with the damping control in the off position and keeping current sensitivity constant. The capillary used has a constant of $m^{2/3} t^{1/6} = 2.035$ $mg^{2/3} sec^{-1/2}$ in 0.5M KNO_3 and 0.01% gelatin at 1.0 V (Vs. S.C.E.).

Various solutions containing 0.5 mM cadmium sulphate, different amounts of sodium thioglycolate (0.05M) and 0.01% gelatin were prepared. Potassium nitrate was added to maintain the ionic strength at 0.5M. The thoroughly mixed solutions were transferred to the cell and deoxygenated for 15 min with nitrogen. The mercury head was adjusted to 29.5 cm and polarograms were run. Necessary corrections for IR drop and residual current were made in determining half wave potentials and diffusion current data respectively.

Results and discussion. A series of c–v curves, obtained as a result of the discharge of Cd^{++} at D.M.E. in TGA media of constant ionic strength, were analysed for determining the number of electrons taking part at the electrode and for ascertaining the reversibility or irreversibility of the electrode reaction. In all cases, except one in which the concentration of TGA was zero, the plots of $\log \bar{i}/(\bar{id} - \bar{i})$ $v.$ $E_{d.e.}$ yielded parabolic curves and indicated considerable shift in $E_{1/2}$ values with the increase in TGA concentration, revealing the irreversible reduction of Cd (II) in presence of TGA and complexation between them; in absence of TGA however, cadmium (II) reduces

[1] L. MEITES, in *Polarographic Techniques* (Interscience Publication, New York 1965), p. 623.