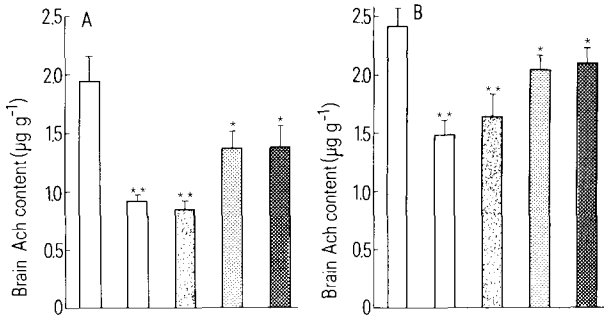
Results and discussion. The principle of measuring Ach turnover by inhibiting its synthesis with HC-3 has been established[4,19] and is based on the fact that HC-3 reduces brain Ach content[17]. Drugs that reduce Ach turnover antagonise the HC-3-induced depletion. Because rapid post-mortem changes in Ach levels can occur the brains were removed from the animals and frozen in less than 1 sec[16]. As can be seen from the figure, HC-3 caused identical reductions in brain Ach in rats pretreated with saline or 6-OHDA. This Ach depletion was partly prevented by morphine which is consistent with an earlier finding[4] that morphine reduces Ach turnover in rat brain. Morphine reduced Ach turnover by exactly the same amount in 6-OHDA-treated rats, which have degenerated catecholamine neurons[10-12], as in saline-treated animals. This finding implies that dopamine is not involved in the action of morphine on Ach turnover. The results are consistent with a direct action of morphine on Ach neurons. An analysis of brain catecholamines confirmed, as shown in the table, that 6-OHDA caused a substantial depletion of noradrenaline together with a reduction in dopamine. Because 6-OHDA neither effects morphine's antinociceptive action in rats[13] nor prevents the action of morphine on brain Ach it is possible that the antinociceptive action of morphine in the rat is partly dependent on cholinergic neurons. Morphine neither reduces Ach release nor induces analgesia in rats with raphe lesions[20].

Morphine reduced Ach turnover to the same extent in normal and 6-OHDA-treated mice. The depletion of catecholamines by 6-OHDA in the mouse was similar to the rat so morphine probably has a direct action on cholinergic neurons. Because 6-OHDA antagonises morphine's antinociceptive action in mice[13] but does not prevent morphine from reducing Ach turnover it is unlikely that Ach has a major, direct role in mediating morphine's antinociceptive action in this species. A reduction in brain Ach with HC-3 only slightly reduces morphine analgesia in mice[21].



Effect of morphine on brain acetylcholine (Ach) turnover in rats (A) and mice (B). Normal and 6-OHDA-pretreated animals received either saline, HC-3 or HC-3 and morphine. Open columns = Ach content of normal animals; closed columns = HC-3 treated; upper left cross-hatched columns = 6-OHDA + HC-3; dotted columns = morphine + HC-3; upper right cross-hatched columns = morphine + 6-OHDA + HC-3. Ach turnover is reflected by the amount of Ach depletion produced by HC-3. Each result is the mean ± SEM obtained with 6 animals. Significance of difference between normal and treated groups: * $p < 0.01$; ** $p < 0.005$.

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Hepatic silver binding protein (Ag BP) from sparrow *(Passer domesticus)*

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Summary. A silver binding protein (Ag BP) has been identified in the liver of sparrows administered a tracer dose of ^{110m}Ag. The protein as purified by gel-filtration shows a major absorption maximum at 260 nm and a minor one at 225 nm. It has a mol.wt of 9500 daltons and is stable when exposed to high temperature (64 °C for 15 min) as well as to acidic pH (2.2).

A low molecular weight protein having high cysteine (upto 30%) and metal (upto 11%) content has been identified in the kidney and liver of a number of mammalian[1-5] as well as other species[6,7]. Proteins named metallothioneins have been shown to bind a number of heavy metals, viz Ag^+, Zn^{++}, Cd^{++} and Hg^{++}. The present interest in metallothioneins arises out of their suggested roles in heavy metal detoxification[8] and normal zinc homeostasis in mammals[9]. Here, we report the identification of a low mol.wt silver binding protein which shows heat stability and low absorption at 280 nm. The silver complex of the protein is also stable at pH 2.2.

Materials and methods. ^{110m}Ag as $AgNo_3$ (sp. act. 168 mCi/g Ag) was obtained from Bhabha Atomic Research Centre,

Trombay, Bombay. Sephadex G-75 (medium), Sephadex G-25 (fine), BSA, pepsin, lysozyme and blue dextran-2000 were obtained from Sigma and Cytochrome-C from V.P. Patel Chest Research Institute, Delhi. Once-distilled water was used throughout.

Male house sparrows (20–25 g) were injected with 2.5 µCi of ^{110m}Ag as $AgNo_3$ in distilled water and sacrificed after 24 h by cervical dislocation. The whole liver was removed, rinsed in ice-cold 0.9% saline until visibly free of blood, blotted, weighed and homogenized. Routinely a 25% homogenate was prepared in 0.01 M Tris-acetic acid buffer (pH 8.0) containing 0.02% sodium azide (Tris buffer). The homogenate was centrifuged at 8000× g in a Janetzki K-24 cold centrifuge at 4 °C for 10 min. The supernatant so obtained was studied for silver binding fractions employing gel-filtration as detailed below.

3 ml of the supernatant having 105 mg protein was applied on a Sephadex G-75 column (59× 1.5 cm) preequilibrated with Tris buffer. Elution was carried out in the same buffer at room temperature and 2.25 ml fractions were collected manually. ^{110m}Ag activity in the fractions was monitored by a γ-ray medical spectrometer having a well-type NaI detector. Protein in the eluate fractions was estimated according to Lowry et al.[10].

Eluate fractions obtained from Sephadex G-75 filtration having high ^{110m}Ag activity per mg protein were pooled. The pooled sample is referred to hereafter as silver binding protein (Ag BP). Electrophoretic homogeneity, UV-absorption spectra, pH stability (heat stability) and mol.wt of Ag BP were studied by the following methods.

Polyacrylamide gel electrophoresis was performed in 15% gels at pH 8.3 and the gels were stained with Coomassie blue[11]. Absorption of UV-radiation by Ag BP at pH 8.0 in Tris buffer was recorded by a Perkin Elmer double beam recording spectrophotometer Model Hitachi 200. To check the stability of Ag BP complex at low pH, 2 ml of Ag BP fraction was passed through a Sephadex G-25 column preequilibrated with 0.187 M acetic acid-formic acid buffer (pH 2.2) and 1.0 ml fractions were collected manually at a flow rate of 30 ml/h. ^{110m}Ag and protein were estimated as described above. To examine the heat stability of Ag BP, the supernatant from the centrifuged homogenate was heated for 15 min at 64 °C, followed by immediate cooling to 4 °C in melting ice. The tubes were then centrifuged at 10,000× g to remove the heat labile fraction. ^{110m}Ag was counted in the heat stable fraction.

Molecular weight of Ag BP was estimated according to Andrews[12] on a Sephadex G-75 column (59.5× 1.5 cm) employing BSA (67,000) pepsin (34,000), lysozyme (17,500) and cytochrome-C (13,000) as marker proteins.

Results and discussion. Liver supernatant proteins, upon fractionation in a Sephadex G-75 column, show two peaks of ^{110m}Ag activity (figure 1). Fractions 14–20 comprised 1 peak in which ^{110m}Ag is associated with proteins which elute at the void volume of the column and presumably have a mol.wt larger than 70,000 daltons. The protein profile in this area clearly shows heterogeneity of this fraction. Further, only 20% of the total ^{110m}Ag present in liver supernatant is bound to this heterogenous fraction. No attempts were made to fractionate this heterogeneous peak any

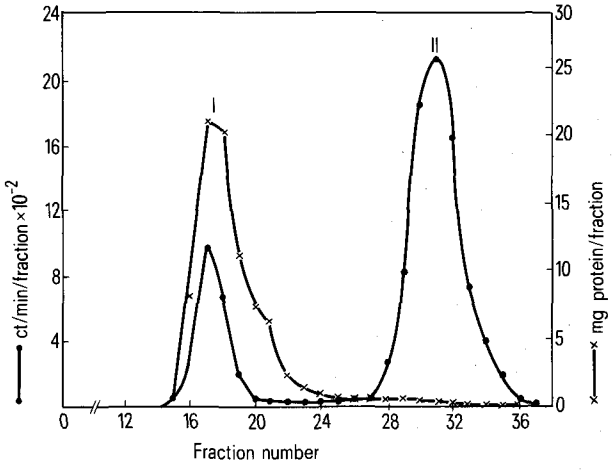

Fig. 1. Gel filtration pattern of liver proteins of house sparrow following administration of ^{110m}Ag.

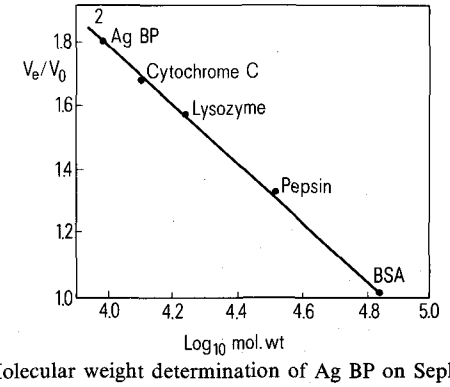

Fig. 2. Molecular weight determination of Ag BP on Sephadex G-75 column.

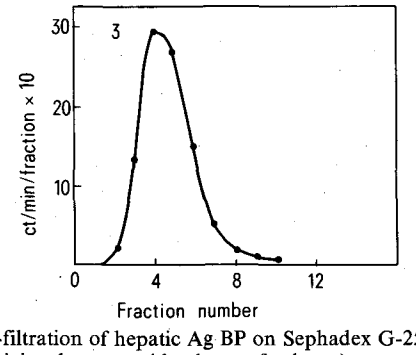

Fig. 3. Gel-filtration of hepatic Ag BP on Sephadex G-25 at pH 2.2 (^{110m}Ag activity elutes at void volume of column).

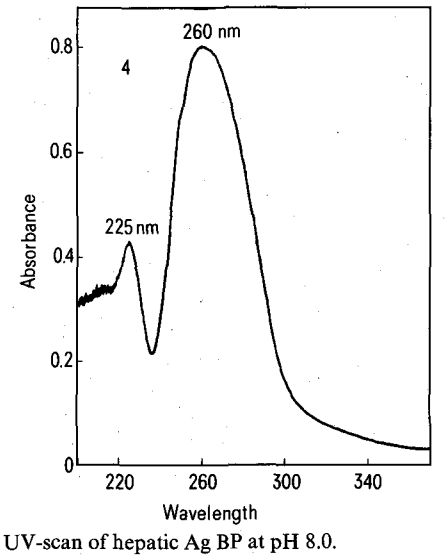

Fig. 4. A UV-scan of hepatic Ag BP at pH 8.0.

more. However, the 2nd ^{110m}Ag peak which spreads from fractions 27 to 36 contains high ^{110m}Ag activity/mg protein. Interestingly no protein peak is discernible in this region, though the fractions do contain protein as estimated by the Lowry method. The protein in the fractions moved as a single band during polyacrylamide gel-electrophoresis.

The gel-filtration estimate of the mol.wt of Ag BP using globular proteins as markers is in the vicinity of 9500 daltons (figure 2). Mol.wt estimates of Cd-Zn containing metallothioneins carried out by a number of workers by a variety of methods have ranged from 6600 to 12,000[13,14]. It was hypothesized that higher estimates were due to the occurrence of dimerization. However, recent work suggests that metallothionein behaves as a linear rather than a globular molecule during gel-filtration; such behaviour leads to a higher estimate of mol.wt when globular proteins are used as markers[14]. Further work is in progress in our laboratory to check the truth of this hypothesis employing Ag BP as a test molecule.

One of the most important properties of metallothioneins studied so far is their heat stability[15]. We have observed that heating liver supernatant at 64 °C for 15 min causes precipitation of only 20% of the total ^{110m}Ag present in the fraction. Heated supernatant, upon fractionation with Sephadex G-75, showed a single ^{110m}Ag peak corresponding to that of the Ag BP of the 2nd peak in unheated supernatant (data not shown).

When Ag BP was allowed to pass through a Sephadex G-25 column preequilibrated at pH 2.2 all of the ^{110m}Ag activity was recovered in the void volume of the column as shown in figure 3. The stability of the Ag BP complex at this extremely acidic pH is suggestive of the presence of much stronger bonds between Ag^+ and protein. In this respect Ag BP differs from Cd-thionein in which Cd^+ dissociates from the protein at pH 2.2 but is similar to Hg-thionein which is stable at this pH[16].

It is known that the binding of metal to protein often causes a shift in absorption maxima as compared to those for the free apoprotein[17]. The UV-scan obtained by us for Ag BP (figure 4) shows 2 absorption maxima, 1 major peak at 260 nm and another at 225 nm. Cadmium-containing and zinc-containing metallothioneins have their absorption maxima at 254 nm and 215 nm respectively. It has been suggested that the absorption maxima observed in cadmium- and zinc-containing metallothioneins are due to the metal mercaptides[17]. However, our observations on the absorption of UV-light by Ag BP need to be investigated further before it can be said with certainity that the observed λmax of Ag BP is due to silver mercaptides. However, the low absorption at 280 nm does suggest a lack of aromatic amino acids in Ag BP.

The properties of avian hepatic Ag BP studied in the present report indicate its similarity to other metallothioneins. Further studies are required to ascertain whether avian hepatic AG BP performs functions similar to those of mammalian metallothioneins.

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Early changes of cyclic nucleotide levels in a mitogenic reaction in the rat mesentery[1]

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Summary. By measuring simultaneously cAMP and cGMP we found a biphasic time course with regard to cGMP and the cGMP/cAMP ratio very early in a mitogenic reaction in vivo. This is a new finding.

Accumulating evidence indicates that the endogenous cyclic nucleotides cAMP and cGMP play a part in the regulation of proliferation[2-4]. These cyclic nucleotides often, but not always[5], show alterations in their intracellular concentrations very early in the process of mitogenesis in a variety of mammalian cells in cell culture[6]. These alterations often are concomitant, reciprocal, and mono-phasic.

The aim of the present investigation was to study simultaneously the concentrations of cAMP and cGMP as soon as possible and at intervals up to 32 min after induction of mitogenesis in intact tissue in vivo. The tissue chosen was the rat mesenteric membrane. Proliferation was induced by i.p. injection of compound 48/80. This caused rapid secretion of mast cells followed by prompt mitogenesis in 2 cell types, namely fibroblast-like and mesothelial-cell-like cells[7,8].

Materials and methods. Animals: male Sprague-Dawley rats (Anticimex AB, Stockholm, Sweden) weighing 170–200 g (mean ± SE was 183.6 ± 2.4 g) fed standard pellets (AB Astra Ewos, Södertälje, Sweden) and water ad libitum were used. The animals were kept under carefully standardized laboratory conditions[7] for at least 5 days before being used in experiments.

Induction of proliferation: compound 48/80 (Sigma Chemical, Co., St. Louis, USA) dissolved in saline was injected i.p. in a single dose of 1 µg/g b.wt (1 ml/100 g) at 09.00 h in unanaesthetized rats. For technical details see[7,8].

At intervals after injection of 48/80 the unanaesthetized