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(54) Title: CERULOPLASMIN AND USES THEREOF IN NEURODEGENERATIVE RELATED CONDITIONS



CERULOPLASMIN AND USES THEREOF IN NEURODEGENERATIVE RELATED CONDITIONS

Background of the invention

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1) Field of the invention

The present invention relates to the use of ceruloplasmin (and/or derivatives thereof) for treating neurodegenerative related conditions encountered during development and in aging. More particularly, the present invention pertains to the use of ceruloplasmin in pharmaceutical neurotrophic compositions and in methods for culturing neuronal cells *in vitro* for promoting the tissular organization of these cells via their aggregation.

15 2) Description of the prior art

Nerve damage may occur through physical injury, which causes the degeneration of the axonal processes and/or nerve cell bodies near the site of injury. Nerve damage may also occur because of temporary or permanent cessation of blood flow to parts of the nervous system, as in stroke or at birth. Nerve damage may also occur because of intentional or accidental exposure to neurotoxins, such as the cancer and AIDS chemotherapeutic agents cisplatin and dideoxycytidine (ddC), respectively. Nerve damage may also occur because of chronic metabolic diseases, such as diabetes or renal dysfunction. Nerve damage may also occur because of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis (ALS), which result from the degeneration of specific neuronal populations.

A number of substances are known to influence neuronal development, including growth factors, components of the extracellular matrix, and cell adhesion and guidance molecules. Although a lot of efforts have been done to obtain factors and compositions for promoting the regeneration of neuronal cells, no ideal

therapeutic agent exist yet. It would therefore be highly desirable to have such a therapeutic agent.

Ceruloplasmin (CP) is a multifunctional copper-glycoprotein of 132 kDa produced by hepatocytes and found in plasma (1.5 - 3 μ M). This protein has important antioxidant and free radical scavenging properties towards reactive oxygen species (ROS) as well as a ferroxidase I activity. In particular, CP was also shown as an important oxygen free radical (OFR) scavenger. Recent studies related to the alterations in the level of ceruloplasmin further support a dominant role of this protein, suggesting possible therapeutic applications. For example, international patent application No WO9825954 relates to the use of modified ceruloplasmin comprising a glycosylphosphatidylinositol moiety and its use for the treatment of toxic level of ferrous iron. Although the ROS scavenging capacities of CP has been shown *in vitro*, none of these studies has suggested the use of CP as neurotrophic factor neither they have shown that CP could act to stimulate the maturation, growth and/or regeneration of neuronal cells.

In view of the above, it is clear that there is a need for a pharmaceutical neurotrophic composition comprising ceruloplasmin (and/or derivatives thereof) for promoting the tissular organization of neuronal cells and also promote the regeneration of neuronal tissues. There is also a need for a composition which would achieve these needs by promoting neuronal cells aggregation.

The purpose of this invention is to fulfil these needs along with other needs that will be apparent to those skilled in the art upon reading the following specification.

SUMMARY OF THE INVENTION

The present invention relates to the use of ceruloplasmin (and/or derivatives thereof) for treating neurodegenerative related conditions. More particularly, the

present invention pertains to the use of ceruloplasmin for promoting the tissular organization of neuronal cells via their aggregation.

According to an aspect of the invention, a pharmaceutical neurotrophic
5 composition comprises a therapeutically effective amount of ceruloplasmin or of a derivative thereof.

According to another aspect of the invention, ceruloplasmin and/or its functional derivative, are used as an active agent in the preparation of a
10 medication for preventing or treating a neurodegenerative disease or for treating an injury to central or peripheral nervous system tissues. Neurodegenerative diseases which could be treated using the method of the present invention include neuronal sequels caused by adverse developmental conditions that may be encountered during gestation or perinatally, Alzheimer's Disease, stroke, trauma,
15 multiple sclerosis, Parkinson's disease, HIV infection of the central nervous system, AIDS dementia, amyotrophic lateral sclerosis, hereditary hemorrhage with amyloidosis-Dutch type, cerebral amyloid angiopathy, cerebral amyloid angiopathy, Down's syndrome, spongiform encephalopathy and Creutzfeld-Jakob disease.

20

According to another aspect of the invention, ceruloplasmin or its functional derivative acts as a transporter for delivering therapeutic agent(s) to a known specific tissue such as the brain. This is achieved by using methods known in the art for coupling a protein such as ceruloplasmin or a functional derivative thereof to
25 a therapeutic agent then administering the "conjugated" or "modified" ceruloplasmin to a patient in need of the therapeutic agent. For instance, such conjugates of ceruloplasmin can be realized with transferrin, which is a large macromolecule, or with other molecules of this type that are able to pass the blood-brain barrier. For other neuronal tissues in various organs, ceruloplasmin or
30 its derivatives can be used as such, or only partly modified, even in the absence of transferrin or other conjugated molecules.

An advantage of the present invention is that it provides effective means for maintaining or stimulating the regeneration of neuronal cells and thereby it permits the treatment of injuries to the brain, the spinal cord and other central nervous system tissue. Another advantage of the present invention it provides a carrier for
5 delivering therapeutic agent(s) to specific tissues such as the brain. A further advantage of the invention is that it improves the efficiency of methods for culturing neuronal cells *in vitro*.

Other objects and advantages of the present invention will be apparent
10 upon reading the following non-restrictive description of several preferred embodiments made with reference to the accompanying drawings.

DESCRIPTION OF THE DRAWINGS

15 **FIGS. 1A and 1B** are pictures showing the morphology of newly differentiated P19 neurons cultured in absence (1A) or in presence (1B) of ceruloplasmin. P19 cells were induced to differentiate to neurons in the presence of retinoic acid (RA). On day 4 of differentiation, cells were trypsinized and plated ($1650 \text{ cells / mm}^2$) on gelatin-coated tissue culture
20 dishes and cultured in the absence (1A) or presence (1B) of $3.8 \mu\text{M}$ CP. Shown are phase-contrast photomicrographs of P19 neurons taken 24 h after plating.

25 **FIG. 2.** Quantitation of the aggregative effect of different concentrations of native CP by measuring unoccupied culture surfaces. P19 neurons were obtained from differentiation of P19 cells and incubated for 24 h in the presence of various concentrations of native CP, as indicated in the legend to Figs. 1A and 1B. Three photomicrographs were taken from each dish and analysed by scoring the surface non occupied by cells.

30 **FIG. 3.** Quantitation of the aggregative effect of different concentrations of native CP by measuring reduction of AlamarBlueTM dye. P19 neurons were

5 obtained from differentiation of P19 cells and incubated for 24 h in the presence of various concentrations of native CP, as indicated in the legend to Fig. 1. Then, AlamarBlue was added to the culture medium (at a final concentration of 10% v/v) and neurons were further incubated for 5 h to allow reduction of the dye. Relative level of reduction was monitored by fluorescence.

10 **FIG. 4.** Influence of cell maturation on the response of P19 neurons to the aggregative effect of CP. P19 neurons were obtained from differentiation of P19 cells with RA and plated on gelatin-coated culture dishes at day 4 of the differentiation protocol, as indicated in the legend to Fig. 1. Different concentrations of native CP were added to the culture medium at 3h (♦), 24h (■) or 48 hours (▲) after the plating of the cells. Neuronal cultures were left for 24 h with CP, and then photographed for analysis of unoccupied surfaces, as explained in the legend to Fig. 2. Three photomicrographs were taken for each condition.

20 **FIGS. 5A and 5B.** Influence of presence (5B) or absence (5A) of serine protease inhibitors on the response of P19 neurons to the aggregative effect of CP. P19 neurons were obtained from differentiation of P19 cells with RA and plated on gelatin-coated culture dishes at day 4 of the differentiation protocol, as indicated in the legend to Fig. 1. Aprotinin (30 µg/ml) and SBTI (100 µg/ml) were added to the culture medium immediately after plating, followed by native CP (3.8 µM). Control dishes containing CP but not the inhibitors were also analysed. Shown are photomicrographs of neurons 24 h after these treatments.

30 **FIG. 6.** Inhibition of CP-induced neuronal aggregation by protease inhibitors as measured by reduction of AlamarBlueTM dye. P19 neurons were obtained and treated with native CP, in the presence (♦) or absence (•) of aprotinin and SBTI, as indicated in the legend to Fig. 5. Then, AlamarBlue was added to the culture medium (at a final concentration of 10% v/v) and

neurons were further incubated for 5 h to allow reduction of the dye. Relative level of reduction was monitored by fluorescence.

DETAILED DESCRIPTION OF THE INVENTION

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This application describes a novel neurotrophic factor. As used herein, "neurotrophic factor" or "neurotrophic composition" refers to any compound (or to any mixture of compounds) that promote the maturation, growth and regeneration of neuronal cells and/or protect the same cells against a variety of different forms of damage. There are these properties that suggest that ceruloplasmin may be useful in treating various forms of nerve damage and neurodegenerative diseases.

More particularly, the present invention describes the use of ceruloplasmin in a pharmaceutical neurotrophic composition and in a method for promoting the tissular organization of neuronal cells via their aggregation i.e. via the modulation of neuronal cells contacts.

As stated previously, ceruloplasmin (CP), is a 132 kDa multifunctional blue-copper plasma protein which is produced by hepatocytes and found in circulating plasma (1.5-3 μ M). Its most known function is the copper transport. Ceruloplasmin also has important antioxidant and free radical scavenging properties as well as a ferroxidase I activity. Another important role has recently been postulated for this protein as a regulator of iron metabolism.

The ceruloplasmin suitable according to the present invention, comprises substantially pure ceruloplasmin purified from blood or produced by recombinant techniques and functional derivatives thereof. As generally understood and used herein, the term substantially pure refers to a ceruloplasmin preparation that is generally lacking in other cellular or blood components.

30

A "functional derivative", as is generally understood and used herein, refers to a protein sequence that possesses a functional biological activity that is

substantially similar to the biological activity of the whole protein sequence. A functional derivative of a protein may or may not contain post-translational modifications such as covalently linked carbohydrate, if such modification is not necessary for the performance of a specific function. The term "functional
5 derivative" is intended to the "fragments", "segments", "variants", "analogs" or "chemical derivatives" of a protein.

The terms "fragment" and "segment" as is generally understood and used herein, refer to a section of a protein, and are meant to refer to any portion of the
10 amino acid sequence.

The term "variant" as is generally understood and used herein, refers to a protein that is substantially similar in structure and biological activity to either the protein or fragment thereof. Thus two proteins are considered variants if they
15 possess a common activity and may substitute each other, even if the amino acid sequence, the secondary, tertiary, or quaternary structure of one of the proteins is not identical to that found in the other.

The term "analog" as is generally understood and used herein, refers to a
20 protein that is substantially similar in function to ceruloplasmin.

As used herein, a protein is said to be a "chemical derivative" of another protein when it contains additional chemical moieties not normally part of the protein, said moieties being added by using techniques well known in the art. Such
25 moieties may improve the protein's solubility, absorption, bioavailability, biological half life, and the like. Any undesirable toxicity and side-effects of the protein may be attenuated and even eliminated by using such moieties. For example, CP and CP fragments can be covalently coupled to biocompatible polymers (polyvinyl-alcohol, polyethylene-glycol, etc) in order to improve stability or to decrease
30 antigenicity. They could also be coupled to proteins (or their chemical derivatives) known to pass the blood-brain barrier via transcytosis across vascular endothelial cells (e.g. transferrin).

The amount of ceruloplasmin and/or functional derivatives thereof present in the neuroprotective composition of the present invention is a therapeutically effective amount. A therapeutically effective amount of ceruloplasmin is that amount of ceruloplasmin or derivative thereof necessary so that the protein acts a neurotrophic factor, and more particularly the amount necessary so that the protein promotes the tissular organization of neuronal cells, increases the aggregation (association) of neuronal cells. The exact amount of ceruloplasmin and/or functional derivatives thereof to be used will vary according to factors such as the protein's biological activity, the type of condition being treated as well as the other ingredients in the composition. Typically, the amount of ceruloplasmin should vary from about 0.01 μM to about 20 μM . In a preferred embodiment, ceruloplasmin is present in the composition of the neuroprotective extracellular medium in an amount from about 0.05 μM to about 10 μM , preferably from about 0.1 μM to about 4 μM . In the preferred embodiment, the neuroprotective composition comprises about 1 μM of highly active ceruloplasmin.

Further therapeutic agents can be joint to the neurotrophic composition of the invention. For instance, the composition of the invention may also comprise therapeutic agents such as modulators of brain functions (neurotransmitters, neuropeptides, hormones, others trophic factors, or analogs of these substances that act by binding to brain receptors (e.g. DOPA in Parkinson's disease) and neurotherapeutic chemical compounds (e.g. antioxidants to diminish or prevent damages caused by oxidative stress).

Further to the therapeutic agents, the pharmaceutical compositions of the invention may also contain preserving agents, solubilizing agents, stabilizing agents, wetting agents, emulsifiers, sweeteners, colorants, odorants, salts, buffers, or coating agents. For preparing such pharmaceutical compositions, methods well known in the art may be used.

The method of preparation of the neuroprotective composition of the invention is very simple as it consists simply in the mixing purified ceruloplasmin and others of component(s) in a buffered saline solution in order to get a homogenous physiological suspension. Suitable saline solution comprises sodium, potassium, magnesium and calcium ions at physiological concentrations, has an osmotic pressure varying from 280 to 340 mOsmol, and a pH varying from 7.0 to 7.4. Preferably, the buffered saline solution is selected from the group consisting of modified Krebs-Henseleit buffer (KH) and phosphate buffer saline (PBS), both at pH 7.4. The homogenous suspension obtained can further be centrifuged and/or filtered to reduce its viscosity and/or eliminated non-soluble particles.

The neurotrophic composition of the invention could be suitable to treat and/or prevent neurodegenerative diseases or treat an injury to central nervous system tissues. Neurodegenerative diseases which could be treated include Alzheimer's Disease, stroke, trauma, multiple sclerosis, Parkinson's disease, HIV infection of the central nervous system, AIDS dementia, amyotrophic lateral sclerosis, hereditary hemorrhage with amyloidosis-Dutch type, cerebral amyloid angiopathy, cerebral amyloid angiopathy, Down's syndrome, spongiform encephalopathy and Creutzfeld-Jakob disease.

The neurotrophic composition could also be involved in the treatment of poisoning or diminution of side effects of drugs (such as chemotherapeutic and immunosuppressive drugs) to the brain and/or to neuronal cells.

The neurotrophic composition of the invention may be administered alone or as part of a more complex pharmaceutical composition according the desired use and route of administration. Anyhow, for preparing such compositions, methods well known in the art may be used.

Using methods known in the art, ceruloplasmin or its functional derivative could also be coupled to one or more therapeutic agent(s). The "conjugated" or "modified" protein it could be administered to a patient in need thereof and thereby

acts as a carrier for delivering therapeutic agent(s) to a known specific tissue such as the brain.

The neurotrophic composition of the invention and/or more complex pharmaceutical compositions comprising the same may be given via various route of administration. For instance, the neurotrophic composition may be administered in the form of sterile injectable preparations, for example, as sterile injectable aqueous or oleaginous suspensions. These suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparations may also be sterile injectable solutions or suspensions in non-toxic parenterally-acceptable diluents or solvents. They may be given parenterally, for example intravenously, intramuscularly or sub-cutaneously by injection or by infusion. Suitable dosages will vary, depending upon factors such as the amount of each of the components in the composition, the desired effect (fast or long term), the disease or disorder to be treated, the route of administration and the age and weight of the individual to be treated.

Other ways that can be considered are rectal and vaginal capsules or nasally by means of a spray. The neurotrophic composition may also be formulated as creams or ointments for topical administration. The neurotrophic composition could be administered *per os* (e.g. capsules) depending of its composition i.e. to do so all composition's components must be absorbable by the gastrointestinal tract. For example CP as such cannot be recommended for oral administration because, as a large molecule, it would not be intestinally absorbed. This may not however apply to smaller and/or functional derivatives of this protein provided their formulation in absorbable forms (e.g. liposomes).

The neurotrophic composition of the invention may be administered alone or as part of a more complex pharmaceutical composition according to the desired use and route of administration. Anyhow, for preparing such compositions, methods well known in the art may be used.

Ceruloplasmin or a functional derivative thereof could also be used in methods for culturing neuronal cells *in vitro*. By providing an effective amount of ceruloplasmin to *in vitro* cultured neuronal cells it will induce the aggregation of neuronal cells and also promote the tissular organization of *in vitro* cultured neural tissues.

As it will now be demonstrated by way of an example hereinafter, the composition of the invention possess a strong neurotrophic activity i.e. the capacity to promote the regeneration of neural tissues by modulating the association (aggregation) of neuronal cells cultured *in vitro*. Although any method and material similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

EXAMPLE:

Modulation of neuronal contacts by ceruloplasmin

Abbreviations : Alpha-MEM, alpha-modified Eagle's minimal essential medium; AE, aminoethyl; CP, ceruloplasmin; degCP, deglycosylated CP; hCP, heat-denatured CP; ECM, extracellular matrix; GPI, glycosylphosphatidylinositol; OFR, oxygen free radicals; RA, retinoic acid; SBTI, soybean trypsin inhibitor.

1. Introduction

1.1 Ceruloplasmin - a multifunctional copper protein

A number of substances are known to influence neuronal development, including growth factors, components of the extracellular matrix (ECM), and cell adhesion and guidance molecules. Ceruloplasmin (CP) is a multifunctional copper-glycoprotein of 132 kDa produced by hepatocytes and found in plasma (1.5 - 3 μ M). The protein is also synthesized in other tissues including the brain where a glycosylphosphatidylinositol (GPI)-anchored form is expressed by astrocytes (Patel and David, 1997; Patel et al., 2000). It is the major copper carrier in

circulation, a scavenger of oxygen free radicals (OFR), and an enzyme displaying significant ferroxidasic and oxidasic activities. These functions are likely implicated in various physiological roles of the protein, such as regulation of copper and iron homeostasis (Bowman, 1993; Mukhopadhyay et al., 1998), induction of
5 angiogenesis (McAuslan et al., 1983), possible modulation of the acute phase response to inflammation (Bowman, 1993) and, demonstrated by our group, protection of heart (Dumoulin et al., 1996) and neurons (Mateescu et al., 1998) against damages caused by oxidative stress conditions, and modulation of potassium ion channels (Wang et al., 1995).

10

1.1.1 Ceruloplasmin and human pathology

Specific CP receptors have been found in mature erythrocytes (Barnes et al., 1985) as well as in membrane preparations obtained from aortic, cardiac, hepatic and cerebral tissues (Stevens et al., 1984; Orena et al., 1986; Frieden,
15 1986; Fischer and Goodie, 1994). However, the mode of interaction of CP with these receptors and the functional significance of this interaction, besides involving the transfer of copper to cells, remain unclear, suggesting the existence of still unknown properties of CP.

20

Inappropriate copper levels in nerve tissues, like those found in the diseases of Menkes (low copper levels) and Wilson (accumulation of copper), lead to severe neurological disorders and neurodegeneration (Wagoonar et al., 1999). Aceruloplasminemia, a genetical deficiency of CP, causes severe intracellular iron accumulation in several organs, including the brain. In the latter organ, iron
25 accumulation is associated with neurodegeneration, probably as a consequence of oxidative stress (Harris et al., 1995; Klomp et al., 1996, Klomp and Gitlin, 1996; David and Patel, 2000). Altered brain iron metabolism and free radical injury are also associated with other neurodegenerative pathologies such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis and Hallervorden-Spatz' disease. Thus, it is possible that a lack of functional CP could contribute to
30 these syndromes (David and Patel, 2000).

1.1.2 Ceruloplasmin biochemistry

The "blue copper" center of CP has a characteristic absorption band at 610 nm. The protein contains six copper atoms per molecule. Three copper atoms are aggregated in a cluster which is the Blue-Copper center of CP. Two others form a diamagnetic pair. The last one is paramagnetic (detectable by electronic paramagnetic resonance or EPR).

An absorbency ratio $A_{610\text{nm}}/A_{280\text{nm}} = 0.040$ was considered in the literature as characteristic of a homogeneous standard pure enzyme. It was reported for CP a high susceptibility at proteolysis, and physiological properties influenced by the molecular integrity. Despite intensive research in various laboratories, many aspects of CP are still unclear. The protein has been the object of many controversies (originated from its high susceptibility at proteolysis) concerning the molecular characteristics and the copper content. Also controversial was its complex physiological role as antioxidant/prooxidant (Gutteridge, 1994; Chahine et al., 1991; Fox et al., 1995). Within the last decade, a continuously growing interest concerns the molecular mechanisms of protection and functions at cellular and tissular level, induced by CP.

It was recently shown that CP structure comprises in six domains. Surprisingly, its configuration appears close to that of clotting Factor VIII. However, the enigma is not ended. The intriguing fact is that CP receptors were identified, localized in tissues strongly involved in oxidative processes (heart) or sensitive to oxidative stress (brain: known to be damaged by the oxidative stress, especially in aging). It is now established the presence of specific ceruloplasmin receptors, with specific localization in aorta and heart, in brain, on erythrocytes and recently reported, on placenta (Fisher and Goodie, 1994; Barnes and Frieden, 1984; Orena et al., 1986, Stevens et al., 1984). Liver endothelium was shown to bind, transport and desialate CP, which is then recognized by galactosyl receptors of hepatocytes. Also it was shown the production of CP by lung, brain (astrocytes), etc. What is the real role of this CP synthesized in extra-hepatic tissues, is still to elucidate.

A questionable aspect is if CP (132 kDa) can be internalized as the whole molecule or as fragments. Chudej et al (1990) reported the transcytosis of exogenous superoxide dismutase (SOD) and even of catalase (240 kDa) from coronary capillaries into dog myocytes. This is a particular case and a complete answer is not yet available for other cell or tissue types. In any case, an interaction of CP with cells was supposed.

1.2 A single-step chromatographic method for the fast purification of ceruloplasmin

Recently, a novel single-step chromatographic method have been reported for the fast purification of ceruloplasmin, a method leading to a purified, electrophoretically homogeneous protein (Wang et al., 1994). Ceruloplasmin is susceptible to proteolytic denaturation and this fast method therefore protects CP against such denaturation by decreasing time of eventual contact with proteolytic enzymes found in plasma or blood. The purification procedure is based on the highly selective retention of CP on Amino-ethyl (AE)-agarose (see Mateescu et al 1999, for details concerning the CP purification schema). Using this procedure, it is possible to obtain CP preparations with ratio $A_{610}/A_{280} = 0.045 - 0.070$ and a very high oxidasic activity. The purification method permits to minimize the risk of protein degradation. In fact it is supposed, following a reexamination of CP spectral properties (EPR [Calabrese et al., 1988]), that CP purified using this procedure is closer to its real native structure than commercial CP obtained by other methods. This method allows to realize an original CP immobilization. The conjugation of CP with biocompatible polymers is important because the immobilized enzyme conjugates show sought-for advantages such as higher stability, lower antigenicity and possibility to continuous use in various devices of potential interest for bioimplants or for organ preservation in view of transplantation.

1.3 *Presentation of the study*

Considering that CP appears as an essential regulator of iron and copper metabolism, that these metal ions must be provided to the brain to sustain its neuronal activity, and that CP receptors have been reported to exist in that organ, the Inventors hypothesized that this protein could modulate neuronal development. This hypothesis using the P19 mouse embryonal carcinoma cell line, since these cells are an established model of neuronal differentiation (Jeannotte et al., 1997; Cadet and Paquin, 2000). This work presents evidences that CP modulates the development of newly differentiated neurons and influence the pattern of organization of neuronal cells.

2. **Materials and Methods**

2.1 *Ceruloplasmin purification, heat-denaturation and deglycosylation*

Ceruloplasmin was purified from bovine plasma following a recently developed method (Wang et al., 1994; Mateescu et al., 1999). This method is based on the fractionated precipitation of plasma with ammonium sulphate and a chromatographic step on aminoethyl (AE)-agarose. The chromatographic material (not commercially available) was realized by treatment of cross-linked agarose beads (CL-Sepharose 6B, Pharmacia, Upsala, Sweden) with 1-chloro-2-ethylamine (Aldrich, Milwaukee, USA) as described (Mateescu et al., 1999). Purified CP was electrophoretically homogenous. Heat-denatured CP (hCP) was obtained by heating a solution containing 2 mg/mL CP in 20 mM potassium phosphate buffer, pH 7.4, at 65°C, overnight. After this treatment, hCP was concentrated about ten fold by lyophilization before being used with cells. Deglycosylated CP (degCP) was obtained by incubating the native protein with N-glycosidase F (New England LabSystem Beverly, MA, USA) at a ratio of 3000 U glycosidase / mg CP, for 24 h, at 37°C. The efficiency of deglycosylation was controlled by electrophoresis with the use of GelCode Glycoprotein Stain (Pierce), a specific dye for carbohydrates (unpublished results).

2.2 *Cell culture*

P19 cells, originally obtained from Dr. M.W. McBurney (University of Ottawa, Ottawa, Ontario, Canada), were cultured and differentiated as described by Jeannotte et al. (1997), with slight modifications. The undifferentiated P19 cells were propagated in complete medium containing alpha-modified Eagle's minimal essential medium (alpha-MEM; Gibco BRL, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Cansera International, Rexdale, Ontario, Canada), 2.5 U/mL penicillin and 2.5 µg/mL streptomycin (both antibiotics purchased from Sigma, Oakville, Ontario, Canada). The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. To achieve neuronal differentiation, P19 cells were seeded in bacteriological-grade petri dishes at a density of 0.9×10^5 cells / mL, and grown as aggregates, during 4 days, in complete medium containing 0.5 µM retinoic acid (RA; Sigma). At day 4, aggregates were trypsinized, and individualized cells (neurons) were transferred to gelatinized tissue culture dishes containing Neurobasal™ medium (Gibco-BRL) supplemented with B27 supplement™ (Gibco-BRL) and 0.5 mM L-glutamine, but no RA. Differentiation to fibroblasts was done by culturing P19 cells as monolayers, for 4 days, in complete medium containing RA. The cells were trypsinized every 2 days and replated in tissue dishes each time. At day 4, cells were switched to complete medium that did not contain RA. Smooth muscle cells and hepatocytes were generously provided by Dr J. De Champlain (Université de Montréal, Montreal, Quebec, Canada) and by Dr F. Denizeau (Université du Québec à Montréal, Montreal, Quebec, Canada), respectively.

2.3 Treatment of P 19 cells with CP and/or other agents

At various times after plating, P19 cells were incubated without (control) or with CP and/or different agents during 24h. After treatment, cells were examined for morphology, viability, dye penetration or apoptosis, as indicated below.

2.4 Cell morphology

Morphological analysis was done using an inverted microscope equipped with phase-contrast objectives, a camera and a photoautomatic apparatus. Photographies were taken with Technical Pan films (Kodak). Morphological

quantitation of aggregation was done by placing a transparent grid made of 5 mm x 5 mm squares over 10.5 x 15 cm-photomicrographs, and counting the empty squares (agregation increased the number of empty square). Three different pictures were systematically obtained for each dish and an averaged number of empty squares from these pictures was used to characterize the dish. The uncovered surfaces in treated cell cultures were expressed relatively to those of control (untreated) cells.

2.5 Cell viability assay

Cell viability was measured with vital dyes after the trypsinization of cells (Bolduc et al., 1997). Briefly, dissociated cells were stained with propidium iodide (10 µg/mL) and acridine orange (1.5 µg/mL), and then counted, with a haemocytometer, under a fluorescent microscope set up to excite for fluorescein. The dead and live cells exhibited a red and green fluorescence respectively.

2.6 Incubation with Alamar Blue™

After exposure to CP, P19 neurons were incubated for 5 h with one volume of Alamar Blue™ (Immunocorp, Montreal, Quebec, Canada) in nine volumes of culture medium. This dye is reduced to a fluorescent derivative by metabolic activity. At end of incubation, the fluorescence of the culture medium was monitored at 590 nm, using 540 nm as the excitation wavelength.

2.7 Apoptosis analysis

Extent of apoptosis in cell cultures was evaluated by using a Nucleosome ELISA kit (Oncogene Research Products, Calbiochem) which allows the quantitation of mono- and oligonucleosomes in cell extracts.

3. Results

3.1 Effect of CP on neuronal morphology

Surprisingly, when we exposed newly differentiated P19 neurons to CP 3.8 µM, we observed a strong aggregative effect of the protein on these cells. Indeed, while neurons cultured in the absence of CP formed well spread cell monolayers

(Fig.1A), those incubated with the protein formed, during the first 24 h of incubation, spherical and compact cell aggregates that adhered loosely to the culture surface and exhibited only few interconnecting neurites (Fig. 1B). Visual examination showed a dose-dependent effect of CP on culture morphology for concentrations varying from 0.038 to 0.38 μ M (Table 1: 0.5 mg/ml of CP = 3.8 μ M of CP). The aggregative effect plateaued at higher concentrations (Table 1). We tested two techniques to quantify this phenomenon of aggregation. First, we calculated the increase in unoccupied culture areas induced by CP, using a transparent grid placed over photomicrographs. The dose-response curve obtained with this technique (Fig. 2) matches the results of visual appreciation (Table 1). The concentration of CP necessary to achieve 50 % of the aggregative effect was 0.038 μ M (Fig. 2). The dose dependent effect of CP was also demonstrated quantitatively by following the extent with which the viability dye, Alamar Blue™, can penetrate cells. Once incorporated in cells, this dye is reduced by cellular metabolism. Since CP treatment did not affect cell viability (see next paragraph) but caused a decrease in dye reduction by cells, one can conclude that aggregation induced by CP prevented access of the dye to cells. Results obtained with this technique (Fig. 3) independently confirmed the concentration-dependent and saturable pro-aggregative effect of CP.

3.2 *Evaluation of neuronal death*

To determine whether the pro-aggregative action of CP was the result of some cytotoxic properties of the protein and/or if aggregation could eventually promote cell death even though CP was not toxic by itself, we evaluated cell viability of P19 neurons exposed to CP and also investigated if treatment induced apoptosis in these neuronal populations. Viability evaluation using fluorescent dyes with individualized cells showed that neurons exposed to native CP, even at saturating pro-aggregative concentrations of the protein, remained entirely viable and were not affected by necrosis (Table 2). Moreover, preliminary results demonstrated that CP did not induce DNA fragmentation characteristic of apoptotic events (Data not shown). These results indicate that CP was not detrimental to neurons.

3.3 *Influence of neuronal maturation on aggregation induced by CP*

We compared the response of P19 neurons incubated with CP at different times after differentiation. Indeed, neuronal maturation continues after plating and P19 neurons develop into a network of cells interconnected with neurites. The sensitivity of newly differentiated neurons to aggregation induced by CP differed when the protein was added at 3, 24 or 48 h after plating. Indeed, although neurons aggregated in all cases, in a saturable fashion, older ones required higher concentrations of CP to achieve an equivalent extent of aggregation (Fig. 4). Consequently, the concentration of CP needed to induce half-aggregation which was approximately 0.05, 0.33 and 0.62 μ M when CP was added at 3, 24 and 48 h after plating, respectively (Fig. 4).

3.4 *Morphological effect of other forms of CP on P19 neurons*

Further experiments have been done to investigate the importance of the protein structural integrity in inducing neuronal aggregation. As showed in Table 1, hCP failed to induce significant aggregation even at high doses of the protein. CP highly irradiated with ^{60}Co (10 Kgy) was also ineffective in promoting neuron aggregation (Table 1). In contrast, degCP was still effective to induce neuronal aggregation although a very slight decrease in potency was observed (Table 1).

The results indicate that the presence of carbohydrates on CP is not necessary for aggregation but the protein still needs to maintain a particular structural feature.

3.5 *Morphological effects of copper on P19 neurons*

5 CP is a protein containing six moderately tight linked copper atoms per polypeptide. It is possible that the aggregative effect of CP on neurons can be caused by copper atoms exposed or released by the protein in the culture medium. However, the copper-released was not the case since copper salts (CuSO₄ and CuCl₂) and the (His)₂Cu²⁺ complex all failed to induce neuronal
10 aggregation, even at copper concentrations equal to or higher than those corresponding to the copper content of the pro-aggregative CP concentrations (Table 1). The aggregative effect of CP was not therefore caused by the release of its copper atoms.

15 3.6 *Morphological effects of other proteins on P19 neurons*

Further studies were conducted to investigate the specificity of the aggregative action of CP. P19 neurons were thus incubated in the presence of other copper proteins (bovine serum amine oxidase and laccase) and of bovine serum albumin. None of them induced aggregation (Table 1). Amine oxidase even
20 caused necrosis when used at 1.1 µM (Table 1), in contrast to CP which was found to be not detrimental to cells when used at higher concentrations (Table 1). This effect can be related to the release of H₂O₂ and aldehydes, products of amine oxidase which both are toxic

25 3.7 *Morphological effect of native CP on non-neuronal cells*

To determine whether the aggregative action of CP on cells was exerted specifically on newly differentiated neurons only, we exposed other cell types to the protein. For these studies, CP was used at 1.5 µM, a concentration more than sufficient to induce neuronal aggregation (Fig. 2). Contrarily to what observed with
30 P19 neurons, CP did not display pro-aggregative effects on undifferentiated P19 cells, on P19 cells differentiated to fibroblasts, on rat smooth muscle cells and hepatocytes, indicating a cell type dependent phenomenon (data not shown).

3.8 *Influence of the culture substratum on CP-induced neuronal aggregation*

In vivo, cells adhere to an ECM by interactions that involve proteins (like integrins) present at the cell surface and proteins of the ECM. One can
5 hypothesize that the aggregative effect of CP stems from the fact that the protein can interfere in cell adhesion to matrix. We thus examined the effect of CP on neurons plated on gelatin, on the ECM proteins fibronectin and laminin, and on poly-D-lysine, a polypeptide that often serves as a synthetic culture matrix *in vitro*. CP induced aggregation of neurons plated on any of these four coating molecules,
10 independently of the type of interactions that could be involved in the attachment of cells to these molecules (not shown). Interestingly, when CP was tested as a coating material, it had an aggregative effect similar to that of soluble CP and failed to promote neuron adhesion (not shown). These results suggest that CP does not induce aggregation by interfering with the interactions of cells with culture
15 matrix proteins.

3.9 *Influence of protease inhibitors on CP-induced neuronal aggregation*

Cell adhesion and migration are finely regulated *in vivo* by proteases and their naturally-occurring inhibitors. As a preliminary work to determine if cellular
20 proteases may be involved in the pro-aggregative effect of CP, we tested the effect of serine proteases inhibitors (aprotinin, soybean trypsin inhibitor [SBTI] and epsilon-amino-n-caproic acid) on CP-mediated neuronal aggregation. Interestingly, the induction of neuronal aggregation by native CP was inhibited by the presence of SBTI and aprotinin in the culture medium (Fig. 5). The
25 AlamarBlue™ test confirmed quantitatively what was observed morphologically (Fig. 6). Indeed, SBTI and aprotinin relieved the restriction imposed on the entry of the dye into neurons by cell aggregation. Contrarily to SBTI and aprotinin, which are non-specific serine protease inhibitors, epsilon-amino-n-caproic acid, which is a plasmin specific inhibitor, did not inhibit the aggregation induced by
30 native CP (not shown), suggesting that aggregation somehow involves neuronal proteases, other than plasmin. These proteases could be secretory or membrane-associated proteins.

4. Discussion

This study demonstrates, for the first time, that CP has a pro-aggregative action on cultured neurons in vitro. This action is specific by several aspects.

5 First, the effect was dose-dependent and saturable, indicating that it must obey to a definite mechanism (Table 1, and Figs 2 and 3). Also, denaturation of the protein abolished aggregation, indicating the requirement for a particular or a native structure of CP (Table 1). Moreover, other plasma proteins, like albumin, or other copper proteins presenting some resemblance with CP, all failed to induce

10 neuronal aggregation (Table 1). In particular, it is worth of mention that laccase is structurally similar to CP by having a blue copper center (Zaitzeva et al., 1996), and that serum amine oxidase, like CP, can act on ionic channels although it differs from CP by being a white copper protein (Wu et al., 1996). On the other hand, the aggregative effect is not a consequence of CP cytotoxicity since we

15 detected practically no sign of cell mortality (Table 2) or apoptosis (Data not shown) in treated neurons. Finally, the effect is cell-type specific since it was observed in neuronal populations, but not in cultures of undifferentiated cells or those of fibroblasts, hepatocytes or smooth muscle cells (Data not shown). All together, these results show that CP, which was recently shown to be synthesized

20 in the brain (review in David and Patel, 2000), can participate in tissular organization of the nervous system, more especially perhaps but not exclusively during development.

The observation that CP exerts an aggregative effect on neurons is

25 physiologically relevant in both neuronal development and regeneration. CP is considered important, even necessary, for neuronal activity itself since it is viewed as a regulator of iron and copper metabolism and since both metal ions are used by metalloenzymes for the synthesis of myelin and various neurotransmitters and neuropeptides. These substances have an established role in ensuring adequate

30 neuron-neuron or neuron-muscle communication via synaptic connections. Other types of neuronal contacts may also be instrumental to brain organization. One can imagine that interactions between neuronal cell bodies help to maintain

positional information. Such information is thought to be required during the migration of neurons to their final destination in the developing brain (Mione and Parnavelas, 1994). For instance, in the neocortex, neurons reside in a highly ordered array of cellular layers that later form distinct functional cortical areas

5 (Mione and Parnavelas, 1994). What we see as an aggregate *in vitro* (this study) could translate into an organized layer *in vivo* if the aggregate interacts with adhesive molecules present in the ECM or on neighbor cells. In the absence or in the presence of insufficient amounts of these molecules, packing may become exaggerated and result in compact cell aggregation. Interestingly, fibronectin and

10 laminin, two ECM proteins that were used as a culture substratum in this work, were not able to inhibit aggregation by themselves (Data not shown). In terms of tissue organization, CP can be therapeutically useful not only to prevent impaired brain development in a perinatal context but also to help regenerating adult neurons affected by degenerative diseases, such as Alzheimer's and Parkinson's

15 diseases, and amyotrophic lateral sclerosis. Indeed, neurodegenerative diseases are characterized by severe neuronal losses and even by cavitations of the neuronal tissue in some instances (Morita et al., 1995; David and Patel, 2000). Therapeutically sustained tissue organization may additionally help stabilizing synaptic connections. In neuronal regenerating strategies, CP would thus

20 beneficially assist the action of neurotrophins - such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) - that were shown to stimulate neurite sprouting and synaptic rebuilding (Lindvall et al., 1994). There is an important reason to provide exogenous CP to persons suffering from neurodegenerative diseases even though this protein is already produced in brain.

25 Indeed, oxidative stress is associated with these diseases and can damage CP which we showed to be a target of reactive oxygen species *in vitro* (Mateescu et al., 1998).

In this work, the aggregative action of CP was observed in neuronal

30 populations only, and not in homogenous cultures of other cell types. However, CP has the potential to influence any heterologous cell interaction involving neurons. This includes neuron-astrocyte and neuron-fibroblast associations, since

brain astrocytes and fibroblasts were shown to produce a form of CP that is anchored to their surface by a GPI group (Patel and David, 1997; David and Patel, 2000), and also neuron-muscle interactions. The latter are suggested by the observations that CP acts on ionic channels present in developing cardiomyocytes (R. Wang et al., unpublished results) and that the protein has a cardiomodulatory effect on isolated adult rat heart, perhaps via an action on peripheral nervous system (Chahine et al., 1991). Heterologous interactions would be another means to assist tissue reorganization.

The mechanisms underlying the aggregative action of CP could involve specific binding sites or receptors at the surface of neurons since the existence of such sites has been demonstrated in membranes prepared from various tissues, including brain and heart (Stevens et al., 1984; Orena et al., 1986; Frieden, 1986; Fischer and Goodie, 1994). One could thus use the protein as a carrier for therapeutic agents, especially those pertinent to neuronal therapies. Specific drug delivery to target tissues is a mean to help prevent undesired spreading of drugs and secondary effects. Besides binding sites, the observation that inhibitors of serine proteases inhibited CP-induced aggregation (data not shown) suggests that a protease(s) can also be involved. Possible explanations to this unexpected finding are that CP can be involved in upregulation, activation and/or secretion of proteases that act on adhesion molecules present at the surface of neurons, or alternately, proteases can produce a bioactive CP fragment that is responsible for the aggregative effect of the protein. There are examples of bioactive peptides released from large extracellular proteins by proteolysis, such as the fragment K5 of plasminogen or various hemorphins produced from hemoglobin (Fruitier et al., 1999).

5. Conclusive remarks

The pro-aggregative action of the protein on neurons appears to be a specific and reproducible property. This action has potential therapeutic applications in the development of nervous system and in the treatment of neurodegenerative diseases. This would add to the other interesting properties of CP as an antioxidant, a cardio- and neuroprotector, and as a modulator of

neuronal and cardiac functions. The possibility that CP can also be used as a drug carrier to neurons opens the way for the design of a very novel therapeutic tool. Indeed, CP (or a bioactive derivative) would be a drug carrier which can enhance the drug effects by its own therapeutic action.

5

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10 Of course, numerous modifications and improvements could be made to the embodiments that have been disclosed herein above. These modifications and improvements should, therefore, be considered a part of the invention.

Table 1. Pro-aggregative effect of various substances on newly differentiated P19 neurons.

	Substance	Concentration	Aggregative effect	Remarks
A:	Native CP	1 mg/mL	++++	- The addition of CP at times varying between 0 to 3h after plating of newly differentiated neurons induced the formation of aggregates that did not exhibit many neuritic outgrowths (Fig. 1B). - The addition of CP one day after the newly differentiated cells were plated, induced the formation of aggregates that exhibited neuritic outgrowths. - The addition of CP two days after the newly differentiated cells were plated, induced a moderate formation of aggregates that still have neuritic outgrowths. - The aggregative effect of CP is influenced by the amount of gelatin used to coat culture surfaces. At high levels, gelatin tends to inhibit the aggregation of neurons.
		0.5 mg/mL	++++	
		0.2 mg/mL	++++	
		0.1 mg/mL	++++	
		0.05 mg/mL	++++	
		0.02 mg/mL	+++	
		0.01 mg/mL	++	
		0.005 mg/mL	+	
		0.002 mg/mL	0	
		0.001 mg/mL	0	
B:	Deglycosylated CP	0.2 mg/mL	++++	- Irradiation is know to induce cross-links between CP molecules. These links could inhibit the activity of CP, change the conformation of the molecules and/or hide specific interaction sites.
		0.1 mg/mL	+++	
		0.05 mg/mL	+	
		0.01 mg/mL	0	
	Irradiated CP	3 Kgy	0.3 or 0.2 mg/mL	
		6 Kgy	0.5 or 0.3 mg/mL	
		10 Kgy	0.3 or 0.2 mg/mL	
	Heat- denatured CP	1 mg/mL	+	
		0.5 mg/mL	+	
		0.2 mg/mL	0	
		0.1 mg/mL	0	
		0.05 mg/mL	0	
C:	CuSO ₄ or CuCl ₂	246 µM	Necrosis	- CP at 0.5 mg/mL (3,8µM) contains approximately 22.8 µM of copper since there are 6 copper atoms /protein molecule.
		49.2 to 1.14µM	0	
	(His) ₂ Cu ²⁺ complex	23 µM to 236 µM	0	
D:	Bovine serum amine oxidase	1 to 0.2 mg/mL	Necrosis	
		0.1 or 0.05 mg/mL	0	
	Laccase	1 mg/mL	Necrosis	
		0.5 to 0.05 mg/mL	0	
	Bovine serum albumin	5 to 0.02 mg/mL	0	

ADDENDUM TO TABLE 1

A) Semi-quantitative evaluation of the pro-aggregative effect of native CP on newly differentiated P19 neurons.

- 5 At day 4 of differentiation, dissociated P19 neurons were plated (1650 cells / mm²) on gelatin-coated dishes and CP added 3 h later. Incubation was resumed for 24 h and culture morphology was then examined under the microscope. 0 = no restriction in cell spreading (morphology resembled that shown in Fig. 1A, control) ; + = slight restriction in cell spreading ; ++ = restriction in cell spreading ;
- 10 +++ = high restriction in cell spreading with a tendency to form aggregates ; ++++ = intense cell aggregation (morphology resembled that shown in Fig. 1B for 3.8 µM CP).

15 *B) Semi-quantitative evaluation of the pro-aggregative effect of different forms of CP on newly differentiated P19 neurons.*

P19 neurons were obtained and treated as indicated hereinbefore, except that deglycosylated CP, irradiated CP and heat-denatured CP were used. The extent of aggregation was scored as indicated hereinbefore.

20 *C) Semi-quantitative evaluation of the pro-aggregative effect of copper on newly differentiated P19 neurons.*

- P19 neurons were obtained and treated as indicated hereinbefore, except that copper salts and copper-histidine complex were used instead of CP. A concentration of 22.8 µM copper ions is equivalent to that of the six copper atoms
- 25 present in the structure of CP 3.8 µM. The extent of aggregation was scored as indicated hereinbefore.

D) Semi-quantitative evaluation of the pro-aggregative effect of albumin and other copper-proteins on newly differentiated P19 neurons.

- 30 P19 neurons were obtained and treated as indicated hereinbefore, except that albumin, amine oxidase and laccase were used instead of CP. The extent of aggregation was scored as indicated hereinbefore.

E) Semi-quantitative evaluation of the pro-aggregative effect of CP on various non-neuronal cells (results not shown).

5 P19 cells and their neuronal or fibroblastic derivatives, as well as aortic smooth muscle cells and hepatocytes obtained from rats were cultured under normal culture conditions. The culture medium was replaced by supplemented Neurobasal medium containing 3.8 μ M CP. The extent of aggregation was scored 24 h later, and expressed as indicated hereinbefore.

10 *F) Influence of the culture substratum on CP-induced neuronal aggregation (results not shown).*

15 P19 neurons were obtained and treated with 3.8 μ M CP as indicated hereinbefore, except that extracellular matrix proteins other than gelatin were also tested. Solutions of gelatin (0.1 and 2%), fibronectin (10 μ g/mL), laminin (10 μ g/mL) or poly-D-lysine (10 μ g/mL) were distributed into the wells of tissue culture plates and incubated during 30 min and also 2.5 h at 22 °C. Wells were thoroughly washed to remove unbound coating material before being inoculated with cell suspensions. The extent of aggregation was scored as indicated hereinbefore.

20 *G) Influence of protease inhibitors on CP-induced neuronal aggregation (results not shown).*

25 P19 neurons were obtained and treated with 3.8 μ M CP as indicated hereinbefore, except that protease inhibitors were present with CP (SBTI, 100 μ g/mL, aprotinin, 30 μ g/mL, and epsilon-amino-n-caproic acid, 6 mM). The extent of aggregation was scored as indicated hereinbefore.

TABLE 2. Viability of P19 neurons cultured in the presence of CP

5

CP concentration (μ M)	Cell viability (%)
0	97 $\pm$ 1.2
0.76	98.4 $\pm$ 1.4
1.5	94.6 $\pm$ 2.2
3.8	94.5 $\pm$ 2.1

At day 4 of differentiation, dissociated P19 neurons were plated (1650 cells / mm²) on gelatin-coated dishes and various concentrations of CP added 3 h later. Incubation was resumed for 24 h and cell viability was then measured using a combination of propidium iodide and acridine orange.

CLAIMS:

1. A pharmaceutical neurotrophic composition characterized in that it comprises a therapeutically effective amount of ceruloplasmin or of a functional derivative of ceruloplasmin.
5
2. The neurotrophic composition of claim 1, characterized in that said composition promote the tissular organization of neuronal cells.
- 10 3. The neurotrophic composition of claim 1 or 2, characterized in that said composition promote the aggregation of said neuronal cells.
4. The neurotrophic composition of any one of claims 1 to 3, characterized in that said composition promote the regeneration of neuronal tissues.
15
5. The neurotrophic composition of any one of claims 1 to 4, characterized in that said neuronal cells are newly differentiated neurons.
6. The neuroprotective composition of any one of claims 1 to 5, characterized in that ceruloplasmin is purified from blood using an one-step affinity chromatography on aminoethyl-agarose.
20
7. The neuroprotective composition of any one of claims 1 to 6, characterized in that ceruloplasmin or its functional derivative are present in an amount varying from about 0.01 μM to about 20 μM .
25
8. The neuroprotective composition of any one of claims 1 to 7, characterized in that it further comprises an agent selected from the group consisting of metal chelators, metal scavengers, proteinic chelators, proteinic scavengers, preserving agents, solubilizing agents, stabilizing agents, wetting agents, emulsifiers, sweeteners, colorants, odorants, salts, buffers, coating agents and antioxidants.
30

9. The neuroprotective composition of any one of claims 1 to 8, characterized in that ceruloplasmin or its functional derivative are coupled to a therapeutic agent.
10. The neuroprotective composition of claim 9, characterized in that said
5 therapeutic agent is selected from the group consisting of neurotransmitters, neuropeptides, hormones, trophic factors, analogs of these substances that act by binding to brain and neurotherapeutic chemical compounds.
11. Use of a composition according to any one of claims 1 to 10, as an active
10 agent in the preparation of a medication for the prevention or the treatment of a neurodegenerative disease, or for the treatment of injuries to brain, spinal cord and other nervous system tissue.
12. Use of ceruloplasmin or of a functional derivative thereof for promoting the
15 tissular organization of neuronal cells.
13. A method for preventing or treating a neurodegenerative disease or for treating an injury to nervous system tissues, comprising the administration to a patient in need thereof of a therapeutically effective amount of ceruloplasmin or of
20 a functional derivative of ceruloplasmin.
14. The method of claim 13, characterized in that ceruloplasmin or its functional derivative are coupled to at least one therapeutic agent.
- 25 15. The method of claim 14, characterized in that ceruloplasmin or its functional derivative acts as a transporter for delivering said therapeutic agent to a known specific tissue.
16. The method of claim 15, characterized in that said specific tissue is the
30 brain, the spinal cord or the peripheral nervous system.

17. The method of any one of claims 13 to 16, characterized in that said therapeutic agent is selected from the group consisting of neurotransmitters, neuropeptides, hormones, trophic factors, analogs of these substances that act by binding to brain and neurotherapeutic chemical compounds.

5

18. The method of any one of claims 13 to 17, characterized in that said neurodegenerative disease is selected from the group consisting of Alzheimer's Disease, stroke, trauma, multiple sclerosis, Parkinson's disease, HIV infection of the central nervous system, AIDS dementia, amyotrophic lateral sclerosis, hereditary hemorrhage with amyloidosis-Dutch type, cerebral amyloid angiopathy, cerebral amyloid angiopathy, Down's syndrome, spongiform encephalopathy and Creutzfeld-Jakob disease.

10

19. A method for culturing neuronal cells *in vitro* comprising providing to *in vitro* cultured neuronal cells an effective amount of ceruloplasmin or of a functional derivative thereof and thereby promote tissular organization of said cultured neuronal cells.

15

20. The method of claim 19, characterized in that it promotes the aggregation of said neuronal cells.

20

35

Fig. 1A

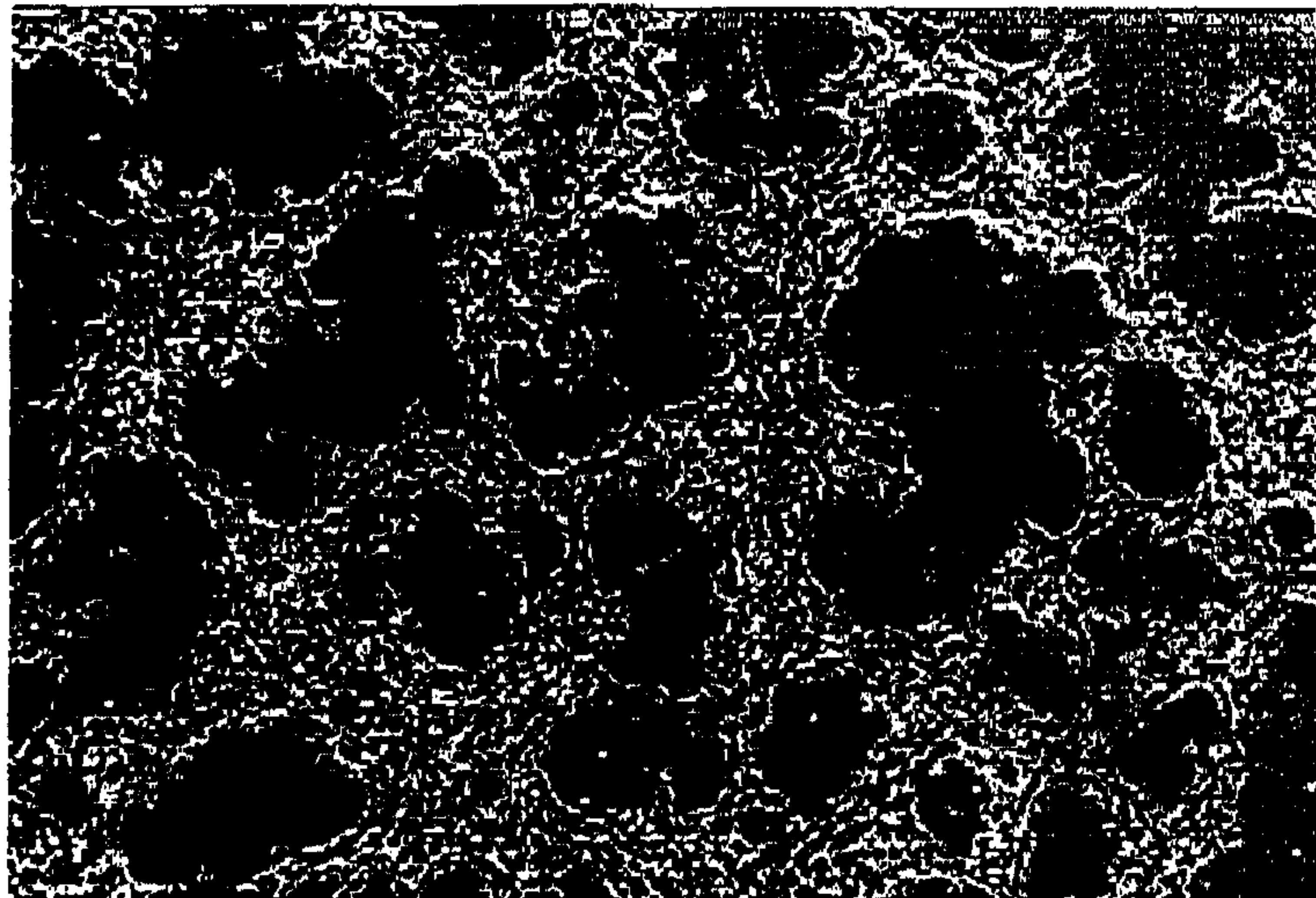
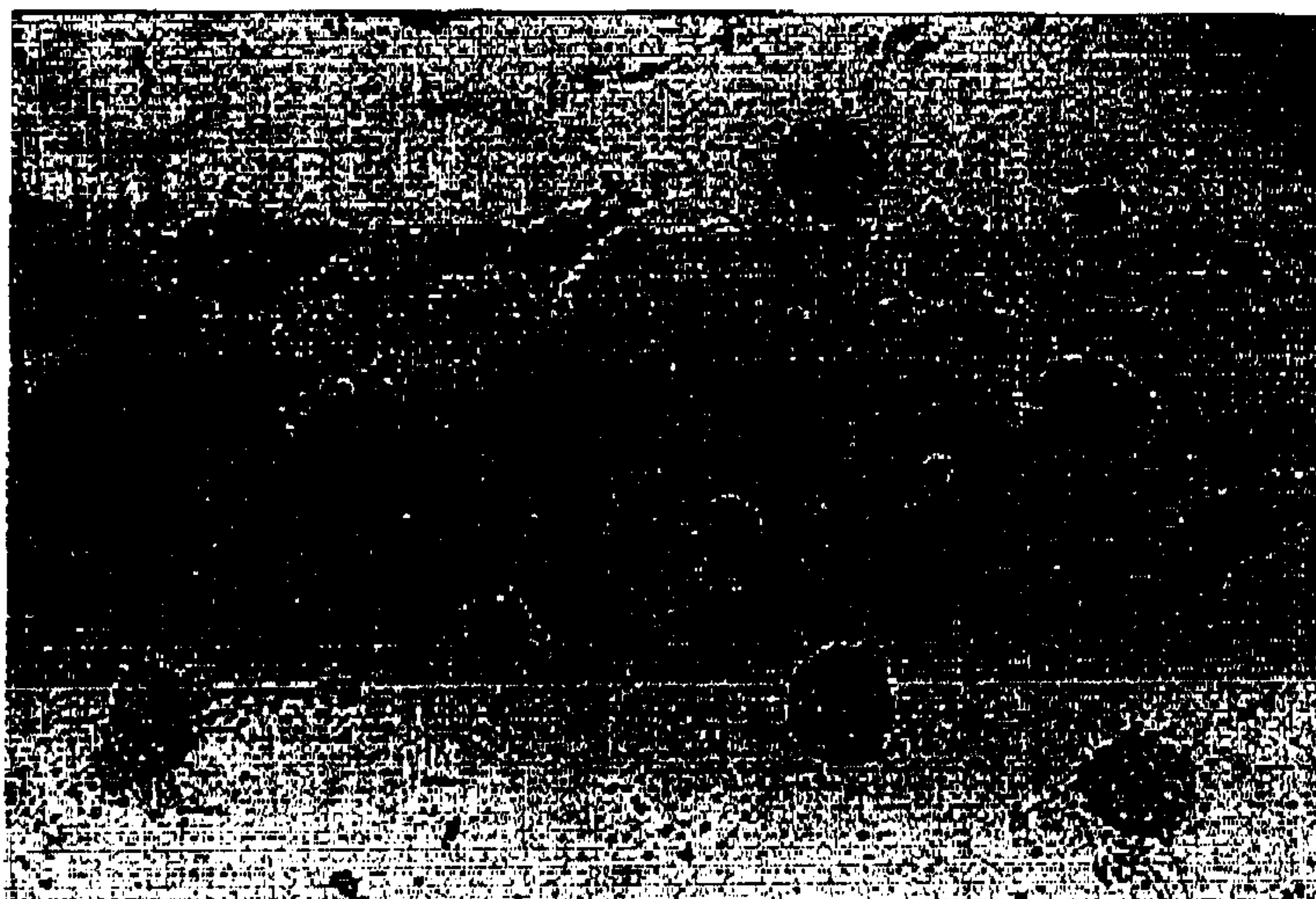


Fig. 1B



36

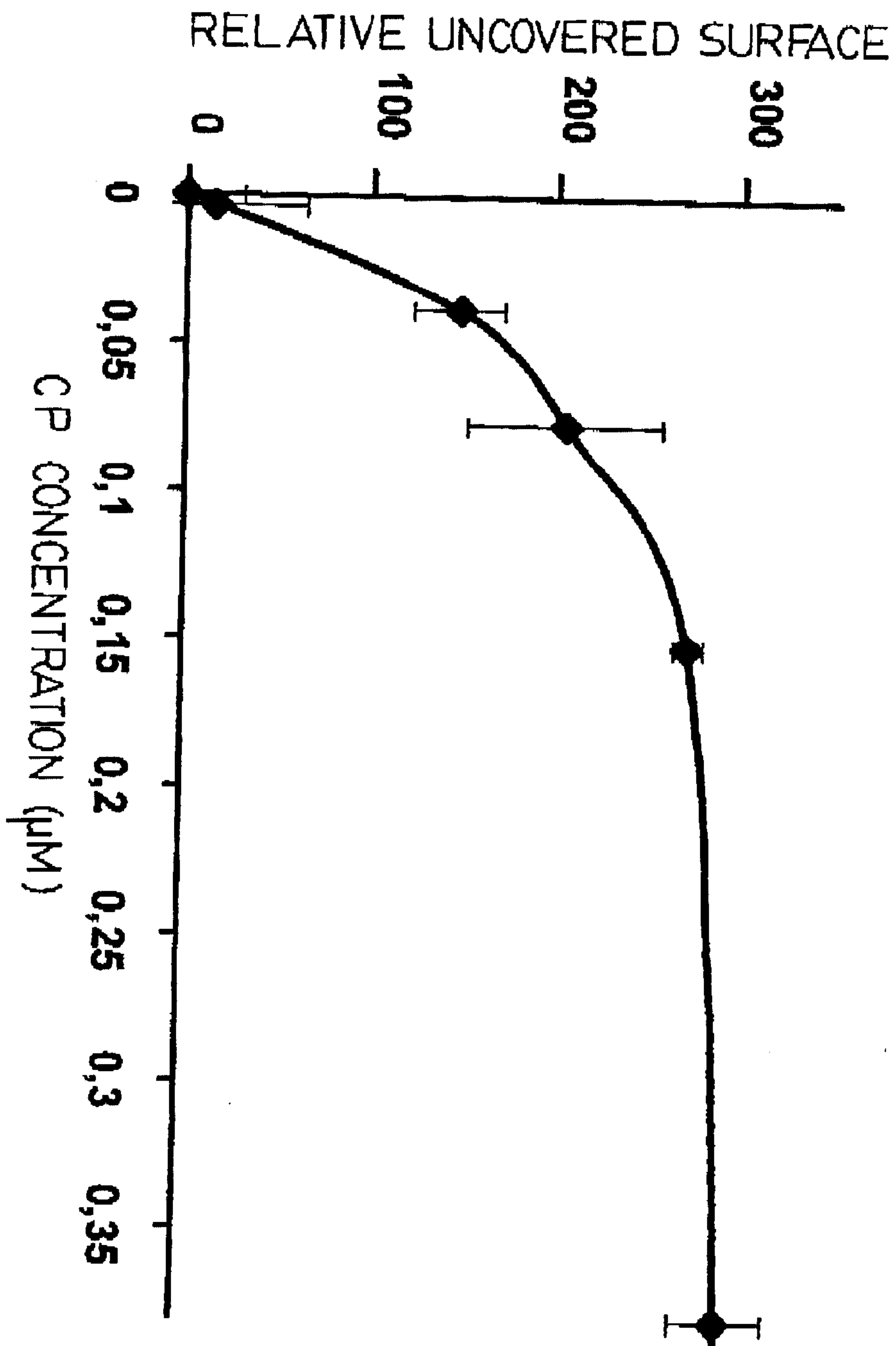


Fig. 2

37

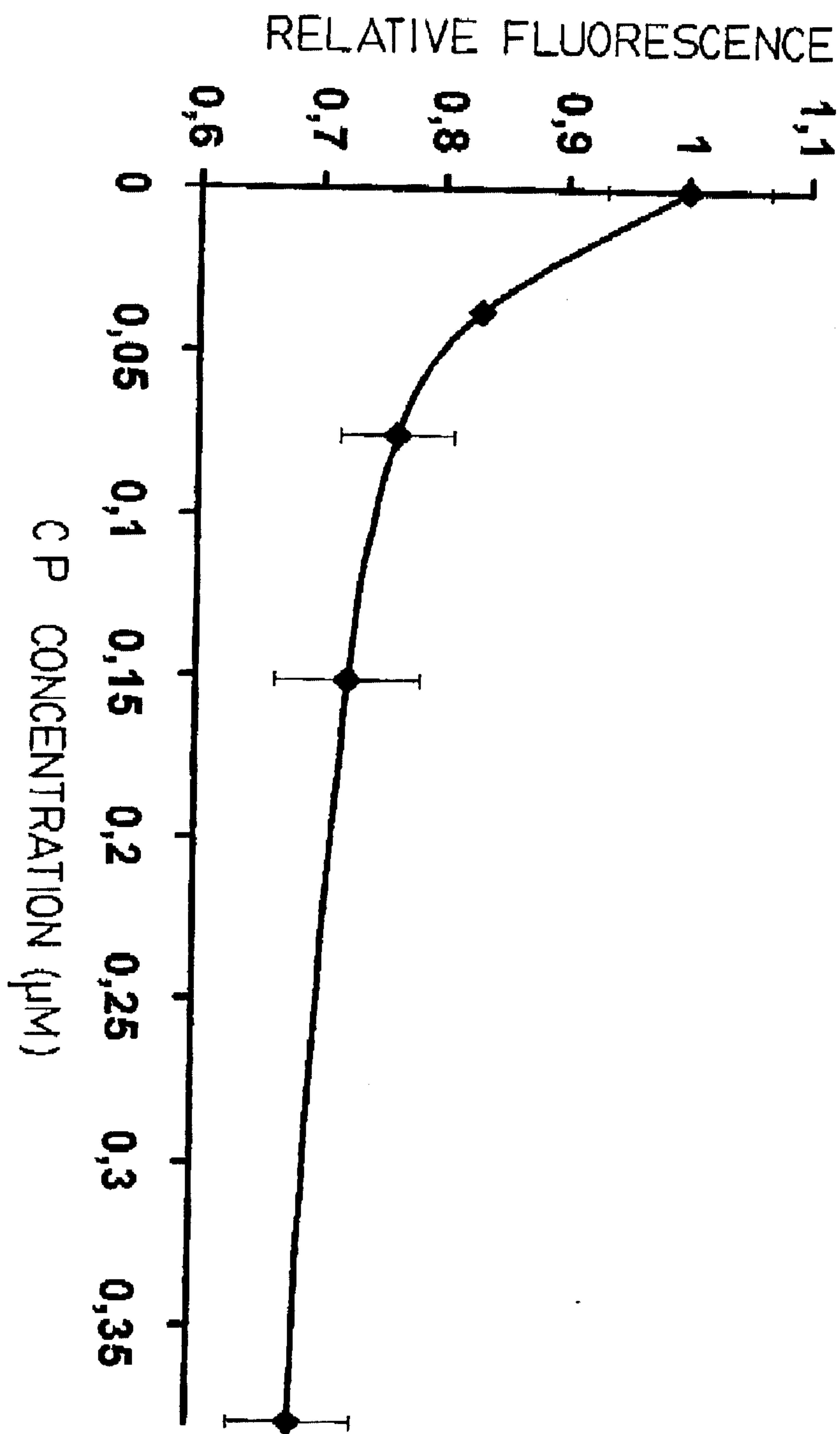


Fig. 3

38

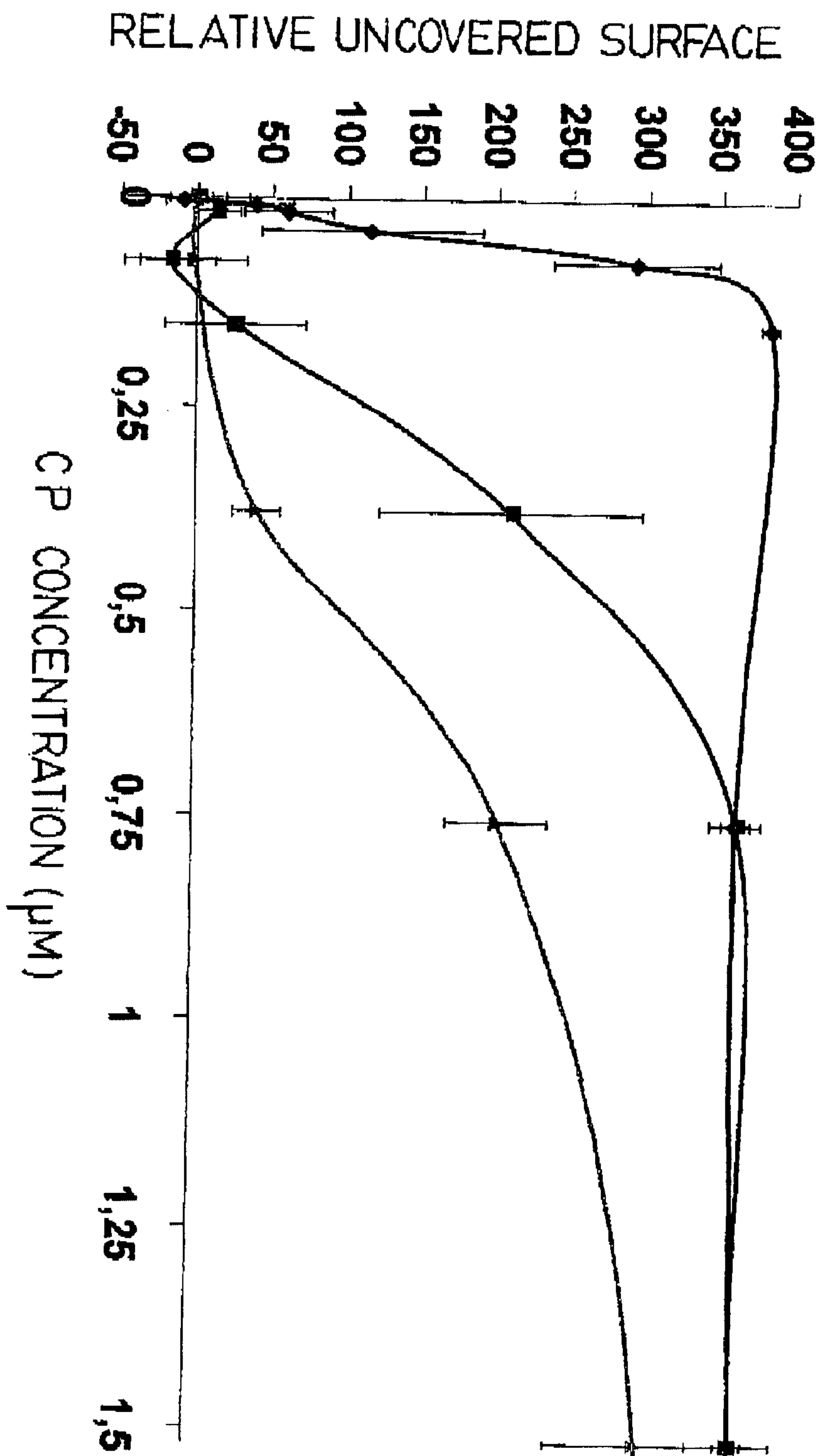


Fig. 4

39

Fig. 5A

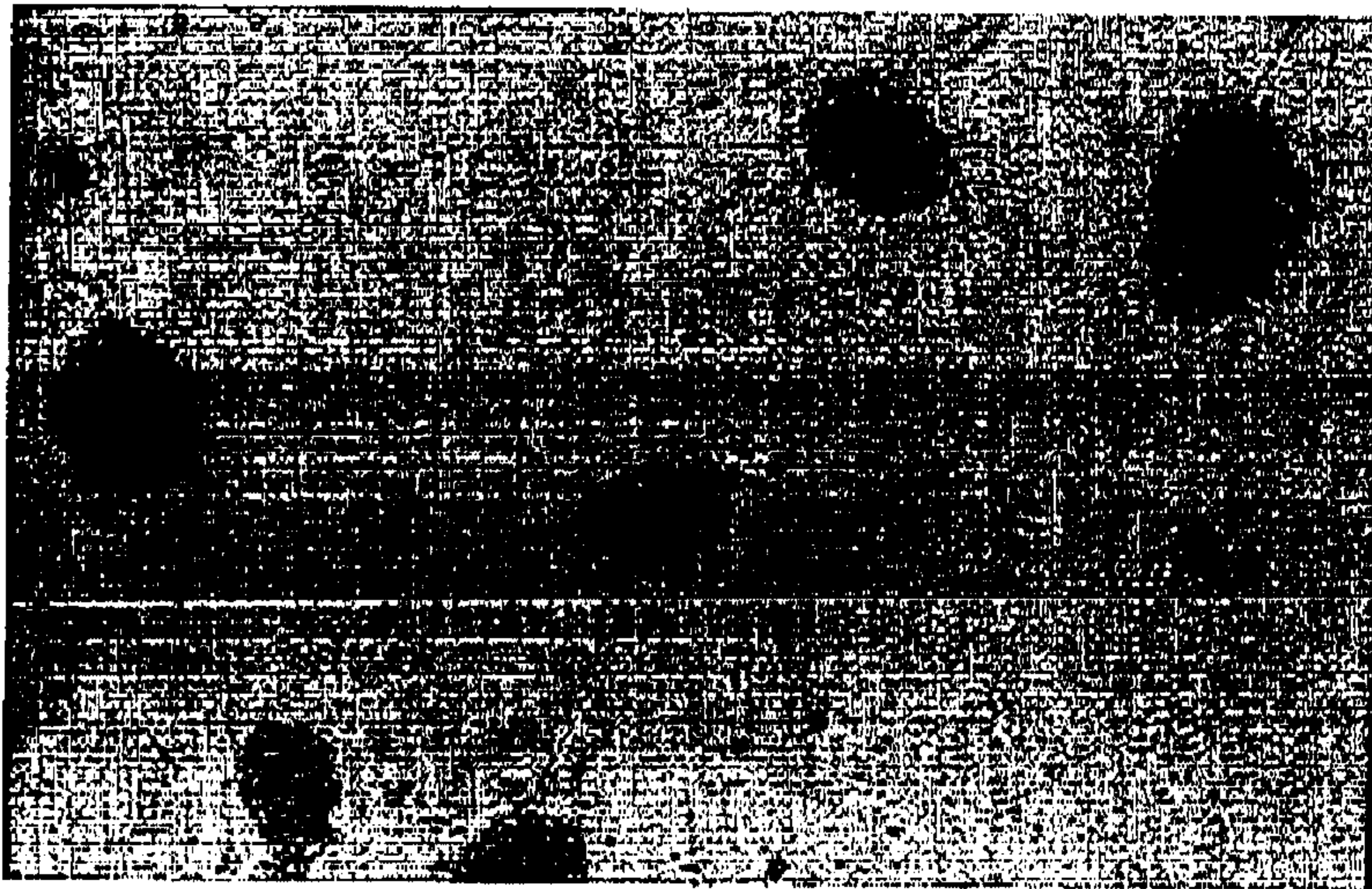


Fig. 5B

