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Hormonally produced changes in caeruloplasmin synthesis and secretion in primary cultured rat hepatocytes

Relationship to hepatic copper metabolism

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Hormonally produced changes in the synthesis and secretion of the serum copper-containing protein caeruloplasmin were studied in primary cultures of rat liver parenchymal cells isolated by the collagenase-perfusion technique. A rabbit antibody directed against rat caeruloplasmin was used to immunoprecipitate labelled caeruloplasmin. Isolated liver cells synthesized and secreted caeruloplasmin over a period of 3 days. Synthesis and secretion of this protein was enhanced when cells were treated with dexamethasone. The accumulation of copper was also moderately enhanced with glucocorticoid treatment. Inclusion of adrenaline in the culture medium resulted in elevated incorporation of copper into newly synthesized caeruloplasmin as well as an increase in ^{64}Cu-labelled caeruloplasmin in the culture medium. However, adrenaline did not seem to increase the secretion of ^{3}H-labelled protein, despite the elevation in secreted ^{64}Cu–caeruloplasmin. This may be due to a large increase in the intracellular pool of ^{64}Cu caused by enhanced accumulation of this metal when adrenaline is included in the incubation medium. Enhanced copper accumulation was also seen when cells were treated with glucagon. Adrenaline-stimulated accumulation of ^{64}Cu could be inhibited by including phenoxybenzamine, an α-adrenergic blocker, in the culture medium. Elevation of extracellular copper caused enhancement in the detection of labelled caeruloplasmin in the medium of cultured cells, probably owing to the ability of this metal to stabilize the protein.

The regulation of copper metabolism in mammals has received recent attention, particularly with respect to changes that occur during an inflammatory response (Brown *et al.*, 1981), certain stresses (Linder *et al.*, 1979*b*) or in the genetic disorders Wilson's disease and Menke's steely-hair syndrome (Mason, 1979). One protein that has been implicated in the control of copper levels in the liver and blood during these conditions is the α_2-globulin caeruloplasmin. In addition to its role in copper metabolism this 131000-dalton glycoprotein has been studied for its multiple potential functions, namely, the mobilization of storage iron (Frieden & Hsieh, 1976), scavenging of superoxide anions (Goldstein *et al.*, 1979), involvement in the acute-phase response (Denko, 1979) and regulation of adrenaline and 5-hydroxytryptamine (serotonin) levels in the serum (Frieden, 1980).

It has become clear that the synthesis of caeruloplasmin may be varied during physiological states that require one or more functions of this protein. Among the factors reported to cause changes in the synthesis of caeruloplasmin are copper (Evans *et al.*, 1970*a*), hormones (Evans *et al.*, 1970*b*) and leucocytic endogenous mediator (Wannemacher *et al.*, 1975). Much controversy surrounds the cause-and-effect relationships of these factors as the result of two distinct problems. First, direct correlations between such factors and the synthesis *de novo* of caeruloplasmin are difficult, owing to the numerous secondary effects involved in

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid; SDS, sodium dodecyl sulphate.

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intact animal experiments. Secondly, measurement of caeruloplasmin levels has relied on methods that involve quantification of oxidase activity, a property known to be dependent on the presence of copper in the protein (Holtzman & Gaumnitz, 1970). We have utilized primary cultures of rat liver parenchymal cells to study caeruloplasmin and copper metabolism because this method eliminated secondary effects brought on by interaction with other organs and/or glands. Also, we have quantified caeruloplasmin synthesized or secreted by these cells by using an antibody directed toward purified rat caeruloplasmin. In the present paper we describe the apparent induction of this protein in liver cells by treatment with various hormones as well as the specific changes in copper content of the liver cell that are concurrent with caeruloplasmin induction and secretion.

Experimental

Materials

[^{3}H]Glutamate (sp. radioactivity 18.8Ci/mmol), [^{3}H]aspartate (10.7Ci/mmol), [^{3}H]threonine (1.1Ci/mmol), [^{3}H]leucine (110Ci/mmol), [^{3}H]glycine (15Ci/mmol), ^{64}Cu (3–6mCi/mg) and ^{3}H-labelled amino acid kits were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). M-199, L15 and amino acid-deficient L15 media, Freund's complete adjuvant and foetal-calf serum were obtained from Grand Island Biological Co. (Grand Island, NY, U.S.A.). Collagenase CLS II was obtained from Worthington Biochemicals (Freehold, NJ, U.S.A.). Hepes, Tes, hormones, fatty acid-free bovine albumin, human caeruloplasmin, penicillin, streptomycin, gentamycin and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cellex-D (Bio-Rad Laboratories) was used for DEAE-cellulose ion-exchange chromatography. ScintiVerse counting solution and all other analytical-reagent-grade materials were obtained from Fisher Scientific Co. (Pittsburg, PA, U.S.A.).

Animals

Male CD-strain rats (250–350g) from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.) were used in all experiments. The rats were housed in stainless-steel suspended wire cages and were fed a commercial laboratory animal diet and tap water *ad libitum*. A 12h dark/light cycle was used.

Primary hepatocyte cultures

Primary monolayer cultures of rat liver parenchymal cells were prepared as described previously (Failla & Cousins, 1978*a*). Approx. 2×10^6 cells were plated on to collagen-coated Falcon tissue culture dishes (15mm × 60mm). Parenchymal cells were allowed to selectively attach to the dishes in M199 medium containing 10% foetal-calf serum. Hepes (15mM), Tes (10mM), insulin (1μg/ml) and antibodies were added as listed below. Subsequently, the medium was changed to either 2 or 4ml of L15 or amino acid-deficient L15 medium containing penicillin (60μg/ml), streptomycin (100μg/ml), gentamycin (50μg/ml), insulin (1μg/ml), albumin (2mg/ml), glucose (1.5mg/ml) and $NaHCO_3$ (5mM). Hormones, metals or isotopes were added as required by experimental protocol.

Preparation of purified rat caeruloplasmin

A modification of the procedures outlined by Deutsch *et al.* (1962) and Morell *et al.* (1968) was used to isolate pure rat serum caeruloplasmin. All procedures were conducted at 4°C. Freshly isolated rat serum (160ml) was diluted 5-fold with 0.05M-sodium acetate buffer, pH5.5, containing 20mM-6-aminohexanoic acid (buffer A). This was applied to two DEAE-cellulose columns (1.5cm × 30cm) connected in series. The columns were washed with buffer A until the A_{280} of the effluent decreased to 0.15. The columns were then eluted with buffer A containing 0.5M-NaCl. The blue fractions were pooled and centrifuged at 25000g ($r_{av.}$ 8.0cm) for 15min. The reddish-white pellet was discarded. The supernatant was dialysed against buffer A overnight and the precipitate that formed in the dialysis bag was pelleted at 25000g for 15min and discarded. The blue supernatant was then applied to a DEAE-cellulose column (1.5cm × 30cm) and washed with buffer A until the A_{280} of the effluent decreased to 0.05. A linear gradient constructed with a lower reservoir containing 200ml of buffer A and an upper reservoir of 200ml of buffer A plus 0.3M-NaCl was used to elute the blue material from the column. Fractions corresponding to A_{280}/A_{610} ratio of less than 50 were pooled. Ethanol/chloroform (9:1, v/v) was added to portions of this solution in a 3:1 ratio. The precipitate formed was recovered by centrifugation at 4000g for 30min. Each blue precipitate was redissolved in 1.5ml of buffer A containing 1.0% (w/v) NaCl. The solutions were centrifuged at 128000g for 15min and the white pellet was discarded. The precipitation steps were repeated once more before pooling the blue solutions and dialysing the pooled material against buffer A overnight. The sample, which exhibited an A_{610}/A_{280} ratio of 0.049, was filtered by positive pressure into sterile tubes and stored frozen in the vapour phase of liquid N_2. The protein concentration of various batches ranged from 1.3 to 2.7μg/μl, as determined by either protein assay (Lowry *et al.*, 1951) with a bovine albumin as standard or by oxidase activity (Ravin, 1961) using

a human caeruloplasmin standard. Electrophoresis of purified rat caeruloplasmin on an SDS/7.5% polyacrylamide slab gel (Laemmli, 1970) gives a major band at 131000 daltons and two minor bands representing proteolytic fragments of caeruloplasmin at 116000 and 20000 daltons (Kingston *et al.*, 1977).

Preparation of antibodies

New Zealand White rabbits received 0.3–0.5 mg injections of rat caeruloplasmin emulsified with Freund's adjuvant over 7 weeks and were bled by heart puncture on week 8. Ouchterlony immunodiffusion of antisera with rat caeruloplasmin gave a single precipitin band. A quantitative precipitin curve was determined (Garvey *et al.*, 1977). A 50 μl portion of antiserum incubated with 27.5 mg of caeruloplasmin produced a precipitation peak corresponding to 64.8 μg of protein.

Immunoprecipitation of rat caeruloplasmin

Synthesis of caeruloplasmin in parenchymal cells was studied with monolayers cultured in 2 ml of L15 medium deficient in leucine, glutamate, aspartate, glycine and threonine, but supplemented with 20 μCi each of the same amino acids labelled with ^{3}H. For experiments on copper-labelled caeruloplasmin, cells received 40 μCi of ^{64}Cu per plate. After the required incubation time, the culture medium was removed by aspiration. The cells were washed twice with 2 ml of ice-cold 10 mM-Hepes-buffered 0.9% NaCl (pH 7.4) containing 10 mM-EDTA to remove residual medium and non-specifically bound ^{64}Cu. The cells were scraped off the plate into 1.5 ml of a buffer, containing 1.0% (v/v) deoxycholic acid, 1.0% Triton X-100, 25 mM-NaCl, 5 mM-$MgCl_2$ and 25 mM-Tris/HCl, pH 7.4, and homogenized with 10 up-and-down strokes in a glass/Teflon Potter–Elvehjem homogenizer. The homogenate was centrifuged for 1 h at 15000 *g* ($r_{av.}$ 5 cm) and 1 ml of the supernatant was used for the immunoprecipitation reaction. For studies on secreted caeruloplasmin, spent medium was removed from the plates and cleared of dead cells and debris by centrifugation. A 1 ml portion of this medium was adjusted to 1.0% deoxycholate, 1.0% Triton-X 100, 25 mM-NaCl, 5 mM-$MgCl_2$ and 25 mM-Tris/HCl, pH 7.4. For immunoprecipitation, 6.25 μg of carrier rat caeruloplasmin and excess antiserum were added to all supernatants. The solutions were incubated at room temperature for 1 h and then at 4°C for 18 h. Precipitates were centrifuged and washed in 1% Triton X-100 containing 0.15 M-NaCl. For direct counting, pellets were dissolved in 4% (w/v) NaOH, pipetted into 7 ml of ScintiVerse counting solution and the ^{3}H content was measured with a Beckman LS 7500 liquid-scintillation counter. For electrophoresis, pellets were dissolved in 50 μl of 8 M-urea containing 2.5 μl of 20% SDS and 1 μl of β-mercaptoethanol. All points represent triplicate plates from a minimum of two separate experiments. Samples were normalized for the decay of ^{64}Cu using a standard decay curve and for any differences in total protein content by measuring a replicate set of plates for protein concentration by the microbiuret procedure (Bailey, 1967).

Electrophoresis and fluorography

SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970). A 7.5% polyacrylamide separating gel was employed (2.5% cross link) with a 3% polyacrylamide (2.5% cross-link) stacking gel. Gels were formed in a 12 cm × 12 cm slab apparatus and run for 4 h at constant current (20 mA). Staining of gels was accomplished by using 0.2% Coomassie Brilliant Blue. For fluorography, SDS/polyacrylamide gels were treated with Enhance (New England Nuclear, Boston, MA, U.S.A.) and dried under reduced pressure on to filter paper. The dried gel was exposed to pre-fogged Kodak X-ray film for 2 weeks at −20°C before developing.

Copper accumulation studies

The accumulation of ^{3}H-labelled amino acids or ^{64}Cu was followed by supplying cells with isotopes and other additions in 4 ml of L15 medium directly after the selective attachment of parenchymal cells to the dish surface. The selection of hormone concentrations was based upon previous investigations on hormonal effects in liver cell culture where functional and metabolic responses to hormone treatment were reported (Pariza *et al.*, 1976, 1977; Veneziale *et al.*, 1976; Failla & Cousins, 1978*b*; Kneer *et al.*, 1979). Concentrations required to yield an effect were determined by constructing dose–response curves for these hormones in liver culture with respect to copper accumulation (results not shown). After the desired incubation time, cells were washed and harvested from the dish and dissolved in 1.5 ml of 0.5% (w/v) deoxycholate containing 0.4% NaCl. This mixture was pipetted into vials for liquid-scintillation counting (Failla & Cousins, 1978*a*) or tubes for γ-counting [Beckman Gamma 4000 spectrometer with 3 inch (7.6 cm) NaI crystal]. The amount of copper (nmol) accumulated by the liver cells was calculated from the total exogenous ^{64}Cu accumulated and the concentration of copper in the medium (determined by atomic-absorption spectrophotometry). All points represent triplicate plates from a minimum of two distinct experiments. Statistical significance was tested by Student's *t* test.

Results

Specificity of rabbit anti-(rat caeruloplasmin) for synthesized rat caeruloplasmin

Polyacrylamide-gel electrophoresis of the immunoprecipitate that develops from the reaction of rabbit anti-(rat caeruloplasmin) with rat caeruloplasmin synthesized in liver culture is shown in Fig. 1. Since rabbit antibodies were raised to intact caeruloplasmin as well as the proteolytic fragments of the protein it can be seen that labelled caeruloplasmin appears not only with the 131000-dalton band but with the 20000-dalton band as well.

Synthesis and secretion of caeruloplasmin by rat hepatocytes

Studies using the rabbit antibody were initiated to examine the synthesis and secretion of caeruloplasmin by primary cultures of rat hepatocytes. Although cultured hepatocytes continued to accumulate labelled amino acids over a period of 72h (results not shown), data in Table 1 show that the amount of secreted [^{3}H]caeruloplasmin declined beyond the first day of incubation. Both the amount of intracellular caeruloplasmin and that found in culture medium appeared to be enhanced when the incubation was carried out in the presence of dexamethasone (Table 1). This hormone was not normally included in these incubations since previous work has demonstrated that it can profoundly affect zinc metabolism in liver cultures (Failla & Cousins, 1978*b*). Therefore, cultures were limited to incubations in the first 24h, where secretion of this protein was maximal.

Hormonal regulation of copper accumulation

A series of hormones was evaluated for possible effects on copper accumulation in cultured hepatocytes (Table 2). Corticosterone and corticotropin tended to increase the accumulation of ^{64}Cu. Dexamethasone increased accumulation to the greatest extent, with a value that was 18% above

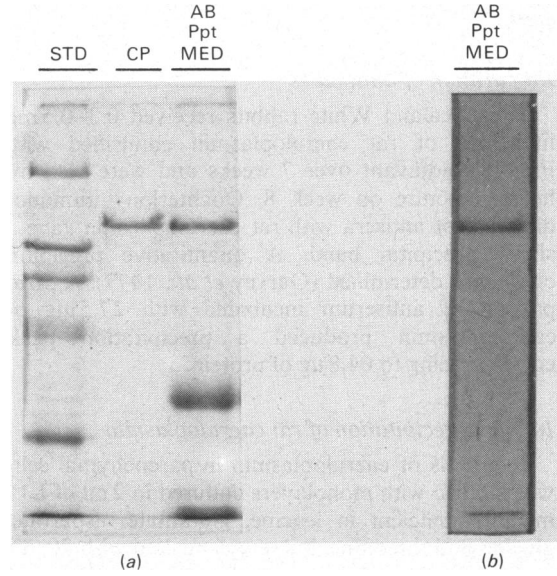

Fig. 1. (*a*) *Coomassie Blue staining of a 7.5% SDS/polyacrylamide gel and* (*b*) *fluorogram of gel shown in* (*a*)
(*a*) Abbreviations used: STD, standards [the molecular-weight markers myosin (200200), β-galactosidase (116500), phosphorylase *b* (94000), bovine albumin (68000), ovalbumin (43000)]; CP, rat caeruloplasmin standard (mol.wt. 131000); AB Ppt MED, immunoprecipitate of spent culture medium containing ^{3}H-labelled amino acids. (*b*) shows a fluorogram of the AB Ppt MED gel from (*a*). The ^{3}H-labelled bands are clearly co-migrating with rat caeruloplasmin.

Table 1. *Time study of caeruloplasmin and secretion in control and dexamethasone-treated hepatocytes*
Three sets of hepatocytes were cultured in normal L15 medium for either 0, 1 or 2 days before receiving 20μCi of ^{3}H-labelled amino acids in amino acid-deficient L-15 medium/plate. At 24, 48 or 72h, cells were harvested from the dishes and either counted for radioactivity directly for total accumulation or prepared for immunoprecipitation of caeruloplasmin as outlined in the Experimental section. In one experiment the cells were incubated with 0.1μM-dexamethasone. Cytosols and spent medium saved from these cultures were also prepared for immunoprecipitation. Values are corrected for differences in cellular protein content. Results are means ± S.E.M.

	^{3}H radioactivity (c.p.m./mg of total cell protein)		
	24h	48h	72h
Control cells			
Caeruloplasmin in cytosol	2778 ± 73††	2269 ± 301	2620 ± 62
Caeruloplasmin in medium	1565 ± 21*,†,**	937 ± 76*	1107 ± 43†
Dexamethasone-treated cells			
Caeruloplasmin in cytosol	3167 ± 52††	—	—
Caeruloplasmin in medium	3132 ± 48**	—	—

* Means are significantly different at $P < 0.01$.
**, † and †† Means are significantly different at $P < 0.001$.

Table 2. *Effect of hormones on ^{64}Cu accumulation*
Liver cells were cultured for 18h with 0.5μCi of ^{64}Cu per plate in L15 medium containing the hormones as listed. Cells were then harvested and counted by gamma spectrometry. Total cell protein was determined by the microbiuret assay as outlined in the Experimental section. Results are means ± S.E.M.

Hormone	Hormone concn. (M)	Accumulation of ^{64}Cu (% of control)	Total cellular protein (mg)
Control		100 ± 1	1.3
Corticosterone	10^{-7}	106 ± 3	1.2
Dexamethasone	10^{-7}	118 ± 2*	1.3
Corticotropin	10^{-8}	105 ± 5	1.3
Oestradiol-17β	10^{-7}	94 ± 6	1.2
Testosterone	10^{-7}	82 ± 3*	1.3
Adrenaline	10^{-5}	148 ± 6†	1.3
Glucagon	5×10^{-9}	112 ± 5*	1.3

* Means statistically different from the control mean at $P < 0.05$.
† Mean statistically different from the control mean at $P < 0.001$.

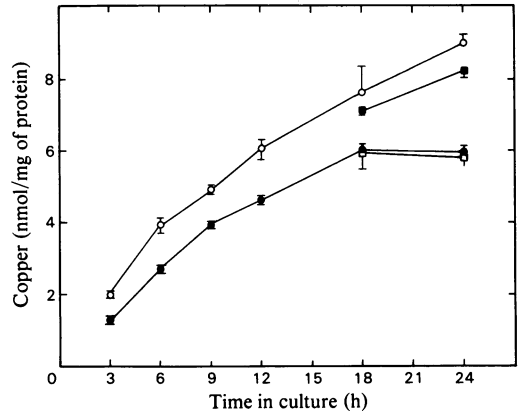

Fig. 2. *Effect of adrenaline on ^{64}Cu accumulation*
Liver cells were incubated with 5μCi of ^{64}Cu in the presence (○) or absence (●) of 10μM-adrenaline. At designated times cells were harvested and accumulated ^{64}Cu was measured. A separate set of cells was incubated concurrently with adrenaline and either 10μM-phenoxybenzamine (□) or 10μM-propranolol (■) and harvested at either 18 or 24h. The S.E.M. ranged from ±0.05 to ±0.85 nmol/mg of protein.

control levels ($P < 0.05$). Oestradiol- and testosterone-treated cells accumulated less ^{64}Cu than control cells.

Data from Table 2 also show that liver cells treated with either adrenaline or glucagon exhibit similarities with respect to increased copper accumulation (increases with adrenaline and glucagon treatment significant at $P < 0.001$ and < 0.05 respectively). Of all the hormones tested, the 48% increase in response to adrenaline was the most pronounced overall change in accumulation in monolayers of hepatocytes. Adrenaline-stimulated ^{64}Cu accumulation fluctuated between experiments but was always an increase of 20–58% of the control accumulation level. Total cell protein was not altered by these treatments.

The enhancement in the accumulation of ^{64}Cu owing to adrenaline treatment was further studied over a time course within the first day of culture (Fig. 2). A statistically significant ($P < 0.001$) differential of ^{64}Cu accumulation was observed very rapidly (within 3h) after the addition of hormone. This differential was significantly different for the duration of the 24h incubation period at confidence levels not lower than 95%. Cells not receiving adrenaline showed a ^{64}Cu accumulation plateau at about 18h of incubation as we observed previously (Weiner & Cousins, 1980). The elevation in copper due to adrenaline could be effectively abolished by incubation with the α-adrenergic inhibitor phenoxybenzamine. A slight inhibition of accumulation was produced by the β-adrenergic inhibitor propranolol.

Adrenaline-stimulated alteration of caeruloplasmin

To investigate the relationship between copper metabolism and caeruloplasmin content of cultured hepatocytes, cells were incubated over a 24h period in media containing ^{64}Cu with or without added adrenaline. At various intervals cell cytosols were prepared and the intracellular caeruloplasmin was immunoprecipitated. Incorporation of ^{64}Cu into caeruloplasmin first increased steadily, then reached a plateau after 12h of incubation (Fig. 3*a*). A difference of at least 92% in ^{64}Cu-labelled caeruloplasmin was detectable between 12 and 24h of culture ($P < 0.05$). To determine if the incorporation of ^{64}Cu into caeruloplasmin occurred concurrently with increased caeruloplasmin synthesis *de novo*, the incorporation of ^{3}H-labelled amino acids into caeruloplasmin was examined in a similar experiment. Adrenaline in culture medium stimulated the incorporation of ^{3}H-labelled amino acids into newly synthesized caeruloplasmin (Fig. 3*b*). This statistically significant difference can be detected as early as 3h ($P < 0.05$) after adrenaline treatment. The major difference between hormone-treated and control cultures appears after 18–24h of incubation ($P < 0.02$ and < 0.001 respectively). These differences do not seem to be related to a pronounced increase in protein synthesis as only minor changes in trichloroacetic acid-precipitable protein are observed as a result of adrenaline treatment (Fig. 3*b*, insert).

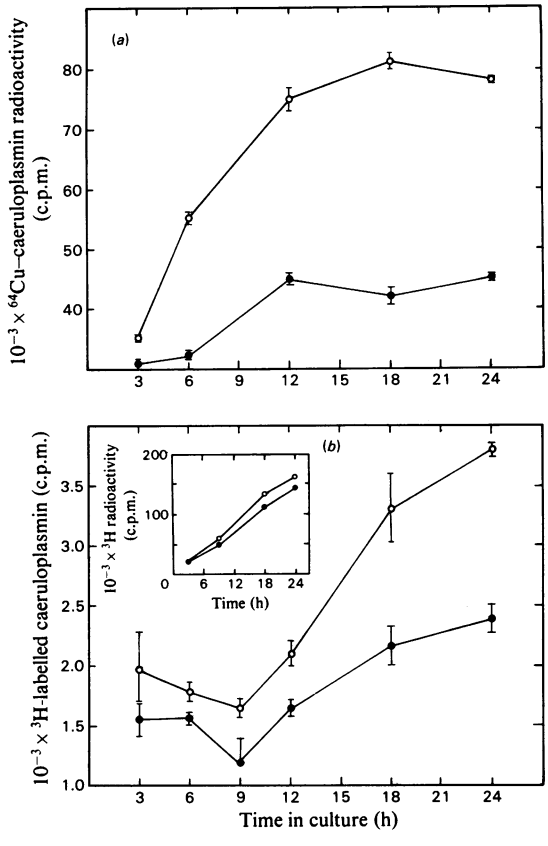

Fig. 3. *Effect of adrenaline on intracellular ^{64}Cu-labelled (a) and ^{3}H-labelled (b) caeruloplasmin*

(*a*) Hepatocytes were given 40μCi of ^{64}Cu and were cultured with (○) or without (●) added adrenaline (1μM). At designated times cells were harvested, homogenized and ^{64}Cu-labelled caeruloplasmin was immunoprecipitated from cytosols as described in the Experimental section. (*b*) Hepatocytes were cultured with 20μCi of ^{3}H-labelled amino acids with (○) or without (●) added adrenaline (10μM) and prepared as described above. The inset shows the ^{3}H precipitated by 10% trichloroacetic acid from the same amount of cell cytosol.

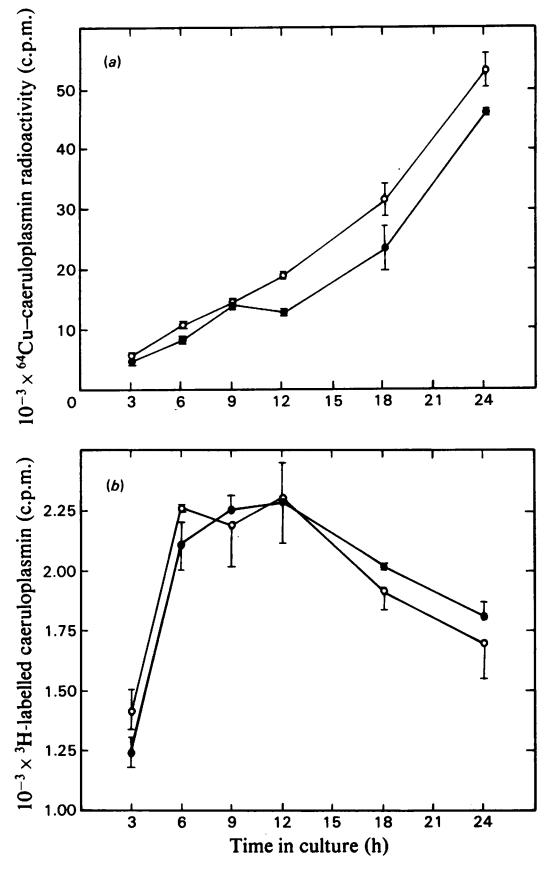

Fig. 4. *Effect of adrenaline on secreted ^{64}Cu-labelled (a) and secreted ^{3}H-labelled (b) caeruloplasmin*

(*a*) Spent culture medium from liver cells incubated with 40μCi of ^{64}Cu in the presence (○) or absence (●) of 10μM-adrenaline was removed after the designated times in culture. Portions were then used for immunoprecipitation of labelled rat caeruloplasmin as described in the Experimental section. (*b*) Spent culture medium from cells receiving 20μCi of ^{3}H-labelled amino acids either with (○) or without (●) adrenaline (10μM) was prepared as described above.

An elevation in the level of ^{64}Cu–caeruloplasmin could be detected in culture medium from adrenaline-treated hepatocyte monolayers (Fig. 4*a*). This elevation was evident by 12h in culture and remained consistently higher (up to 35%) for the duration of the incubation period. The difference was statistically significant ($P<0.05$) between 12 and 24h. Elevated ^{64}Cu–caeruloplasmin in the culture medium after adrenaline treatment may reflect either an increased amount of secreted ^{64}Cu–caeruloplasmin or an increase in the intracellular pool of ^{64}Cu–caeruloplasmin, which is then secreted at a constant rate. The latter possibility seems more likely, based upon the results presented in Fig. 4(*b*). Profiles of ^{3}H-labelled caeruloplasmin secretion in both hormones-treated and control cultures were not statistically different.

The L15 medium used in these experiments normally contains low concentrations of copper (1–5μM). We have determined that addition of exogenous copper at about 18μM facilitates the maximum accumulation of this metal by hepatocytes. It was observed that ^{3}H-labelled caeruloplasmin in culture medium declined when the medium was not supplemented with copper and that addition of 18μM-copper or higher prevented the

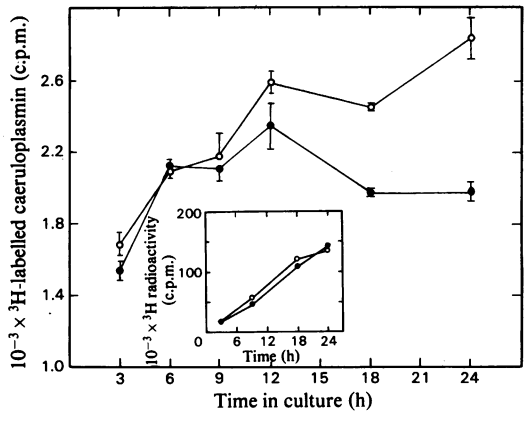

Fig. 5. *Effect of copper on secreted ^{3}H-labelled caeruloplasmin*

Medium from cultures containing 20μCi of ^{3}H-labelled amino acids with (○) or without (●) added copper (50μM) was collected at various times. The labelled caeruloplasmin was immunoprecipitated as described in the Experimental section. The insert shows the ^{3}H precipitated by 10% trichloroacetic acid from the same volume of medium.

decline in measured caeruloplasmin (Fig. 5). This phenomenon was not the result of a general increase in protein synthesis, since added copper did not cause an increase in the amount of trichloroacetic acid-precipitable ^{3}H-labelled material (Fig. 5, insert).

Discussion

The α_2-globulin, caeruloplasmin, has been reported to carry out a wide range of functions *in vivo*. Therefore, it is of considerable interest that many factors have been found to influence levels of caeruloplasmin (oxidase activity) in both the serum and the liver. Of these factors, hormones have received widespread attention (Meyer *et al.*, 1958; Johnson *et al.*, 1959; Evans *et al.*, 1970*b*; Pekarek *et al.*, 1972; Evans, 1973; Schenker, 1977). Adrenaline has been reported to cause increases in both serum oxidase activity and copper levels (Evans, 1973). We have also found that in liver cell culture adrenaline caused large increases in the accumulation of ^{64}Cu by hepatocytes. This is concurrent with large increases in the levels of ^{3}H-labelled caeruloplasmin in the cell cytosol. It is unclear whether the increased copper accumulation is a consequence of the elevation in newly synthesized caeruloplasmin. Phenoxybenzamine was effective in reducing the elevation in copper caused by adrenaline. α-Blockers have been shown to inhibit amino acid transport into cultured liver cells (Pariza *et al.*, 1977), a process that may be important to the uptake of copper by the hepatocyte (Mason, 1979). Adrenaline can inhibit amino acid incorporation into protein and this inhibition can be reversed by phenoxybenzamine but not by propranolol (Mandl *et al.*, 1982). The relationship of these results to the effects of adrenaline on copper metabolism are at present unclear.

When cells are treated with glucocorticoids, particularly dexamethasone, a distinct elevation in ^{3}H-labelled caeruloplasmin was detected in the culture medium. Glucocorticoids have previously been shown to have generalized effects on maintenance of protein synthesis and secretion in cultured hepatocytes, which may be related to an enhancement of cell viability (Ernest *et al.*, 1977; Tanaka *et al.*, 1978; Williams *et al.*, 1978; Haars & Pitot, 1979). Dexamethasone, however, was not effective in promoting accumulation of ^{64}Cu as a result of metallothionein induction in liver cells (Weiner & Cousins, 1981; Cousins & Weiner, 1982), a mechanism shown to cause copious accumulation of ^{65}Zn (Failla & Cousins, 1978*a*,*b*). The ability to enhance copper–caeruloplasmin secretion may account for this finding.

It has been previously reported that the presence of copper in caeruloplasmin increases its longevity in the serum of animals (Holtzman & Gaumnitz, 1970). Although adrenaline enhanced ^{64}Cu accumulation by liver cells it did not profoundly alter the stability of caeruloplasmin in culture medium by conversion of the apo-form into the metalloprotein. A decline in the amount of detectable ^{3}H-labelled caeruloplasmin was noted in the medium during 12–24h of culture whether or not adrenaline was included in the incubation. This paradox seems to be related to the low copper concentration normally found in the L15 medium. We observed that hepatocytes can accumulate ^{64}Cu more efficiently when exogenous copper sulphate is added to the medium up to the saturation point of about 18μM. Supplementation of copper to hepatocytes at this concentration or higher resulted in enhanced detection of [^{3}H]caeruloplasmin in cytosols and spent medium. These findings are in agreement with previous reports that hepatocytes are the primary site of serum caeruloplasmin degradation (Gregoriadis *et al.*, 1970) and that the decay rate is dependent on the presence of copper in the protein (Holtzman & Gaumnitz, 1970).

While the stabilizing effects of copper on the half-life of caeruloplasmin in the serum may be a factor in these results, the induction of caeruloplasmin synthesis by exogenous copper (Evans *et al.*, 1970*b*) still remains a possibility. Our studies using isolated liver cells rule out any secondary effects that may elevate caeruloplasmin due to

increased haemolysis from a copper injection (Linder *et al.*, 1979*a*). Although our data support the contention that copper can enhance detection of the protein, more information is required on the direct action of copper on caeruloplasmin gene expression.

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