3.5–5.5%) granulocytes. Viability assessed by dye exclusion was over 95%. 2. The response of rabbit lymphocytes to varying concentrations of PHA are summarized in table 1. Even if the response varied from animal to animal, a concentration of PHA of 1:40 or 1:80 was found to be optimal for rabbit lymphocytes of both strains examined under the culture conditions described. These concentrations were used in the further experiments of this study. 3. Results of PHA stimulation of rabbit lymphocytes and of human lymphocytes cultured under the same conditions are presented in the figure. Stimulation varies from animal to animal, but the stimulation of rabbit lymphocytes is consistently better at lower concentrations compared to human cells. 4. Results of the allogeneic stimulation of rabbit lymphocytes are summarized in table 2. It shows clearly that rabbit lymphocytes can be stimulated by allogeneic cells. The responses observed with allogeneic cells is consistently lower than with mitogen (PHA) which is also well known from other animals. The response is also lower than usually observed with human lymphocytes in a similar system.

Discussion. The results presented here demonstrate that rabbit lymphocytes can be reproducibly separated from small quantities of whole blood on a 1.09 Ficoll-Ronpacon density gradient. This sedimentation technique yields a quite pure suspension of viable lymphocytes (90–93%) with about 4% monocytes and a contamination of less than 6% granulocytes. The number of cells harvested is also fairly reproducible, about 20×10^6 cells out of 8 ml whole blood. This allows various tests from a single sample of blood. The quality of the cells permits reproducible cultures either for stimulation with PHA or with allogeneic cells. Results are comparable to those published by other authors using other techniques[5,6].

As recently demonstrated by Bell[12], the feature of rabbit T-lymphocytes appear to be quite similar to those of human T-cells. T-cells of the rabbit are PHA responsive. Rabbit lymphocytes stimulated by PHA show a variation from animal to animal, as expected from other experiments and from comparison with the human system. This appears to make functional tests of T-lymphocytes of the rabbit possible which are important for studies on allogeneic bone marrow transplantation. The amount of PHA needed for optimal stimulation of rabbit lymphocytes appears to be quite similar to the human one[10], about 0.5 µl–0.25 µl of stock solution per culture.

Compared to human controls, the response of rabbits is usually lower, despite identical cell concentrations and culture conditions. This has already been shown by other investigators and is also known from other species[6,8,9]. The reason for this is not yet understood. This difference appears particularly important in allogeneic lymphocyte stimulation where it may be difficult to compare results of animals and man[10,11]. The fact that the stimulation with allogeneic lymphocytes is lower than PHA stimulation of T-cells, is also well known in man. Here again the similarity of rabbit T-lymphocytes is evident. It is well known[13] that about 40% of human T-lymphocytes will be stimulated by PHA whereas less than 5% respond to PPD, even after sensitization, or to allogeneic cells. This may explain the difference in H_3-thymidine incorporation.

In conclusion: We have shown that stimulation of rabbit lymphocytes by mitogens or allogeneic cells is possible on cells separated on a Ficoll-Ronpacon density gradient of 1.09 from small quantities of peripheral blood. Culture results are fairly reproducible and comparable to those previously published using different techniques. This separation technique allows repeated tests of rabbit T-lymphocyte function, as well as histocompatibility testing using mixed lymphocyte cultures.

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Interphase studies with a simplified method of silver staining of nucleoli

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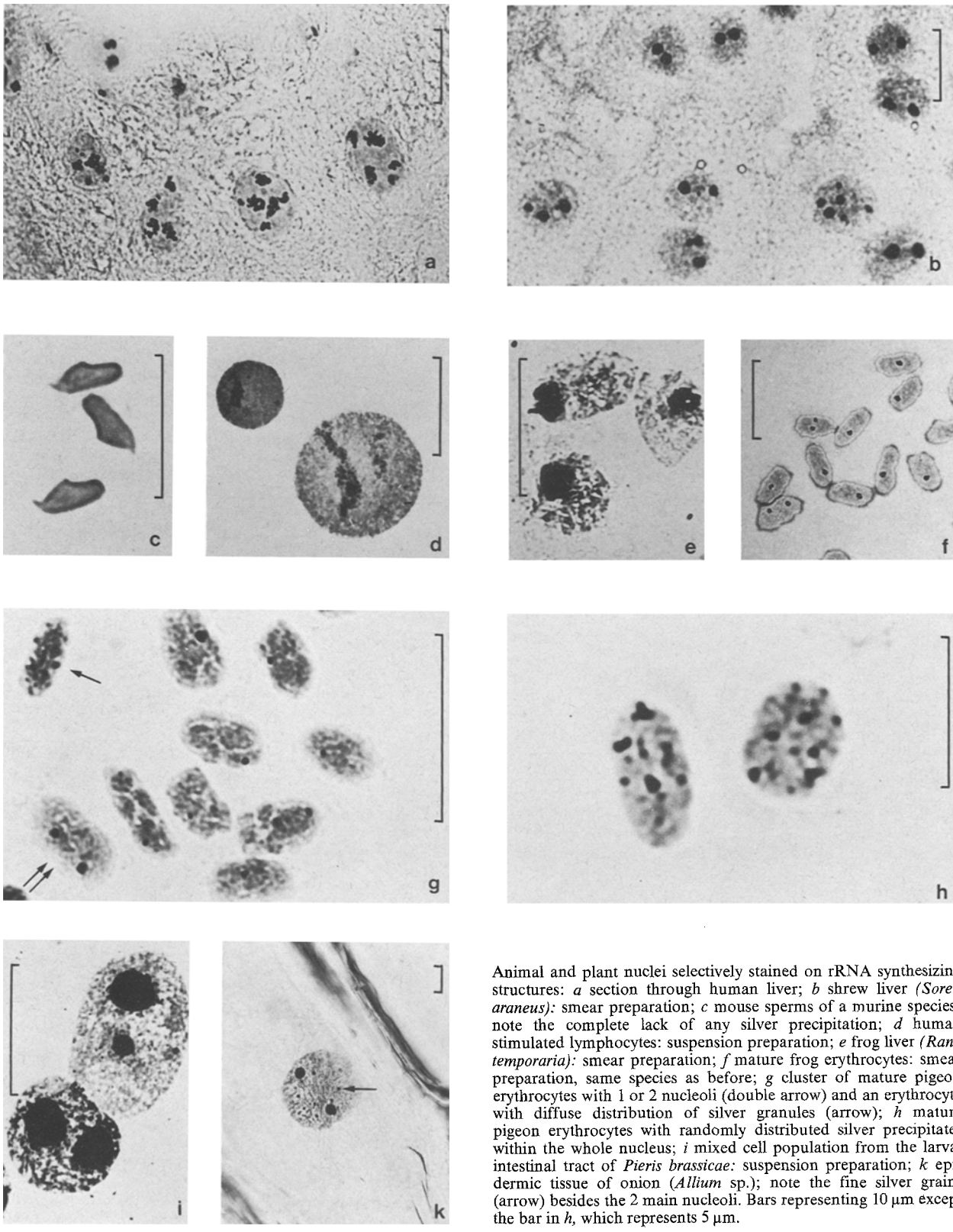
Summary. A simple silver staining method is presented providing a rapid and reliable technique for the selective staining of nuclear structures synthesizing ribosomal RNA (18S and 28S RNA).

Many techniques for the selective staining of nucleoli and RNA-rich structures are available. Their application depends on the use either of basic dyes[2–4], or basic dyes plus cationic surfactant[5], or silver-salt solutions[6,7]. More recently developed silver staining methods[8–10] have been widely used to detect nucleolar organizing regions (NORs) in the karyotypes of man and other organisms. There is evidence from studies in somatic cell hybrids that those NORs are stained by silver which were actively engaged in the synthesis of rRNA during the preceding interphase[11,12]. Furthermore, the silver staining patterns obtained from preparations of early embryonic stages[13], and those from stages of male gametogenesis[14], provide evidence that silver staining reflects the function of ribosomal genes rather than their mere presence.

However, with the exception of these latter studies there are very few applications of this method to interphase cells. In a study about the nature of Ag-staining, Schwarzacher et al.[15] made it most plausible that the Ag-granules are mainly associated with the fibrillar component of the nucleolus. A positive direct correlation between the amount of silver precipitates over the nucleolar regions and the rate of rRNA synthesis was clearly demonstrated by Hofgärtner[16].



Animal and plant nuclei selectively stained on rRNA synthesizing structures: *a* section through human liver; *b* shrew liver *(Sorex araneus):* smear preparation; *c* mouse sperms of a murine species; note the complete lack of any silver precipitation; *d* human stimulated lymphocytes: suspension preparation; *e* frog liver *(Rana temporaria):* smear preparation; *f* mature frog erythrocytes: smear preparation, same species as before; *g* cluster of mature pigeon erythrocytes with 1 or 2 nucleoli (double arrow) and an erythrocyte with diffuse distribution of silver granules (arrow); *h* mature pigeon erythrocytes with randomly distributed silver precipitates within the whole nucleus; *i* mixed cell population from the larval intestinal tract of *Pieris brassicae:* suspension preparation; *k* epidermic tissue of onion (*Allium* sp.); note the fine silver grains (arrow) besides the 2 main nucleoli. Bars representing 10 µm except the bar in *h,* which represents 5 µm.

In the present paper, various types of tissues of animal, as well as of one plant species, were tested on ribosomal gene activity by employing a new technique which provides a simple and rapid way for selective silver staining of nucleoli in interphase cell nuclei.

Materials and methods. Various tissues of several animal species and of one plant species were used (for details see legend of the figure). The best results were achieved using a methanol: acetic acid mixture (3:1) as fixative. Formaline-fixed sections or smear preparations must otherwise be

pretreated with hot trichloroacetic acid (TCA: 5% w/v) for about 30 min.

Description of the staining procedure is as follows: 7 parts of an $AgNO_3$ solution (50%; w/v in distilled water) are mixed with one part of 0.2% formic acid (in distilled water; adjusted to a pH of 2.5 with sodium formate), immediately pipetted on to the prepared slide and covered with a coverglass. Excessive background is avoided by applying very small quantities of silver solution and blotting with filter-paper. Incubation in a moist chamber follows until the preparation becomes a yellow to brown colour (2–5 min). Then the coverglass is removed by rinsing with distilled water and the slide is air-dried. To prevent disappearance of the yellow colour of the chromatin and of the silver precipitates, the slides should be mounted for monitoring and photographing only in distilled water. An additional stabilization of the silver staining pattern can be attained by incubating the preparation in alkali (e.g. in 0.01 N NaOH for about 2 min). Adjustment of the pH of the formic acid developer to 2.7 makes the method suitable for silver staining of NORs in metaphase.

Results and discussion. After successful staining, black silver precipitates have formed on the nucleolar regions, whereas the surrounding chromatin and some cytoplasmic structures appear in various yellow to brown shades (figure, a, b, e). In some preparations, the chromatin is less homogeneously stained and shows varying numbers of brown granules (figure, d, e, g).

Surprisingly, mature frog erythrocytes, which were shown not to synthesize ribosomal RNA and to contain an almost completely inactivated nucleus[17,18], exhibit 1 or 2 silver blocks per nucleus (figure, f). This corresponds to the single pair of NORs shown to occur in all European species of Ranidae[19]. Apparently, the silver-binding components remain associated with the inactive micronucleoli of mature frog erythrocytes[20] in a similar way as how they stay with the synthetically inactivated NORs of metaphase chromosomes. On the other hand, a low rate of residual activity of the ribosomal genes in the mature frog erythrocyte cannot be excluded with certainty. Furthermore, extranucleolar lampbrush loops were found silver positive in *Triturus cristatus* by Varley and Morgan[21], which suggests that sites of synthesis of nonribosomal RNA show affinity for the silver stain.

A different pattern of distribution of silver blocks is observed in pigeon erythrocytes (figure, g, h). Most of the nuclei exhibit several small silver grains randomly distributed across the nuclear area. Only a few cells (approximately 2–3%) show one or two larger blocks (figure, g), corresponding presumably to the NORs on the microchromosomes of birds (own unpublished observation), associated with the micronucleoli detected by electron microscopy in the mature chicken erythrocyte[22]. Whether or not this cell type represents a less mature stage of erythropoesis (reticulocyte) remains to be investigated. It can also not be decided if the more diffuse distribution of silver grains (figure, h) in the mature pigeon erythrocyte bears any relationship to the sites of RNA synthesis observed in the mature hen erythrocyte[23,24]. The RNA species synthesized by the mature hen erythrocyte do not seem to be related to ribosomal RNA[25].

Mature sperm cells contain a totally inactivated nucleus. As expected, this stage failed to show silver precipitation (figure, c). On the contrary, the highly active nucleoli of cells from larval stages of insects[26] exhibit a correspondingly strong reaction with the silver-salt solution (figure, i).

As representative for plants, interphase nuclei of epidermic tissues of onion (*Allium* sp.) were tested for ribosomal gene activity. *Allium* species exhibit 1 to 2 pairs of nucleolus organizing chromosomes[27], and it has been shown by Maggini et al.[28] that there is an exact relationship between the NOR number and the maximum number of nucleoli in interphase nuclei of *Allium cepa*. The cell shown in the figure, k, represents 2 distinct nucleolar regions probably formed by 2 NORs. Besides these nucleoli, small silver granules within the nucleus (see figure, k, arrow) possibly indicate further ribosomal gene activity, or the presence of silver-stainable components deriving from the main nucleoli. However, there is no ultrastructural evidence for nucleolar regions outside the main nucleoli in *Allium* microspores[29]. Thus, these fine silver granules may reflect a special functional state of the nucleus of epidermic cells of *Allium*.

In summary the silver staining method presented could serve as a useful tool for investigating ribosomal gene activity and function, as well as their distribution within various types of animal and plant cells.

Note added in proof: recent observations have shown that the fixation-step with methanol/acetic acid can be omitted in air-dried blood smears.

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