results do not, however, exclude the possibility that there might be coupling between the synthesis of certain proteins and the synthesis of some particular fraction of the ribonucleic acid.

Whether protein synthesis can be suppressed without affecting RNA synthesis remains undecided, since the amino acid analogue, somewhat paradoxically, inhibited RNA synthesis much more readily than protein synthesis. Both thienylalanine and azaguanine suppressed cytoplasmic RNA synthesis more rapidly than nuclear RNA synthesis, thus indicating that there is no rigid coupling between these two processes either. No dissociation of nuclear from cytoplasmic protein synthesis was observed: thienylalanine produced no change in the rate of either nuclear or cytoplasmic synthesis over 12 hr., and azaguanine reduced the rate of synthesis in nucleus and cytoplasm to about the same extent.

SUMMARY

1. Radioautographic methods were used to study the effect of β-2-thienylalanine and 8-azaguanine on the synthesis of protein and ribonucleic acid in the nucleus and cytoplasm of the rat connective-tissue cell.

2. With β-2-thienylalanine it was possible to reduce the rate of ribonucleic acid synthesis in the cytoplasm to about 10% of the normal value, and in the nucleus to about 50% of the normal value, without affecting either nuclear or cytoplasmic protein synthesis.

3. With both β-2-thienylalanine and 8-azaguanine, cytoplasmic ribonucleic acid synthesis was much more rapidly suppressed than nuclear ribonucleic acid synthesis.

4. The results indicate that there is no mandatory coupling between the synthesis of ribonucleic acid and the synthesis of protein, as measured by the overall rates of the two processes, either in the nucleus or in the cytoplasm.

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The Purification of Human Caeruloplasmin

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Almost all of the copper in plasma is bound to a single protein. Holmberg & Laurell (1948) isolated a cuproprotein from porcine and human plasma, and because of its blue colour they called it caeruloplasmin. It had a molecular weight of about 151 000 and contained 0·34% of copper, which corresponded to eight atoms of copper per molecule. Like a number of other copper–proteins, it is an enzyme, having oxidase activity, though its physiological substrates, if any, are unknown. In Holmberg & Laurell's method of preparation (1948) caeruloplasmin was precipitated from serum with the globulin fraction by ammonium sulphate, dissolved in water and dialysed. Some impurities were precipitated at pH 6·2 and 5·5 and other impurities by two treatments with 50% alcohol–chloroform at room temperature; caeruloplasmin was extracted with sodium chloride solution from the resulting precipitate, which was mostly denatured protein. Details of percentage yields were not given. Scheinberg & Morell (1957) used caeruloplasmin prepared from Cohn's fraction IV by methanol–chloroform fractionation, but details of the method were not given. Most published work on caeruloplasmin has been related to various disease processes. There is little published on the structure and properties of caeruloplasmin, mainly because of the labour and difficulties in preparing purified material in sufficient quantity. Recent interest in caeruloplasmin has been concerned with its deficiency in hepatolenticular degeneration (reviewed by Cumings, 1959) and its possible significance in mental disease (e.g. Melander, 1960).

Recently, the preparation of caeruloplasmin of unspecified purity was briefly described by Hjerten (1959), who used chromatography on hydroxylapatite and by Steinbuch & Quentin (1959) who used protein precipitation by 6:9-diamino-2-ethoxyacridine (Rivanol).

A method of preparing caeruloplasmin from the G 2 fraction, consisting mainly of α- and β-globulin, which is obtained in the fractionation of plasma by the method of Kekwick & Mackay (1954) is described in this paper. It entails extraction with sodium chloride solution, denaturation of lipoproteins and precipitation by ether at pH 4·8 and at pH 5·4. This is followed by fractional precipitation at low salt concentrations and anion-exchange chromatography on diethylaminoethylcellulose. As the method does not require dialysis in the early stages, when volumes are large, it is particularly suitable for application on a large scale. A preliminary account has already been published (Curzon & Vallet, 1959).

EXPERIMENTAL

In the initial large-scale stages, special washing of apparatus to prevent contamination by copper was impracticable. In the chromatographic experiments and in the determination of oxidase activity and copper content the apparatus was washed in chromic oxide–sulphuric acid, following by water, 30% (v/v) hydrochloric acid, water and ion-exchange resin-purified water (Elgastat deionizer; Elga Ltd., London, S.W. 19). All solutions were made with ion-exchange resin-purified water, except in the earlier large-scale stages in which once-distilled water was used.

Determination of protein. Rough protein estimations were made with $E_{1\,cm.}$ at 280 mμ. Triplicate N determinations by a micro-Kjeldahl method were done on some of the fractions.

Determination of pH. This was done with a 23 A pH meter (E.I.L., Richmond, Surrey).

Determination of caeruloplasmin. (*a*) Oxidase-activity method. Caeruloplasmin oxidizes *p*-phenylenediamine and related substances to coloured products. Methods of estimation using this property are briefly described by Akerfeldt (1957), Houchin (1958) and other workers. Our method is a modification of one briefly described by Curzon (1958).

A freshly prepared 1·7 mM solution of *N*-dimethyl-*p*-phenylenediamine (DPD) in the form of the oxalate salt was made and held at 37°. Water (1 ml.) and 1 ml. of a suitable dilution of the test material in 0·1 M-sodium chloride was added to 2 ml. of 0·2 M-sodium acetate buffer, pH 5·50, and incubated for 5 min. at 37°. A portion (1 ml.) of the DPD solution was then added to the test mixture and the purplish-red colour allowed to develop for 15 min. A blank was run with 1 ml. of 0·1 M-sodium chloride instead of 1 ml. of the test solution. Reaction was stopped by adding 2 ml. of ice-cold 0·3 mM-sodium azide, freshly prepared from a stock 10 mM solution kept at 4°. $E_{1\,cm.}$ was read at 550 mμ. When the activity of plasma or of the more impure fractions was being measured, $E_{1\,cm.}$ decreased after addition of azide and readings were taken at 4, 6, 8 and 10 min. and extrapolated to zero time. With the purer fractions this was not necessary, E varying by less than 3% during 30 min. after addition of the azide. When activity was determined in plasma, an azide solution in 10% sodium chloride was used to clear a slight turbidity which developed during incubation. With purified material there was a linear relationship between $E_{1\,cm.}$ and amount of caeruloplasmin for values up to $E_{1\,cm.}$ 0·5. Duplicate determinations varied from their mean by an average of ±1%. A unit of activity was arbitrarily defined as the activity of an amount of caeruloplasmin giving an $E_{1\,cm.}$ of 0·10 under the above conditions.

(*b*) Determination with $E_{1\,cm.}$ at 605 mμ. Caeruloplasmin absorbs in the visible range with a peak at 605 mμ. Addition of potassium cyanide causes fading of the colour. The difference between $E_{1\,cm.}$ at 605 mμ before and after addition of potassium cyanide was used to determine the caeruloplasmin (Scheinberg, Morell, Harris & Berger, 1957). This procedure was found useful for estimation of the caeruloplasmin content of the purer fractions. The method is less sensitive than the oxidase estimation but is less affected by foreign substances. The ratio of the difference between $E_{1\,cm.}$ at 605 mμ, before and after addition of potassium cyanide, to $E_{1\,cm.}$ at 280 mμ gives an estimate of caeruloplasmin purity in relation to protein content.

Determination of copper. (*a*) Total copper. To determine copper it is necessary first to break the caeruloplasmin–copper bonds. A volume (3 ml.) of a solution containing about 4 μg. of total copper was heated in a boiling-water bath for 5 min. After cooling, 1 ml. of aq. 30% (w/v) trichloroacetic acid was added and the mixture heated as before. After cooling, the precipitate was centrifuged and extracted with 2 ml. of 15% (w/v) trichloroacetic acid, and the solution was heated again for 5 min. in boiling water. The supernatant after centrifuging was added to the first trichloroacetic acid supernatant and copper determined by the bis*cyclo*hexanone oxalyldihydrazone method of Peterson & Bollier (1955).

(*b*) Non-caeruloplasmin copper. This was determined by the method of Scheinberg & Morell (1957).

Preparative procedures. The preparation was carried out in a cold room at 4° except where otherwise stated. The precipitation vessels and general methods of handling were similar to those described by Kekwick & Mackay (1954). Fraction V was purified on anion-exchange columns with diethylaminoethylcellulose (DEAE, Kodak Ltd). Columns were prepared as described by Sober, Gutter, Wyckoff & Peterson (1956) except that sodium acetate buffers were used instead of sodium phosphate buffers. The columns were run at room temperature. Before material was applied, it was dialysed against the starting buffer. Salt and pH gradients in the concentration of the eluting buffer were established, by means of a magnetically stirred, constant-volume mixing chamber, except in the run with hydroxylapatite, when an apparatus giving a linear gradient was used (Broman, 1958). Eluate fraction volumes were roughly controlled by a photoelectric drop counter. The protein content of eluate fractions was estimated by $E_{1\,cm.}$ at 280 mμ and caeruloplasmin by $E_{1\,cm.}$ at 605 mμ. Pooled eluate fractions were concentrated when necessary by ultrafiltration, with filtration shells (Membranfiltergesellschaft-Sartorius-Werke, A. G. Gottingen). Though ultrafiltration and dialysis of eluted material took 5–6 days no significant decrease of purity occurred.

METHODS AND RESULTS

Method of caeruloplasmin purification

The stages in the purification are as follows:

(1) Extraction of caeruloplasmin from G2 by 0·07 M-sodium chloride at pH 7·0, dissociation of extracted lipoprotein by the method of McFarlane (1942) and precipitation of contaminant protein by excess of ether.

(2) Precipitation of caeruloplasmin at pH 4·8.

(3) Precipitation of impurities at pH 5·35 and a second extraction by the McFarlane method.

(4) Reprecipitation of caeruloplasmin at pH 4·8.

(5) Fractional precipitation by decreasing salt concentration.

(6) Purification on a DEAE column.

Ether was used in many of the stages to decrease protein solubility. Stages (2) and (4) result in concentration rather than purification of caeruloplasmin. They are necessary to reduce working volumes in the large-scale work described here but presumably could be omitted in smaller-scale work. Recovery and purification data from stages (1)–(5) of two fractionations are summarized in Table 1. In practice, batches of G2 from 47–192 l. of citrated plasma were used. For convenience, the purification of caeruloplasmin from 100 l. batches is described.

Fraction G2 of plasma. This fraction, which is obtained from the plasma of freshly collected blood, is a greasy greenish-yellow precipitate, contains about 14% of the total nitrogen of the plasma and is rich in lipoprotein. Aqueous suspensions of G2 have pH about 5. The citrated plasma used for the preparation of G2 had an apparent oxidase activity of 10·4 units/ml. of citrated plasma (average of three batches). Activity was decreased in the presence of citrate. Corrected for the effect of the amount of citrate present, the activity of the plasma was about 14 units/ml.

Stage 1: extraction of caeruloplasmin from G2 by sodium chloride solution at pH 7·0. Caeruloplasmin was extracted from the G2 precipitate by sodium chloride solutions. More active material is extracted at pH 7·0 than at pH 5·0, though the amount of contaminating protein is rather greater at pH 7·0. The sodium chloride extracts were thick suspensions which separated extremely poorly. They could be clarified by filtration through graded series of filter pads with Hyflo-Supercel (Johns-Manville Co. Ltd.), but the process was very time-consuming. Difficulties in clarification were mainly due to the large amount of suspended lipoprotein present. Freezing below −25° in the presence of excess of ether (McFarlane, 1942) was used to dissociate the lipoprotein, much contaminant protein also being precipitated by the excess of ether.

About 3·4 l. of G2 precipitate (obtained from 100 l. of citrated plasma equivalent to 71 l. of undiluted plasma) was suspended in 18 l. of 0·07 M-sodium chloride. The pH of the suspension was brought from 5·10 to 6·97 by slowly adding 280 ml. of 0·5 M-sodium hydroxide over 2 hr. The suspension was poured into Winchester bottles, shaken well with 0·5 vol. of ether and frozen below −25°. After thawing, the clear-orange upper ethereal layer was removed by suction and discarded and the slightly cloudy-green aqueous layer (fraction I) drawn off from beneath the poorly packed denatured lipoprotein layer at the ether–water interface. Attempts were made to pack the denatured material more tightly and thus to recover caeruloplasmin from it. A tighter pack was obtained at slightly acid pH, below 6·1, but much of the activity remained in it. At pH 6·1 an amount of

Table 1. *Stages in the partial purification of caeruloplasmin*

Results of two separate purifications are given. The citrated plasma from which G2 was obtained had an oxidase activity of 1400 × 10^3 units (corrected)/100 l. and a Cu/N ratio of 0·10 μg./mg.

Fraction		Vol. (l.)	10^{-3} × Total oxidase activity (units)	Activity/ml. $\frac{}{E_{1\,cm.}\ (280\ m\mu)}$	$\frac{Cu}{N}$ (μg./mg.)
I.	Ether-treated NaCl extract of G2 from 100 l. of citrated plasma	(i) 14·6	414	2·7	—
		(ii) 16·6	543	2·8	0·9
II.	pH 4·8 ppt.	(i) 1·62	389	3·5	—
		(ii) 3·08	445	—	—
III.	pH 5·35 supernatant	(i) 4·44	364	9·7	—
		(ii) 4·51	368	8·5	—
IV.	Second pH 4·8 ppt.	(i) 0·74	304	9·9	—
		(ii) 4·00	245	—	—
V.	0·045 M-NaCl ppt. in NaCl–sodium acetate	(i) 0·13	194	21·0	—
		(ii) 0·153	147	23·5	—
V.	Above fraction dialysed against 0·1 M-NaCl	(i) 0·156	156	18·5	—
		(ii) 0·149	165	22·9	9·4

active material equivalent to 10% of that present in fraction I remained in solution after centrifuging but was much contaminated with other proteins. It was accordingly decided to use fraction I alone.

Stage 2: precipitation of caeruloplasmin at pH 4·8. Fraction I was brought to pH 4·8 by the addition of M-acetic acid with stirring over a 2 hr. period at a temperature between 0° and −2°. After stirring for a further 1 hr. and standing overnight at −2° the almost clear supernatant was discarded, and the pale-blue sludge packed by centrifuging at −2° for 1 hr. at 500 *g*. The precipitate was washed with 15 vol. of 4 mM-sodium acetate buffer, pH 4·8, saturated with ether at 4°, and again centrifuged. The packed precipitate had a volume of about 500 ml. and was dissolved in 1 l. of 0·1 M-sodium chloride (fraction II).

Stage 3: precipitation of impurities at pH 5·35. Tests showed that much contaminating material was precipitated from fraction II saturated with ether at pH 5·35. Residual lipoprotein not precipitated by the first treatment with ether below −25° would tend to be precipitated under these conditions, almost all plasma lipoproteins having an isoelectric point in the range pH 5·3–5·4.

The ether-saturated material was also frozen below −25° to remove from solution residual traces of lipoprotein, susceptible to this treatment, that had come through the first stage.

Fraction II, a deep-blue solution, was adjusted to pH 5·35 by adding 90 ml. of 0·1 M-sodium hydroxide slowly with stirring during 45 min. Ether (to 45%, v/v) was added and, after shaking well, the material was stored overnight below −25°. After thawing, the mixture was centrifuged at 0° for 1 hr. at 500 *g*. The clear ether layer was discarded and the cloudy-blue solution drawn off by suction from beneath a thick blue layer. The solution was filtered through a FCB pad (T. B. Ford Ltd.) with Hyflo-Supercel, the pad was washed with 0·1 M-sodium chloride adjusted to pH 5·35 with acetic acid, until the washings were no longer appreciably blue, and the washings added to the filtrate, the resultant solution being designated filtrate A. The loosely packed thick-blue layer had a volume of 520 ml. To it were added 2·3 l. of 0·32 M-sodium chloride saturated with ether (the final sodium chloride molarity being about 0·28 M) and the suspension was stirred for 30 min. After centrifuging at 4° for 45 min. at 800 *g*, the cloudy-blue supernatant was mixed to a thin paste with Hyflo-Supercel and filtered through a FCB pad. The pad was slowly washed with 0·28 M-sodium chloride–0·01 M-sodium acetate buffer, pH 5·4, saturated with ether, until the washings were no longer appreciably blue. The filtrate and washings were bulked with filtrate A to give fraction III.

Stage 4: reprecipitation of caeruloplasmin at pH 4·8. To the whole of fraction III saturated with ether was added 10·7 l. of water, with stirring, to bring the sodium chloride molarity to about 0·07 M. The resulting solution was then saturated with ether at 4° and placed in a cooling bath at 0° to −2°; 25 ml. of M-acetic acid was added with stirring for 2 hr. to bring the pH to 4·8. The precipitate was centrifuged and washed as in the first pH 4·8 precipitation and taken up in 340 ml. of 0·1 M-sodium chloride. The turbid-blue solution was partly cleared by filtration under pressure, through a FCB pad with Hyflo-Supercel. After filtration, the pad was washed with 0·1 M-sodium chloride until the washings were no longer blue. The molarity of the sodium chloride in the filtrate, after addition of the washings of the filter, was about 0·086. This solution (fraction IV) was deep blue and pH 4·9.

Stage 5: fractional precipitation by decreasing salt concentration. It was noticed that when water was added to a small sample of fraction IV a pale-blue precipitate appeared. After centrifuging this and adding more water to the supernatant a strong-blue precipitate appeared. This suggested the possibility of further purification by fractional precipitation at decreasing salt concentrations. An experiment on a small sample of fraction IV (Table 2) showed that this was in fact so and indicated suitable conditions for further purification.

The whole of fraction IV was brought to about 0·02 M-sodium chloride by the addition, with

Table 2. *Fractional precipitation of fraction IV at decreasing salt concentrations*

	Approx. concn. of NaCl (M)	Total activity*	Activity/1 ml. $E_{1\,cm.}$ (280 mμ)
(*a*) Fraction IV (10 ml.)	0·085	4540	9·9
(*b*) Fraction IV (10 ml.) + 20 ml. of water → ppt. 1	0·028	605	4·7
(*c*) Supernatant from ppt. 1 + 20 ml. of water → ppt. 2	0·017	1280	12·1
(*d*) Supernatant from ppt. 2 + 50 ml. of water → ppt. 3	0·0085	1560	20·4
(*e*) Supernatant from ppt. 3 + 100 ml. of water → ppt. 4	0·0042	420	21·7
(*f*) Supernatant from ppt. 4 (pH 5·0)	—	880	7·1

* Activity figures refer to precipitates, except for figures on lines (*a*) and (*f*), which refer to the initial solution and final supernatant respectively.

stirring, of 2·9 l. of water. Throughout all procedures in this section, the pH was not allowed to rise above 4·8–4·9, being adjusted when necessary by addition of a few drops of M-acetic acid. The precipitate was centrifuged at 4° for 25 min. at 800 *g* and washed repeatedly with 0·02 M-sodium chloride, the washings being added to the supernatant. Even after repeated washings the precipitate was still appreciably blue. More activity could be recovered from it by redissolving in 0·086 M-sodium chloride and reprecipitating at 0·02 M-sodium chloride. The supernatant was added to the main supernatant and more water added to bring to 4·5 mM-sodium chloride. On standing overnight almost all of the active material of the solution precipitated in a compact layer, from which most of the 15 l. of supernatant was drawn off by suction. The precipitate was centrifuged for 30 min. at 800 *g*. An additional 3% of active material could be centrifuged from the 20 l. of slightly cloudy supernatant under the same conditions. The bulked precipitates were washed with 5 vol. of 4·5 mM-sodium chloride, centrifuged down and dissolved in as small a volume as possible of 0·05 M-sodium chloride–0·05 M-sodium acetate. The resultant clear, deep-blue solution after dialysis against three changes of 0·1 M-sodium chloride (fraction V), was pH 5·5. $E_{1\,cm.}$ (605 mμ)/$E_{1\,cm.}$ (280 mμ) was between 0·016 and 0·022. A typical preparation possessed 40·3 μg. of total copper/ml., of which 32·6 μg./ml. was caeruloplasmin copper.

Stage 6: further purification of fraction V by chromatography on diethylaminoethylcellulose. Preliminary batch experiments showed that caeruloplasmin in fraction V was absorbed by DEAE from 0·05 M-sodium acetate buffers of from pH 4·8 to 5·8, and eluted by 0·05 M-sodium acetate buffers–0·3 M-sodium chloride in the same pH range. Columns were therefore run under a variety of conditions within the above range. A preliminary run with gradient from 0·05 M-sodium acetate buffer, pH 5·2, to 0·05 M-sodium acetate buffer (pH 5·2)–0·25 M-sodium chloride yielded three main protein peaks in the eluate, the first with the eluate front. Only

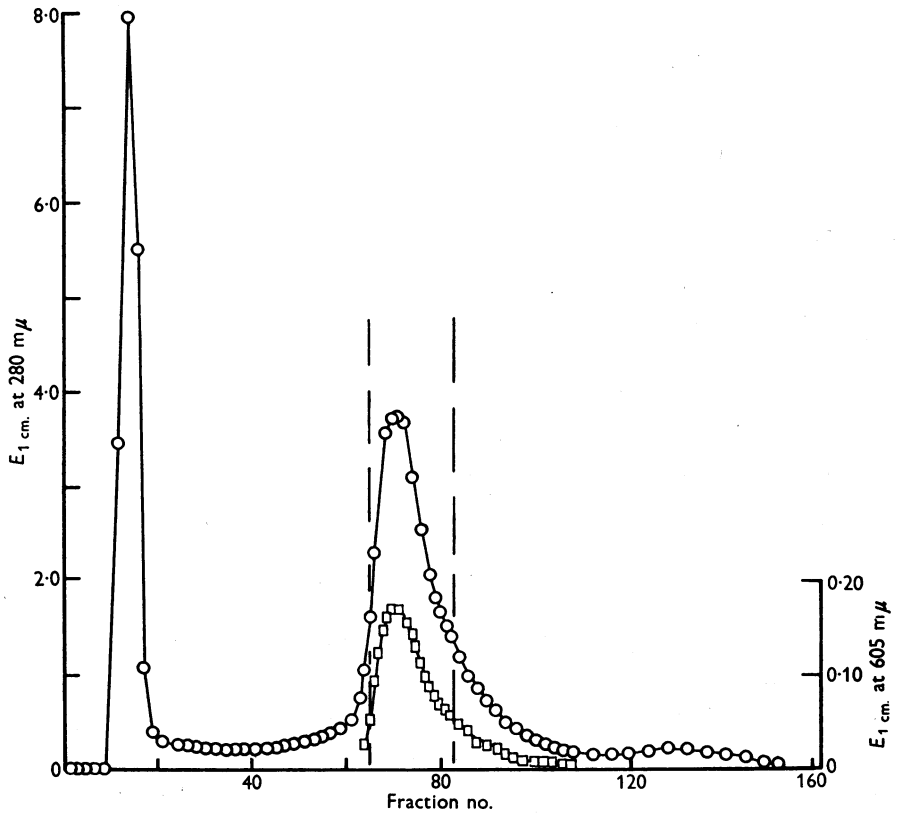

Fig. 1. Effluent diagram of 37 ml. of fraction V on a 42 cm. × 2·5 cm. DEAE column at room temperature. The DEAE was previously equilibrated with 0·05 M-sodium acetate buffer (pH 5·7)–0·1 M-sodium chloride. Protein was eluted with a gradient from this buffer to 0·05 M-sodium acetate buffer (pH 5·2)–0·25 M-sodium chloride with a 1000 ml. mixing chamber. The height of the buffer level above the top of the column was 34 cm. Eluate fractions of about 10 ml. were collected. ○, $E_{1\,cm.}$ at 280 mμ; □, $E_{1\,cm.}$ at 605 mμ. Fractions between broken lines were rechromatographed.

the third peak was blue and had DPD oxidase activity. There were appreciable quantities of protein in all fractions between the second and the caeruloplasmin peaks. The results suggested that with higher initial ionic strength the peaks of contaminant protein would be eluted together and the time necessary for elution of the caeruloplasmin peak would be considerably decreased. Also a higher initial pH, i.e. one further from the isoelectric point of caeruloplasmin, would hold it on the column more tightly and thus perhaps facilitate its separation from protein eluted between peaks 2 and 3. Where columns were run slowly with a small pressure head the recoveries of applied caeruloplasmin were poor, suggesting that caeruloplasmin was unstable under column conditions and indicating that columns should be run as quickly as possible. Though eluate flow through DEAE columns is slow under conditions suitable for separation of most plasma proteins, it is relatively fast at the high salt concentrations used for caeruloplasmin purification. DEAE chromatography under the conditions shown in Fig. 1 resulted in a high degree of purification. Most of the contaminant protein was eluted with the eluate front. Fractions 66–84 inclusive contained 85% of the total caeruloplasmin of the second peak with an average $E_{1\,cm.}$ (605 mμ)/$E_{1\,cm.}$ (280 mμ) of 0·043. Recovery of caeruloplasmin applied to the column was 80–95%. When elution was continued after the emergence of the main caeruloplasmin peak, a third slight but definite peak appeared associated with oxidase activity.

Fractions 66–84 were bulked and, after dialysis against the starting buffer, were chromatographed again on a similar DEAE column. Only slight further purification was obtained, the highest $E_{1\,cm.}$ (605 mμ)/$E_{1\,cm.}$ (280 mμ) being 0·044. The minor active peak was again observed. It contained about 5% of the total protein eluted and 2–3% of the total oxidase activity eluted. It probably represents either an active artifact or indicates that caeruloplasmin exists as an equilibrium mixture. The properties of the purified caeruloplasmin were investigated after dialysis against 0·1 M-sodium chloride or water and concentrating by ultrafiltration where necessary.

Partial purification of caeruloplasmin with diethylaminoethylcellulose by batch method. The results of using DEAE suggested that it could perhaps be used at an earlier stage than fraction V. Small-scale experiments were performed on fractions III and V. The caeruloplasmin was absorbed on to DEAE from 0·05 M-sodium acetate buffer (pH 5·7)–0·08 M-sodium chloride and eluted by 0·05 M-sodium acetate buffer (pH 5·2)–0·25 M-sodium chloride. In both cases the eluted material had $E_{1\,cm.}$ (605 mμ)/$E_{1\,cm.}$ (280 mμ) 0·033, indicating a considerable degree of purification. Thus batch treatment by DEAE is a possible alternative to fractional precipitation by decreasing salt concentration (stage 5). Attempted purification of fraction II under similar conditions failed, possibly owing to interaction between caeruloplasmin and the large amounts of contaminant protein present in this material.

Properties of caeruloplasmin

Stability on storage. Only partial stability studies were made. Dilute aqueous solutions of purified caeruloplasmin lost about 10% of their oxidase activity in 10 days when stored in glass at −25°. Similar losses occurred in 0·1 M-sodium chloride. Repeated freezing and thawing resulted in rather greater losses (Fig. 2). Preparations stored at −25° decreased in extinction in the visible range, though there was no significant change in the shape of the spectrum. A preparation of fraction V having 1000 units of activity/ml., stored at 4° in a 0·1 M-sodium chloride, lost 30% of its activity in 70 days. Activity was completely lost after heating for 5 min. in a boiling-water bath. After 5 min. in a bath at 80°, however, the blue colour was not destroyed in a solution at pH 5·5.

Stability on freeze-drying. When fraction V was frozen and dried at temperatures above −25° it tended to lose activity and to change from a pure blue to a rather more greenish colour. Decreased absorption at the 605 mμ peak occurred together with increased absorption in the 360–560 mμ range. There was no change in absorption at 280 mμ.

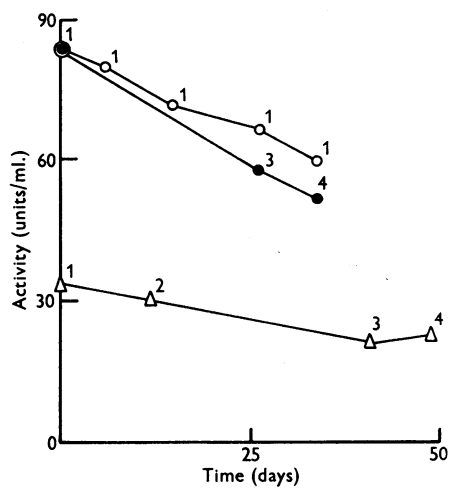

Fig. 2. Loss of activity of purified caeruloplasmin during storage at −25°. ○, ●, Dialysed against water; △, dialysed against 0·1 M-sodium chloride. Numbers above points show how many times the material was frozen and thawed.

High protein concentration and the presence of sodium chloride both resulted in higher stability. Another important variable appears to be the rate of initial freezing of the caeruloplasmin. A preparation of fraction V frozen from 0·1 M-sodium chloride in an acetone–solid carbon dioxide bath at −77° and then dried below −25° showed no significant change in activity or spectrum on reconstitution. When purified caeruloplasmin obtained by DEAE chromatography was dried under similar conditions 32 % of the activity was lost and spectral change occurred (Fig. 3). Thus it is probable that the contaminant protein in fraction V had some stabilizing effect on caeruloplasmin. The purified caeruloplasmin damaged by freeze-drying was run on a DEAE column under conditions essentially identical with those in Fig. 1. No new protein peaks appeared, though $E_{1\,cm.}$ (605 mμ)/$E_{1\,cm.}$ (280 mμ) was decreased from 0·044 to 0·040 in the purest fractions. It appears that the damaged caeruloplasmin behaves rather similarly to the undamaged protein under these conditions. $E_{1\,cm.}$ (605 mμ)/$E_{1\,cm.}$ (280 mμ) ratios of individual fractions suggest that the damaged material is eluted slightly more slowly than intact caeruloplasmin. When a preparation of fraction V which had been damaged by freeze-drying was purified on a DEAE column under conditions as in Fig. 1, two runs were necessary to attain purification to $E_{1\,cm.}$ (605 mμ)/$E_{1\,cm.}$ (280 mμ) 0·044 whereas only one run was necessary with undamaged fraction V material to attain a ratio of 0·043.

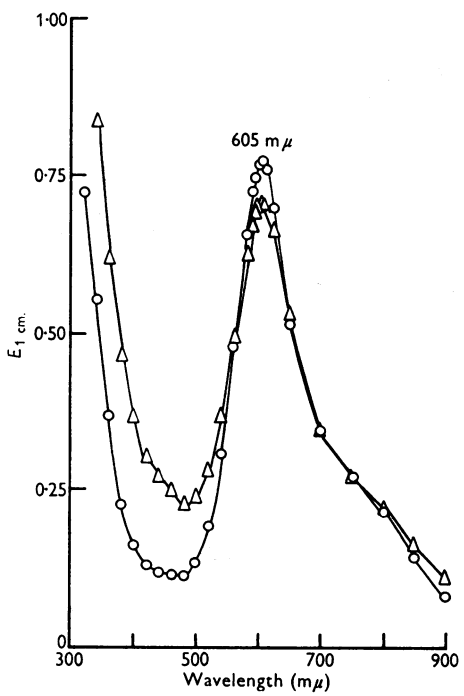

Fig. 3. Spectrum of purified caeruloplasmin: ○, before freeze-drying; △, after freeze-drying, with 68% recovery of activity.

Electrophoresis. Starch-gel electrophoresis of purified material was kindly done by Dr B. P. Hughes by a method similar to that of Smithies (1955). The gel was made transparent and scanned by the method of Fine & Waszczenko-Z (1958). A single, though unusually broad, band was obtained. There was no evidence of asymmetry.

Chromatography on hydroxylapatite. Broman (1958) showed that human serum gave two peaks having caeruloplasmin activity when run on a hydroxylapatite column. Purified caeruloplasmin run under Broman's conditions also gave two active peaks, the elution pattern being very similar to that found with serum. On both hydroxylapatite and DEAE chromatography the activity/protein ratio of the major peak was twice that of the minor peak.

Absorption spectrum. The spectrum in the visible range is shown in Fig. 3. Residual absorption at 605 mμ of material decolorized by cyanide was about 1 % of that of the best untreated preparations. In general, absorption in the 300–500 mμ range decreased as purity increased. Holmberg & Laurell's (1948) finding that mg. of copper/100 ml. is five times $E_{1\,cm.}$ (605 mμ) was confirmed approximately, factors of 4·8–5·0 being found. Ratios of absorption at various wavelengths in the visible range were almost identical to those published by Scheinberg & Morell (1957). $E_{1\,cm.}$ (605 mμ)/$E_{1\,cm.}$ (280 mμ) was 0·044, which is slightly higher than the ratio found by the last-named authors. In the ultraviolet range caeruloplasmin gave a peak at 277–278 mμ; $E_{1\,cm.}$ (278 mμ)/$E_{1\,cm.}$ (260 mμ) was 1·72, which indicates no significant amount of nucleotide impurity to be present.

Copper/nitrogen ratios. Two preparations had caeruloplasmin copper/nitrogen ratios of 0·0197 and 0·0203 (w/w) respectively. This is in reasonable agreement with a copper/nitrogen ratio of 0·0211 (w/w) calculated from Holmberg & Laurell's finding of eight atoms of copper/caeruloplasmin molecule and a molecular weight of 151 000, which they obtained by an ultracentrifuge method. A protein/nitrogen ratio of 6·25 has been assumed.

Enzyme properties. The purified caeruloplasmin had 21 units of oxidase/μg. of caeruloplasmin copper and an optimum pH in acetate buffers of 5·5–6·0 under conditions of oxidase-activity determination as described in the Experimental section. Adrenaline and 5-hydroxytryptamine were also destroyed, in agreement with Holmberg & Laurell (1951) and Porter, Titus, Sanders & Smith (1957) respectively. 5-Hydroxyindoleacetic acid was converted into a more polar indolic substance which no

longer gave the typical 5-hydroxyindole reaction of Udenfriend, Titus & Weissbach (1955). The pH optimum for this oxidation under the above conditions was 4·5–5·0, which is about one pH unit lower than pH optima with other substrates. The oxidase was strongly inhibited by cyanide and azide ions. Ethylenediaminetetra-acetic acid (EDTA) also inhibited it; thus in a typical experiment 0·01 and 1·0 mM-EDTA inhibited the enzyme 13 and 32% respectively. The sharp inhibition by 1·0–10·0 μM-EDTA reported by Broman (1958) with caeruloplasmin contaminated with metal ion was not observed, the percentage inhibition increasing steadily with log concentration of EDTA. This indicates that the traces of contaminant copper present were insufficient to affect activity against *NN*-dimethyl-*p*-phenylenediamine oxalate. Some of these findings will be described more fully elsewhere.

DISCUSSION

Our method of purifying caeruloplasmin is suitable for application on a large scale. The method differs from that of Holmberg & Laurell (1948) at most points, particularly in the omission of the drastic 50% ethanol–chloroform denaturation. A drastic denaturation of copper-free proteins is commonly used in the preparation both of caeruloplasmin and other cuproproteins (Mann & Keilin, 1939; Porter & Folch, 1957). Although caeruloplasmin is apparently stable to high concentrations of organic solvents and is more stable to heat than most proteins, it is unstable under certain conditions during freeze-drying. Similar findings have been made with other cuproproteins. Thus freeze-drying caused haemocyanin to become insoluble, with loss of oxygen-binding capacity and loss of the property of becoming blue on exposure to the atmosphere (Litt & Boyd, 1958). Moreover, Frieden & Maggiolo (1957) found that ascorbic acid oxidase was partly destroyed by freeze-drying. Thus it appears that a number of copper–proteins have similar stabilities, e.g. towards organic solvents and freeze-drying. The instability to freeze-drying may be related in some way to copper-bound water being an essential part of the molecule, its removal resulting in irreversible changes. Mahler (1958) interprets the properties of the complexes of the copper–enzyme uricase with substrates and inhibitors in terms of a copper–aquo complex.

Investigations suggest that the small second peak with oxidase activity found both on DEAE and hydroxylapatite chromatography may be an active artifact or may indicate that caeruloplasmin exists as an equilibrium mixture. Activity/protein ratios are consistent with the minor peak's representing a modification of the protein, in which only half of the active centres are intact or available.

The general properties of the purified caeruloplasmin compare well with those found by other workers (Scheinberg & Morell, 1957; Holmberg & Laurell, 1948). The discrepancy between our finding that 5-hydroxyindoleacetic acid is a substrate and the negative results of Martin, Eriksen & Benditt (1958) is probably due to the fact that we tested at the optimum pH (4·5–5·0) whereas Martin *et al.* worked at pH 6·0.

The development of methods for the large-scale purification of caeruloplasmin is of some importance. Biochemical studies of caeruloplasmin in the 10 years since Holmberg & Laurell's isolation have been few, largely because of the scarcity of purified material. Knowledge of the nature and physiological significance of caeruloplasmin is of interest not only with respect to the more general problems of metal–protein interaction and the involvement of metals in enzyme activity but also because of the light it may throw on Wilson's disease and the biochemistry of mental disease.

After this paper had been submitted for publication a paper was published on the preparation of caeruloplasmin from Cohn fractions IV 1 and IV 1, 4 (Sanders, Miller & Richard, 1959). The method was similar at some stages to that described here, batch and column purification on DEAE being used.

SUMMARY

1. A method for the purification of caeruloplasmin from the G 2 fraction of human plasma is described. Protein precipitation at various pH values and concentrations of sodium chloride in the presence of diethyl ether is followed by chromatography on diethylaminoethylcellulose.

2. Partly purified caeruloplasmin is stable to freeze-drying only when rapid initial freezing is used. Highly purified caeruloplasmin is partly inactivated when freeze-dried under the same conditions.

3. Purified material has a copper/nitrogen ratio of 0·0197–0·0203 (w/w) and gives a single broad symmetrical peak on starch-gel electrophoresis. The values of the copper/nitrogen ratio agree reasonably with a molecular weight of 151 000 and eight atoms of copper/molecule.

4. On hydroxylapatite and diethylaminoethylcellulose chromatography, in addition to the main caeruloplasmin peak, a trace of a second peak with oxidase activity is eluted. The significance of this is discussed.

5. Some enzymic properties of purified caeruloplasmin are briefly described.

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The Transmission of Sugars across the Goat Placenta

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Fructose is known to occur in the foetal blood of a number of species, which include the sheep and the goat, and in the seminal plasma of most species. The presence of a considerable concentration of fructose in foetal blood and its virtual absence from the maternal blood indicate that the placenta must be impermeable to fructose in the direction foetus to mother. On the other hand, Huggett, Warren & Warren (1951) showed that, in the sheep, glucose rapidly passed between the bloods of ewe and foetus in both directions.

In the work to be described in this paper, goats have been used as experimental animals, and sufficient experiments similar to those of Huggett *et al.* (1951) on sheep have been performed to demonstrate that the placentae of these two species behave in an analogous manner. Further, a number of other sugars have been used in order to obtain some information as to the specificity of the permeability properties of the placenta.

A preliminary account of this work was given by Walker & Warren (1955).

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METHODS

Animals. In most cases the date of mating of pregnant goats was known. For the remaining animals the stage of pregnancy was deduced from a graph of foetal age against foetal crown–rump length (R. J. Harrison, personal communication). The goats were kept in the animal house or in open-air pen for a period of from 2 days to several weeks. They were fed on hay and greenstuff and were allowed an unlimited supply of water.

Surgical procedure. This was based upon that used by Barklay, Haas, Huggett, King & Rowley (1949) and Huggett *et al.* (1951).

Anaesthesia was initially induced by injection of sodium pentobarbitone (Veterinary Nembutal, supplied by Abbott Laboratories Ltd., London) into a convenient vein in the forelimb of the goat. Between 8 and 18 ml. was required, depending upon the size of the goat. Thereafter, 1–2 ml. of the anaesthetic was given intravenously as indicated by variations in muscle tone. Administration of the anaesthetic during the course of an experiment did not appear to have any effect on the blood-sugar levels.

Cannulae were inserted into the jugular vein (for infusion of the supplementary doses of the anaesthetic and injection of the sugar solution) and carotid artery (for obtaining maternal-blood samples). Clotting in the cannulae was avoided by the liberal use of heparin. The goat was