(final concentration 10^{-5} M), and the reaction mixture was incubated for 10 min at 25 °C. After stopping the reaction by chilling, the slices were collected, washed and prepared for scintillation counting; 100 µl aliquots of the uptake medium, and of the release medium after the release incubation were also counted. From these data, the tissue/medium ratios after uptake and the percentage of label released in 10 min into the medium were calculated.

Results and discussion. As the tissue/medium ratios (table 1) show, there is a small but significant inhibition of the uptake of both 5HT and NA into rat cortex slices by MBP at concentrations above 10^{-5} M. This inhibition is about twice as strong for NA as for 5HT. At lower MBP concentrations, no significant effect on the uptake of either neurotransmitter was observed. The apparently significant value of 11.5% inhibition of 5HT uptake by 10^{-6} MBP is probably an artefact due to the small number of experiments. When the subcellular distributions of 5HT and NA after uptake in the presence of MBP are compared (table 2) 2 different patterns of inhibition are found. For 5HT, 10^{-4} M and 5×10^{-5} M MBP inhibit transmitter uptake into the synaptosomes and induce a corresponding increase in the supernatant, which contains the glial as well as the neuronal cytoplasmic material. In the total tissue, the decrease in uptake is very small. Thus, MBP produces a shift in the uptake of 5HT from the synaptosomes to the cytoplasmic material. This is particularly evident when the cpm are calculated as percentage of the total cpm recovered from the gradient. With 10^{-4} MBP the reduction from 51.5% (controls) to 38.3% in the synaptosomes can be compared with an increase from 37.7% (controls) to 50.6% in the supernatant.

The pattern for NA is clearly different. 5×10^{-5} M and 1×10^{-5} M MBP both induce a larger and significant decrease in cpm per mg in the whole tissue. This loss of accumulated labelled NA is found not only in the synaptosomes, as for 5HT, but also in the supernatant. Since this reduction in uptake is larger in the synaptosomes than in the supernatant, there is an (apparent) small increase in the percentage of labelling in the supernatant But as with 5HT, MBP concentrations below 10^{-5} M have no effect on NA uptake.

Raising the K^+-concentration to 56 mM in the presence of 2.7 mM Ca^{++} induces a rapid and intensive release of all 3 neurotransmitters tested from rat cortex slices (table 3). Addition of 10^{-5} M MBP to the release medium had no effect on the release of any of the 3 substances. These results lead to the conclusion that the site of action correlated with the bioelectric blocking activity of MBP[4] is not presynaptic, i.e. that there is no interference with uptake or release of these neurotransmitters at synaptic terminals by the electrophysiologically active concentrations of MBP. This is evident from the finding that a concentration of 5×10^{-6} M MBP, which totally abolished the spontaneous firing rate of mouse Purkinje cells in culture[5], was ineffective in our experiments, where interference with pre-synaptic biochemical mechanisms was tested. Only high MBP concentrations showed slight activity. Other mechanisms of action of MBP must be considered. For instance, it is possible that MBP acts on biochemical processes in the mitochondria, or by blocking the Na/K pump[5,11]. Since the experiments with various selective antagonists of NA, DA and Gaba have led to the conclusion that the postsynaptic receptors for these neurotransmitters are not involved in MBP action[5], and if we exclude presynaptic action, then the suggestion of Carnegie[3,4] that MBP interacts with 5HT at its postsynaptic receptor site is still a possible explanation.

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Quantification of melanin in hepatic and cardiac lipofuscin[1]

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Summary. Melanin pigment in liver and heart tissue, obtained at autopsy from patients, was isolated and quantified. The quantity of melanin extracted was directly proportional to lipofuscin granule counts. Infrared and electron spin resonance spectrographs of the isolated pigments from liver and heart showed absorption characteristics identical to those of known melanins. The pigment was absent in fetal and neonatal life, increased in brown atrophy of the heart and liver, and diminished in livers with fatty metamorphosis.

Previous spectroscopic analyses have demonstrated a melanin component in cardiac lipofuscin granules in addition to its larger lipid fraction[3-6]. In this report, we describe the isolation, identification and quantification of melanin in heart and liver tissues obtained at post mortem from patients of different ages, dying of various causes. The amount of pigment obtained using this method was compared with the number of lipofuscin granules determined by microscopic examination.

Materials and methods. Heart and liver tissues were obtained at autopsy from patients ranging in age from 5 months (fetus) to 90 years. 5 g (wet weight) of tissue was cut into small pieces; 30 ml of concentrated hydrochloric acid was added and left at room temperature. After 1 week, the mixture was centrifuged at $1000 \times g$ in an International centrifuge for 6 h. The supernatant was removed and the acid digestion of the pellet was repeated. 25–30 ml boiling chloroformmethanol (v/v $^1/_2$) mixture was added to the pellet and incubated

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in a water bath at 70 °C for 4 h. This suspension was recentrifuged for 2 h at 1000 × g and the supernatant discarded. Extraction of this pellet with chloroform-methanol was repeated until the supernatant obtained after centrifugation was clear. The brown-black pigment obtained was dried in a desiccator containing phosphorous pentoxide and then weighed. Infrared, ultraviolet (UV) and electronparamagnetic resonance spectroscopy were performed as previously described[6]. B-16 mouse melanoma and substantia nigra melanin were used as controls and subjected to the identical extraction procedure.

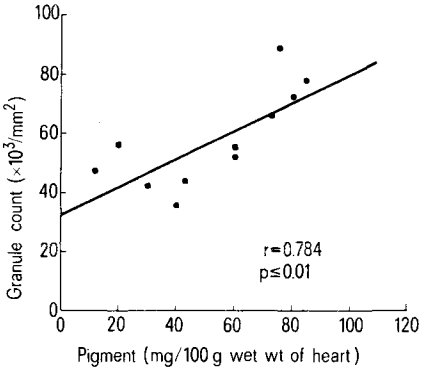

Fig. 1. Correlation of the concentration of extracted melanin pigment from hearts with the granule counts from the same tissue.

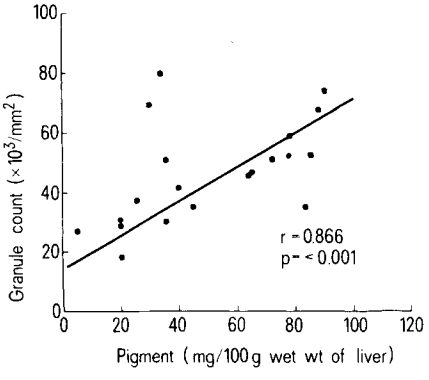

Fig. 2. Correlation of the concentration of extracted melanin pigment from livers with the granule counts from the same tissue.

Lipofuscin granules were counted in ten random areas of microscopic sections stained with Fontana-Masson silver method[7]. These sections were cut 5 nm in thickness from tissues embedded in paraffin. These granules were counted using a Nikon binocular microscope with 15 × ocular and 40 × objective lens, with a Bausch and Lomb micrometer. The surface area of the liver lobule or myocardium counted was 1.56×10^4 μm² (central 25 squares). The granule concentration was determined on the basis of the number of single granules present and the number of granules clustered together per mm². Each cluster contained approximately 2–6 granules. It was noted that as the granule concentration increased, the number of granule clusters also increased.

Results. The black pigment obtained by the preceding extraction procedures from both heart and livers of normal and brown atrophy patients was identified as melanin by its spectral characteristics, i.e. they had major absorption peaks identical to melanoma and substantia nigra melanin by infrared (3400 cm^{-1}, 2900 cm^{-1} and 1650 cm^{-1}) and electron paramagnetic resonance spectroscopy[6, 8, 9]. The amount of extracted melanin correlated with the granule count in the normal tissues (correlation coefficients, heart, r = 0.784; liver, r = 0.866) (figures 1 and 2). Figures 3 and 4 show the quantity of melanin pigment extracted from normal hearts and livers as a function of age. No pigment could be isolated from neonatal and fetal tissues. Although there appeared to be an increase in melanin pigment in both heart and liver during the first 3 decades of life, the correlation coefficients (r = 0.492 and r = 0.400, respectively) were not significant. We determined the quantity of melanin pigment in one heart weighing 250 g and one liver weighing 960 g which had gross and histological characteristics of brown atrophy. The content of melanin pigment was very high: 90 mg/100 g wet weight of heart tissue and 210 mg/100 g wet weight of liver tissue, and the granule counts were 81×10^3/mm² and 163×10^3/mm², respectively. On the other hand, 4 livers with fatty metamorphosis (age range 32–64 years) had greatly reduced amounts of melanin (4.5 + 2.2 mg/100 g, mean + S.E.).

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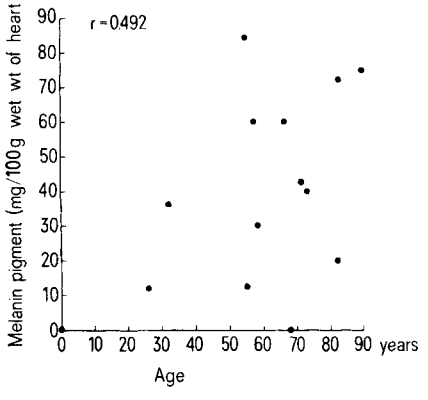

Fig. 3. Effect of age on the concentration of melanin pigment in the heart.

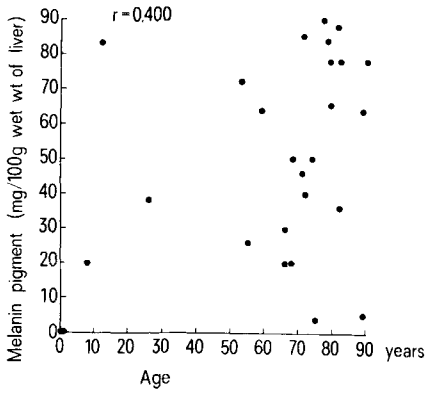

Fig. 4. Effect of age on the concentration of melanin pigment in the liver.

Discussion. When the lipid and protein components of lipofuscin granules are removed, a black insoluble residue remains which has the same chemical and spectral characteristics of known melanin isolated from other sources[6]. The quantity of melanin isolated from normal hearts and livers and those with brown atrophy correlated well with the microscopic counts of lipofuscin granules in these tissues. This suggests that lipofuscin pigment contains a melanin component.

Strehler et al.[10] observed by histochemical studies, that the number of lipofuscin granules in the heart increased with age. In our study, although the melanin pigment in control hearts and livers appeared to increase during the first 3 decades of life, the overall correlation of melanin pigment with age was not significant in either the heart or the liver. Melanin is absent in fetal liver and heart, decreased in fatty metamorphosis of the liver and increased in brown atrophy of the heart and liver. The absence of lipofuscin in fetal organs has been previously reported[11] and suggests that an adequate period of time is necessary for the synthesis of this pigment. In fatty metamorphosis there is liver cell destruction and increased cellular turnover. The newly formed cells may also not have had sufficient time to synthesize lipofuscin granules which could account for our observation of decreased melanin in livers with fatty metamorphosis. On the other hand, in brown atrophy, there is a marked increase in the lipofuscin granule count as well as melanin concentration. Large quantities of melanin pigment also have been found in the black livers obtained from mutant Corriedale sheep[12–14], Howler monkeys[15,16] and patients with Dubin-Johnson syndrome[12,14]. Further studies are needed to determine the pathogenesis and function of this visceral pigmentation.

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The effects of acetylation on the binding region of cartilage proteoglycans to hyaluronic acid[1]

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Summary. Proteoglycans in cartilage are found as aggregates and as monomers. Evidence has been obtained indicating that hyaluronic acid, normally present in this tissue, binds together monomers into large molecular weight aggregates. In this investigation, the interacting region of the protein backbone has been studied. The results unequivocally demonstrated that the epsilon amino groups of lysine are involved in hyaluronic acid binding to proteoglycans and that their blocking by acetylation either prevents reaggregation or disaggregates the high mol.w aggregates.

The chondroitinsulfate proteoglycans are characteristic components which occur in cartilage as aggregates of a mol.wt ranging from 30×10^6 to 100×10^6 dalton. These aggregates are made up by proteoglycan monomers of an average mol.wt of 2 to 2.5×10^6 dalton, by 2 link proteins[2] and by hyaluronic acid[3]. Recently, evidence has been obtained demonstrating that the main function of hyaluronic acid consists of binding together proteoglycan monomers into aggregates of large molecular size[3]. This interaction appears, therefore, to be of fundamental importance for aggregation and thus for the organization of proteoglycans in cartilage extracellular matrix. Certain features of this phenomenon are now known in some detail, e.g. the general position of the interacting side(s) in the polysaccharide-free region of the core protein of the monomer[4], the minimum size of a hyaluronic acid segment capable of interacting with the core protein[5], and the function of the link proteins which do not promote aggregate formation per se, but they seem to stabilize the whole molecular system, preventing its dissociation under the stress of ultracentrifugal forces[6].

The present experiments were devised to study the chemical characteristics of the hyaluronic acid-proteoglycan interaction, and attempts are made to identify the sites on the protein backbone of the proteoglycan involved in the linkage with hyaluronic acid.

Materials and methods. Viscosimetric and chromatographic analyses of the A_1 preparations demonstrated that the sample were high molecular weight compounds (fig. 1a) and that about 50% of the material was excluded from the gel suggesting that a large proportion was in an aggregated form (fig. 2-1). On acetylation, the drastic drop in viscosity of these samples, indicating a decrease in hydrodynamic size of the proteoglycans and their chromatographic behaviour on Sepharose 2B, demonstrates that they were all eluted in the included volume and provides evidence for their disaggregation (figs. 1b and 2-2. In this regard, it is interesting to note that the size of the acetylated samples corresponded to that of the A_1D_1 preparations (figs. 1c and 2–4) and was also similar to the A_1

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