sured by ^3H thymidine incorporation whereas higher doses were inhibitory[10]. Our own unpublished data using the TEEM test show a dose-dependent stimulatory effect for aprotinin ending in a plateau at higher doses[11].
In the present study aprotinin stimulated the spleen cells from normal and tumour-bearing animals to the antigens used. The magnitude of the responses was greatest in animals whose immune response was suppressed by tumour. In this case the response of spleen cells became equivalent to that of spleen cells from normal animals. The spleen cell stimulation after the addition of aprotinin is not specific to tumour cells since it also occurred with PPD even though tumour cells were not present in the initial suspension.

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Chemical studies on the silver staining of nucleoli

H. Kling, L. Lepore, W. Krone, J. Olert and G. Sawatzki[1]

Abt. Humangenetik and Abt. für Transfusionsmedizin, Universität Ulm, P.O. Box 4066, D-7900 Ulm (Federal Republic of Germany), 14 May 1979

Summary. The uptake of Ag-ions by isolated nucleoli of rat liver cells was studied. Nucleolar proteins separated by electrophoresis were examined for selective silver-staining. A mechanism for a preferential Ag-staining of the nucleoli is discussed.

Nucleoli of interphase nuclei and nucleolus-organizing regions of metaphase chromosomes (NORs) that were active during the preceding interphase can be specifically stained with silver salt solutions. The high affinity of the nucleolus for silver was first noticed at the beginning of this century[2-6]. The improvement of the silver staining methods[8,9] and the applications of electron microscopy has resulted in the assignment of the material with high silver-affinity to the fibrillar component of the nucleolus[10,11]. Acidic nucleolar proteins are considered to be the most likely candidates responsible for silver staining, since proteolytic digestion abolishes the specific staining of nucleoli and NORs with silver[8-11].
The present study was undertaken to investigate quantitatively the reaction of isolated nucleoli with Ag-ions. Saturation values were determined in isolated nucleoli and under conditions of silver staining. The influence of the pH was studied. Electrophoretically separated components of 3 extracted nucleolar protein fractions were examined for preferential silver staining. Atomic absorption spectrophotometry was used to determine the amount of bound silver.
Materials and methods. For the determination of the amounts of silver taken up by the nucleoli under saturation and staining conditions, the Ag-loaded nucleoli were filtered on acetate filters (pore size: 0.2 µm; Schleicher and Schüll) with hydrophobic border. The loaded filters were disintegrated in acid-digestion bombs (Parr Instrument Co Moline, Ill. USA) with nitric acid (sp. wt 1.4 g/ml) at 160 °C for 75 min. Silver content was determined in an atomic absorption spectrophotometer (AAS 400, Perkin Elmer) by the graphite tube method. Instrumental parameters: Dry(°C/sec) 100/30; ash (°C/sec) 490/20; atomize (°C/sec) 2400/10; wave length: 278 nm; injection volume (µl) 20.
Nucleoli were isolated from fresh rat liver cells[12]. The preparations of nucleoli were analyzed for RNA[13], DNA[14] and protein[15]. Photometric determination of the amount of nucleoli was based on the relationship: 1 mg nucleoli/ml = 10.0 OD at 260 nm[16].
For saturation with Ag^+, the nucleoli were suspended in 0.05 M Na-acetate buffered $AgNO_3$ solutions (pH 5.3; containing 0.17 M sucrose and 5 mM Na-acetate) with different Ag^+-concentrations (figure 1). Saturation values at different pH were determined in 0.05 M Na-acetate (pH 4.0 and 5.0), 0.05 M boric-acetate (pH 6.0 and 7.0) and 0.05 M Na-borate (pH 8.0 and 8.9) buffered 0.28 M sucrose + 5 mM Mg-acetate at a concentration of 150 µg Ag^+/mg nucleoli. The incubation time was 2 min at 0-4 °C (figure 2).
For silver treatment under staining conditions 2.0 mg nucleoli were incubated in 8 ml of a staining solution − 7.0 ml 50% $AgNO_3$ (w/v) + 1.0 ml Na-formate buffer -

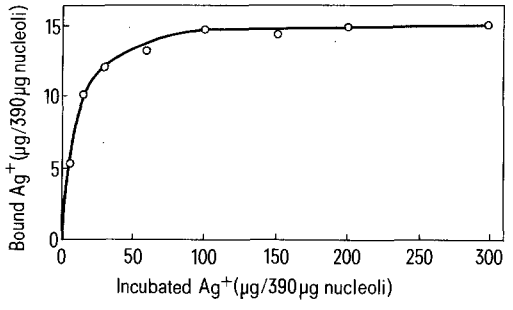

Fig. 1. Saturation of nucleoli with increasing amounts of Ag-ions.

at 60 °C[17,18] for about 2 min. The intensity of staining was checked by light microscopy. Then 4.0 ml ice-cold 0.34 M sucrose was added and the nucleoli were homogenized and filtered.

Extraction of nucleolar proteins was performed with 0.14 M NaCl, 0.3 M NaCl[19] and 6.0 M urea-0.5 M guanidine-hydrochloride solutions[20]. Electrophoresis of the extracted protein fractions was performed on 10% polyacrylamide slab gels (4.5 M urea) with a 4% stacking gel (4.0 M urea). For visualization of the separated proteins, the slab gels were first incubated in 7% acetic acid to remove all urea, followed by an incubation in an ice-cold silver staining solution (10 min). The slab gels were developed at 60 °C.

The extracted nucleolar protein fractions were dialyzed against water and lyophilized; smears of equal amounts of the protein fractions were developed on a slide with silver staining solution at 60 °C.

To test the hypothesis that carboxyl-groups are the target candidates of silver staining, esterification of the carboxyl-groups in nucleoli was carried out in methanol saturated with diazomethane for 30 min at 20 °C.

To study the silver reactions of CM-Sephadex C25, Sephadex G10, SP-Sephadex C25, Cellex N1, Cellex CM and of Ag-acetate crystals, small amounts of these substances were developed on slides at 60 °C.

Results and discussion. Saturation experiments were performed to determine the maximum uptake of Ag-ions by isolated nucleoli. Figure 1 shows a typical saturation experiment performed at pH 5.3. A saturation value of 38.1 µg Ag/mg nucleoli was obtained. Various preparations of nucleoli yielded saturation values in the range of 32–38 µg Ag/mg nucleoli. This variability can be attributed to differences of the composition of the various nucleoli preparations with respect to the ratios of DNA:RNA:protein. The RNA contents fall into the range between 1.06 to 1.75 times the amount of DNA, and the protein contents vary between 4.45 to 6.8 times the amount of DNA. Increasing the excess of Ag-ions up to 8.0 mg/mg nucleoli does not bring about additional silver-binding. Saturation of nucleoli with Ag-ions is complete within 2 min under our experimental conditions. Incubation for up to 60 min does not cause further alterations of the amount of silver bound to the nucleoli. Ag-saturated nucleoli are nearly colourless, their colour changes to black upon exposure to day light.

Methanol-acetic acid fixed nucleoli, as occurring in cytological preparations, do not show any changes in the uptake of Ag-ions at pH 5.3. Extraction of nucleoli with 0.14 M NaCl causes significant diminution of the nucleolar size and a reduction of staining with methylene blue but not with silver-ions. These nucleoli which have lost about 30% of their proteins by extraction show a reduction of silver-ion uptake of 20%, but no reduction in silver staining.

Among the components of the nucleolus the proteins with functional groups such as carboxyl-, amino- and sulfhydryl groups show Ag-affinity[21], but DNA and RNA[22,23] are also able to bind silver-ions with some preference for the GC pairs in the DNA[24]. A dependence of Ag-binding on the pH can therefore be expected for the nucleoli. As shown in figure 2 the amount of Ag-ions bound by the nucleoli is a linear function of the pH within the range measured. The maximum amount of Ag-ions taken up at pH 8.9 is 4 times that bound at pH 4.0.

Isolated nuleoli take up higher amounts of silver under staining conditions than during the saturation experiments. This increase of Ag-uptake may not be assumed to be due to additional saturation of other groups, but by a formation of growing silver nuclei. The experiments reveal that at pH 5.3, when nucleoli are covered with dark blocks, the silver content is about 900 µg Ag/mg nucleoli.

An important question is whether the results obtained with isolated nucleoli can be compared with those from non-isolated nucleoli. Alterations taking place during the nucleolar isolation procedure do occur; for instance the ratio of DNA to RNA and protein can vary. It is therefore difficult to evaluate the consequences of such variations on the Ag-staining of isolated nucleoli. Assuming that it is not a defined nucleolar protein, but rather a certain arrangement of the active silver-groups that is responsible for preferential Ag-staining, the destruction of this arrangement could influence the velocity of silver nuclei formation. Such destruction probably has no important consequences for the single Ag-active groups, so that the pH dependence of the nucleolar Ag-uptake may hardly change. Pretreatment of neural tissue[25] with diazomethane produces a reduction of Ag-staining, whereas in the nucleolar regions of Hela cells Ag-staining disappears completely[18]. Isolated nucleoli also show reduced Ag-staining after pretreatment with diazomethane but it is difficult to determine whether this reduction is caused by esterification of carboxyl-groups alone, or if it is due, in part, to shrinking of the nucleoli. It is probable that in these experiments an esterification of the carboxyl-groups of the nucleolar proteins occured, because after short treatment with dilute NaOH the nucleolar regions of the Hela cells regain their silver-staining. If methylation of OH-groups had taken place, this would not be expected. It is known that formate ions are able to reduce the Ag-ions of silver acetate to elementary silver[26]. Our investigations showed, for instance, that silver acetate crystals are covered rapidly with silver during the staining procedure. It is possible that formation of CO occurs after decomposition of Ag-formate which reduces

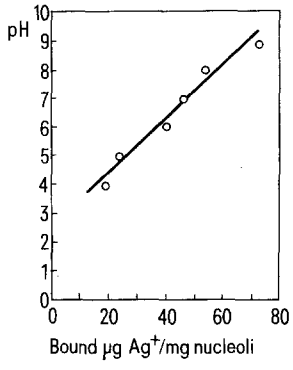

Fig. 2. Effect of pH on the saturation of nucleoli with Ag-ions.

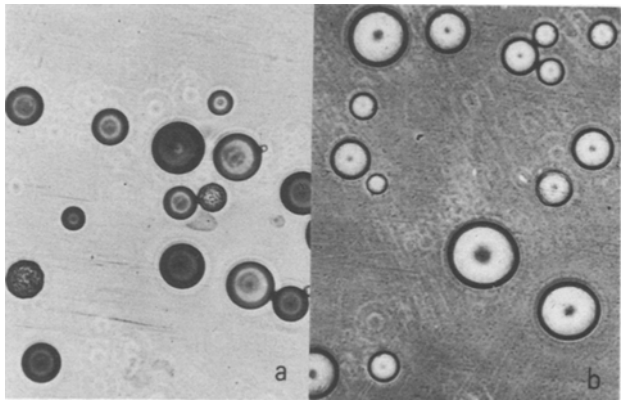

Fig. 3*a* CM-Sephadex C25 after silver staining. *b* Sephadex G10 after silver staining.

the Ag-ion[27]. Gaseous CO alone is able to produce silver blocks in the nucleolar regions of Hela cells[18]. The Ag-ions bound to the carboxyl-groups appear to be reduced very easily by formate ions. This can be demonstrated with Carboxyl-Sephadex which under staining conditions precipitates silver faster than Sephadex G10 or HSO_3-Sephadex. Carboxyl-cellulose reacts in a similar fashion.

Another possibility for the preferential silver staining, is that nucleoli contain specific proteins which are able to reduce Ag-ions. To test this, nucleoli were extracted with salt and urea-guanidine hydrochloride solutions to obtain 3 protein fractions. Smears of these fractions did not show any difference in the silver staining ability. By polyacrylamide gel electrophoresis of the 3 protein fractions and subsequent silver staining of the gel, patterns of brown bands were obtained with corresponded with all bands stained with Coomassie blue.

In summary, the investigations show that a preferential silver staining of nucleoli and NORs need not necessarily be caused by a special silver affinity protein. Carboxyl-groups alone, which are active at the pH of the staining conditions, could separate silver. A corresponding accumulation or arrangement of carboxyl-groups of nucleolar proteins could favour the formation of silver nuclei in the nucleoli and NORs and they could therefore cause an equivalent rapid Ag-staining.

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Autoradiographic localization of ^{3}H-angiotensin II in rat adrenal glands

M. Coutard and M.J. Osborne-Pellegrin

Department of Cardiovascular and Renal Physiology and Pharmacology, INSERM U. 7, Hôpital Necker, 161 rue de Sèvres, F-75015 Paris (France), 24 April 1979

Summary. Autoradiographic studies of rat adrenal gland approximately 1 min after intra-aortic injection of ^{3}H-angiotensin II show that radioactivity concentrates in the zona glomerulosa. This concentration is reduced by concomitant administration of excess unlabelled angiotensin II.

The adrenal glands are a well known target tissue for angiotensin II. A stimulatory effect of this hormone on aldosterone secretion from the zona glomerulosa has been reported[1], and angiotensin II has also been shown to stimulate adrenocortical corticosterone release[2] and adrenomedullary catecholamine release[3]. Angiotensin receptors have been studied by biochemical methods in isolated zona glomerulosa cells[4,5] and adrenal homogenates[6–8], but the precise localization of angiotensin II within the adrenal gland after in vivo injection has not been reported. The autoradiographic approach has been used previously to localize specific angiotensin II binding sites within the kidney[9]. Here we have studied the localization of ^{3}H-angiotensin II in rat adrenal glands, after its injection in vivo, using 2 different autoradiographic methods.

Materials and methods. Adult, male Wistar rats were bilateraly nephrectomized 48 h before the experiments since previous studies[8] have shown that this procedure, as well as eliminating endogenous angiotensin II, also increases adrenal angiotensin binding sites, thus providing optimal conditions for their autoradiographic detection. ^{3}H-angiotensin II (2 batches of sp. act. 70 and 46 Ci/mM respectively) was injected under pentobarbital anaesthesia via a cannula placed in the carotid artery, the tip of which just entered the aorta.

Autoradiographic procedures. Method I. 1 min after ^{3}H-angiotensin II injection (400 ng, 600 ng, 1 µg) adrenals were fixed by intra-aortic perfusion of buffered glutaraldehyde at 2.3%, post-fixed in 2% OsO_4 and embedded in epon. 1.5-µm semi-thin sections were mounted on slides and coated with autoradiographic emulsion (stripping film AR.10 Kodak). After 6 weeks exposure at room temperature, autoradiograms were developed, fixed and stained with toluidine blue.

Method II. Rats received an intra-aortic injection of 25, 50 or 100 ng of ^{3}H-angiotensin II either alone or in presence of excess unlabelled angiotensin II. 45 sec after the injection, the adrenal glands were removed and quickly frozen in