

CHARACTERIZATION AND LOCALIZATION OF THE RNA SYNTHESIZED IN MATURE AVIAN ERYTHROCYTES

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SUMMARY

RNA present and synthesized in mature hen erythrocytes *in vivo* and *in vitro* has been studied with electron microscopic, autoradiographic, and biochemical methods. The average hen erythrocyte contains a minimum of 0.02–0.04 μg RNA which is predominantly, if not exclusively, located in the nucleus. The incorporation of [^3H]uridine into this RNA is very low but is clearly demonstrable *in vivo* and *in vitro* using different incubation media. The specific efficiencies of the incorporation assays are compared. The incorporated radioactivity is contained in RNA as shown by its selective sensitivity to hydrolysis in mild alkali and with ribonuclease and is not hybridized to DNA as shown by Cs_2SO_4 gradient centrifugation of total hen erythrocyte nucleic acids. LiCl precipitation as well as gel electrophoresis on agarose under denaturing and non-denaturing conditions has revealed that this labelled RNA is of high-molecular weight and covers a broad range from ca 0.25 to 5.0 million D, with gel-electrophoretically detectable peaks at positions corresponding to 1.8, 1.0 and 0.5 million D. The pattern obtained is virtually identical after the different incorporation conditions and was also similar to that obtained in reticulocyte cells of animals made anaemic with phenylhydrazine. The incorporation is inhibited by actinomycin D with a sensitivity characteristic for mRNA formation and is also reduced in the presence of α -amanitin. The labelled RNA remains within the confinements of the nucleus and does not seem to be transported into the cytoplasm. It appears to be preferentially enriched in the interchromatinic channels. It is concluded that mature hen erythrocytes are capable of transcribing a small part of their genome into pre-mRNA at a very reduced level but that this RNA is not only excluded from functioning in translation but also from nucleocytoplasmic transport. The findings are discussed in relation to the controversial literature in this field, and possible functions of this RNA are hypothesized.

Various forms of cell differentiation are characterized by a progressive cessation of the synthesis of most, if not all, classes of RNA. In some late stages of such differentiation processes one even notes a general breakdown of RNA-containing structures and the mature cell is almost emptied of RNA. The formation of the red blood cell, in particular in animals with nucleated erythrocytes, has become of special importance in studies of the pro-

grammed switch-off sequence of the transcription of most, and finally perhaps of all, genes. It has been shown that during erythropoiesis the nuclear content and the nuclear envelope is greatly reduced, the chromatin condenses and contains an additional special histone, the nucleolus disappears, concomitant with the cessation of ribosome formation, the number of transcribed genes decreases, leaving the transcription of one class of genes in the

late erythroblast, namely the codons of the precursor molecules of globin mRNAs [4, 17, 25, 27, 29, 41, 48, 54, 57, 59]. Fully mature erythrocytes are believed to be cells completely inactive in RNA formation [15, 16, 31, 48, 49, 54] and without significant amounts left of the common cytoplasmic structures such as ribosomes and endomembranes [27, 30, 67]. There have been, however, also indications in the literature of the presence of some minor quantities of RNA in the fully mature avian erythrocyte as well as of some continued RNA synthesis [4, 36, 38, 39, 41, 42, 55, 62, 67], corresponding to findings of active RNA polymerases and low but significant template activity in these cells [1, 2, 9, 12, 25, 40, 50, 52, 56] (see however, [19]); for amphibian erythrocytes see [28, 37]. In our studies of the hen erythrocyte nucleus we became interested in the possible existence and synthesis of some RNA in a cell that apparently is late in the process of a 'dead end' differentiation and has lost the machinery of protein synthesis.

MATERIAL AND METHODS

Animals

In all experiments white Leghorn chicken (HNL-Nick-Chick, 6–8 weeks old, ca 250 g body wt) or adult egg-laying hens were used.

Preparation of cells

Blood was prepared either from decapitated chicken [67] or by wing vein puncture. Enrichment and purification of mature erythrocytes was done by low speed centrifugation [66]. Blood was allowed to drop into physiological saline that was 2% with respect to the anticoagulate Liquemin (Liquemin 5000; Hoffmann-La Roche AG) and was centrifuged for 6 min at 100 g. Erythrocytes were washed twice at low centrifugal speed in physiological saline, 'buffy coat' components and top layer erythrocytes were removed with pipettes, and only the very bottom layer of red blood cells was collected. In some experiments designed to study the incorporation of nucleosides *in vitro* this was followed by three

washes in minimal essential medium (MEM) containing Hanks' salts. Blood enriched in immature red cells (mostly late erythroblasts) was obtained from chicken treated with phenylhydrazine [3, 66]. Counting of blood smears from anaemic hens showed that usually more than 70% cells of the population were erythroblasts, in contrast to the purified erythrocytes from normal hens which contained less than 2% not fully mature cells (mostly polychromatic erythroblasts [34]).

Incorporation of [³H]uridine

Incorporation in vivo. One to 3 ml of [³H]uridine (5, 6-[³H]uridine; 41 to 43 Ci/mmole; The Radiochemical Centre, Amersham, UK) was concentrated by rotary evaporation (25°C), resuspended in 0.5 ml physiological saline and injected into the wing vein. To obtain blood at different time intervals (30 to 180 min) a fine needle (Braunula, O/G20, Braun, Melsungen, BRD) was inserted and fixed in the wing vein. Prior to the first bleeding 1 ml Liquemin was injected. Blood samples were collected with a heparinized syringe and suspended in defined volumes of physiological saline (2% Liquemin). Erythrocytes were washed and purified as described above at 4°C. The cell-free serum (obtained after centrifugation of the 100 g supernatant at 10 min, 3000 g) was collected for determinations of radioactivity.

Incorporation in vitro. Two sets of experiments were performed.

(a) [³H]uridine was concentrated by evaporation. Liquemin was injected into the wing vein. After 10 min 1 ml blood was obtained by puncturing the wing vein and immediately added to the uridine so that the final concentration was 0.25 mCi/ml. The blood was incubated for 30 min at 38°C. After dilution of the sample with 5 ml ice-cold physiological saline erythrocytes were purified by centrifugation in the cold at low speed as described above.

(b) Erythrocytes were purified as described and resuspended in MEM containing 0.06 to 0.25 mCi/ml of [³H]uridine. The erythrocytes (10⁷ to 10⁸ cells/ml) were incubated at 38°C between 30 and 180 min. After incubation the erythrocytes were spun for 10 min at 3000 g in the cold. The concentration of the [³H]uridine in the supernatant was determined by measuring the radioactivity in aliquots. Pellets were washed twice in MEM medium by centrifugation in the cold. The erythrocytes were then homogenized in cold (4°C) 10% TCA with a Potter-Elvehjem homogenizer. After 30 min precipitation the material was washed several times with 10% ice-cold TCA on Whatman GF/C glass fibre filter disks. The filters were incubated overnight in 2 ml NCS (Nuclear Chicago solubilizer; Amersham/Searle Corp., Arlington Heights, Ill.) at 40°C, 10 ml toluene-based scintillation fluid was added and radioactivity was determined in a liquid scintillation spectrometer (Betasint 5000; Berthold-Frieseke, Karlsruhe). Counting efficiency was corrected for hemoglobin quenching.

Experiments with inhibitors

of RNA synthesis

Blood was obtained from decapitated hens, and erythrocytes were purified as described above. The final pellet of red blood cells was resuspended in MEM medium to 1×10^8 cells/ml. To 1 ml samples of erythrocyte suspension actinomycin D (AMD; Serva, Heidelberg) or α -amanitin or its methyl derivate (kindly provided by Drs T. Wieland and R. Govindan, Max-Planck-Institut für Medizinische Forschung, Heidelberg) was added to give final concentrations of 0.1, 0.5, 1, 10, 50, 100, 200 $\mu\text{g/ml}$ AMD and 1, 5, 10, 50, 100, 400, 500 $\mu\text{g/ml}$ amanitin, respectively. After an incubation at 38°C for 30 min 0.1 mCi [^3H]uridine was added to each sample, followed by a further incubation (30 min) at 38°C. Finally, the samples were spun at 3000 g, and the erythrocytes were washed twice by resuspension and centrifugation in MEM. TCA-insoluble radioactivity was determined as described above.

RNA extraction

The erythrocytes were lysed in a medium consisting of 0.05 M Tris-HCl (pH 7.6), 1% NaCl, and 2% (w/v) sodium tri-isopropyl naphthalene sulphate, using a motor-driven Potter-Elvehjem homogenizer. Extraction of the nucleic acids was done according to Parish & Kirby [43], as modified by Loening [32]. The ethanol-precipitated nucleic acids were centrifuged at 3000 g for 10 min in the cold, the pellet was suspended in 0.15 M Na-acetate buffer (pH 6.0) containing 0.5% sodium dodecyl sulfate (SDS), and 2 vol ethanol were added. After 24 h incubation at -20°C, the pellet was washed in 70% ethanol, containing 0.1 N NaCl, in order to remove traces of the detergent, and the resulting pellet was resuspended in 0.05 M Tris-HCl (pH 7.4), containing 2 mM magnesium acetate, to a DNA concentration of 200 to 400 $\mu\text{g/ml}$. DNase I (RNase-free; Worthington, Freehold, N.J.) was added to give a final concentration of 20 $\mu\text{g/ml}$, and the solution was kept at 37°C for 45 min. SDS was then added to 0.5%, and the solution was incubated with pre-digested pronase (1 mg/ml; nuclease-free; Calbiochem, San Diego, Calif.) for 30 min at 37°C. RNA was extracted by shaking with an equal volume of a mixture consisting of one part chloroform and one part phenol-cresol-8-hydroxyquinoline. The aqueous phase was precipitated by adding 2 vol of ethanol. In most cases considerable amounts of DNA remained; therefore, a second DNA-digestion with DNase had to be performed. The RNA was then twice reprecipitated from Na-acetate-SDS buffer and its purity was judged from its absorbance spectrum. The ratios of absorbancies at 260/280 and 260/230 nm were 2.0 and 2.6, respectively. The presence of small amounts of DNA in the final preparation was also routinely checked by electron microscopy of spread preparations without addition of denaturing agents (for details, see [23]).

In order to selectively precipitate high molecular weight RNA a defined amount of RNA was dissolved in 0.1 M Tris-HCl (pH 7.2), and the same volume

of 4 M LiCl was added. The mixture was stored at 2°C for 48 h and then centrifuged at 3000 g for 20 min. The pellet was resuspended in Na-acetate-SDS buffer, and the absorbance at 260 nm was measured. An aliquot was taken for determination of radioactivity. Finally, the RNA was again precipitated by adding ethanol.

Gel electrophoresis

Gel electrophoresis was carried out either under non-denaturing conditions in cylindrical 1% agarose gels with the buffers used by Daneholt & Hosick [18] or under denaturing conditions in 1% agarose gels containing 80% formamide (see also [11]). For preparation of formamide-agarose gels, formamide (Merck, Darmstadt) was stirred with 5% Amberlite MB-1 ion exchange resin (Serva, Heidelberg) for about 2 h until a specific conductivity of about 10 μMho was attained. The resin was removed by filtering through a Whatman glass fibre filter GF/C. The deionised formamide was diluted with 0.1 N sodium phosphate buffer (pH 6.8) to 80% resulting in a final phosphate concentration of 20 mM. Agarose (Seakem, Springfield, N.J.) was dissolved in 80% formamide by heating. After cooling the solution was transferred into glass tubes (15 cm long 6 mm inner diameter) and left overnight at about 2°C. To obtain a flat surface, the gels were inverted prior to use. The running buffer was aqueous 20 mM phosphate, pH 6.8.

The ethanol precipitated RNA was centrifuged, the pellet dried in vacuo and finally suspended in 20 μl of 80% buffered formamide containing 10% sucrose. The solution was layered on top of the inverted gel under a column of 80% buffered formamide. Electrophoresis at 30°C was for about 3 h at 10 V/cm, until the bromphenol blue marker had reached the end of the gel. As internal molecular weight standards, *E. coli* rRNA was co-electrophoresed assuming a molecular weight of 1.1 and 0.56×10^6 D.

TMV-RNA was used as a high molecular weight reference (molecular weight approx. 2×10^6 D; e.g. [8, 33]). Gels were scanned at 260 nm in a Gilford spectrophotometer. Gels containing formamide had to be washed before scanning for at least 2 h in distilled water. Radioactivity was determined in 1.1 mm gel slices after overnight incubation in toluene based scintillation fluid containing 5% NCS by liquid scintillation (see above).

Alkaline hydrolysis of RNA

Samples of RNA were hydrolysed for 20 h in 0.3 N KOH at 37°C. After addition of 60% ice-cold TCA to a final concentration of 10% the material was allowed to precipitate for 30 min at 4°C. The material was washed twice on Whatman GF/C glass fibre filter disks with cold 10% TCA. Counting of adsorbed radioactivity was performed as described above.

Examination of sensitivity of [³H]uridine incorporated into erythrocytes to ribonuclease

Samples of RNA diluted in 1×SSC (pH 7.0) were incubated for 30 min at 37°C after addition of RNase (pancreatic ribonuclease; Boehringer, Mannheim) to a final concentration of 100 µg/ml. Prior to use, the RNase was dissolved in 1×SSC adjusted to pH 5, heated for 10 min to 80°C, cooled and adjusted with NaOH to pH 7.0. After incubation the samples were made 10% with respect to TCA, washed with ice-cold 10% TCA on filters and radioactivity was counted.

Cs₂SO₄-gradient centrifugation

Total nucleic acids, i.e. without DNase-digestion, extracted from mature erythrocytes were centrifuged in Cs₂SO₄ and Cs₂SO₄-DMSO gradients (for details see [61, 65]). The nucleic acids were dissolved in 0.01 M Tris-HCl (pH 7.2) and were adjusted with solid Cs₂SO₄ to a density of 1.550 g cm⁻³. Refractive index-density relations for Cs₂SO₄ gradients which were 10% with respect to DMSO were taken from the study of Williams & Vinograd [65]. Nucleic acid solutions were run at concentrations from 20 to 150 µg per gradient in a WKF-Hitachi ultracentrifuge (ultracentrifuge P 65; Weinkauf, Brandau) at 40 000 rpm and 20°C for 55 h in a 10×12 ml angle head rotor [20]. Gradients were fractionated with an apparatus modified from that originally described by Flamm et al. [20]. The absorption of the 0.2 ml fractions at 260 nm and the radioactivity was determined. The density of the fractions was calculated by measuring the refractive index in a refractometer (Zeiss, Oberkochen).

Isolation of nuclei and chemical determinations

Nuclei were isolated as described by Zentgraf & Franke [66]. Determinations of phospholipids, RNA, DNA, and proteins were performed as indicated in our previous communication [67].

Light and electron microscopy and autoradiography

Labelled or unlabelled erythrocytes were fixed with glutaraldehyde and OsO₄, washed, dehydrated and embedded in Epon 812 as described elsewhere [22, 67]. For light microscopy, 1 µm thick sections were covered with emulsion (Kodak NTB 2 and NTB 3), exposed for 4 to 6 weeks and developed in Kodak D 19. Light micrographs were taken in bright field after staining with toluidine blue (0.25%; Tris-HCl, pH 6.3; 1.5 min). For electron microscopy a modification of the method described by Granboulan [26] was used (for details see [23]). Ultrathin sections from the same blocks as were used for the light microscopic autoradiography were covered with Ilford L4 emulsion and exposed for 12 to 16 weeks. After

developing (Kodak D 19; 3.5 min; 20°C) and fixing, the sections were stained with uranyl acetate (2% aqueous solution) and lead citrate.

Localization of RNA was performed according to the method described by Bernhard [6]. Small pieces from various tissues including bone marrow and leg muscle were fixed with glutaraldehyde (0.5, 1.0, or 3.0%), dehydrated, and embedded in Epon 812 as described [21]. Ultrathin sections were stained with uranyl acetate (1 or 4% aqueous solution; 1 or 2 min) washed and treated with neutral EDTA-solutions (0.2 M) for various times. After rinsing with distilled water the sections were stained with lead citrate (for details of method see also [21]). Electron micrographs were taken with a Siemens Elmiskop 1A or 101 or with a Zeiss EM 10.

RESULTS

Estimation of minimal RNA content of mature hen erythrocytes

The literature contains controversial data as to the amount of RNA present in the nucleus of the avian erythrocyte (ranging from zero to 0.12 pg, cf [17, 19, 41, 67]). A good part of this discrepancy might come from the difficulties in determining small amounts of RNA in the presence of an excess of DNA by colorimetric determinations using the orcinol reaction or by the preferential alkaline hydrolysis according to the Schmidt-Thannhauser scheme. In order to examine the existence of hen erythrocyte RNA and to estimate the minimal amount of RNA present in the average mature erythrocyte we have extracted the total nucleic acids from large numbers of erythrocytes (2.5×10¹⁰ per experiment) and performed determinations by A₂₆₀ and orcinol and diphenylamine reactions (see Material and Methods) after repeated digestions with DNase and re-extractions with the phenol-cresol mixture. The final preparation contained 218 µg of RNA as could be determined colorimetrically, in the presence of only minor quantities of DNA retained, and from the degradation by pancreatic ribonuclease. Having controlled the recoveries of labelled

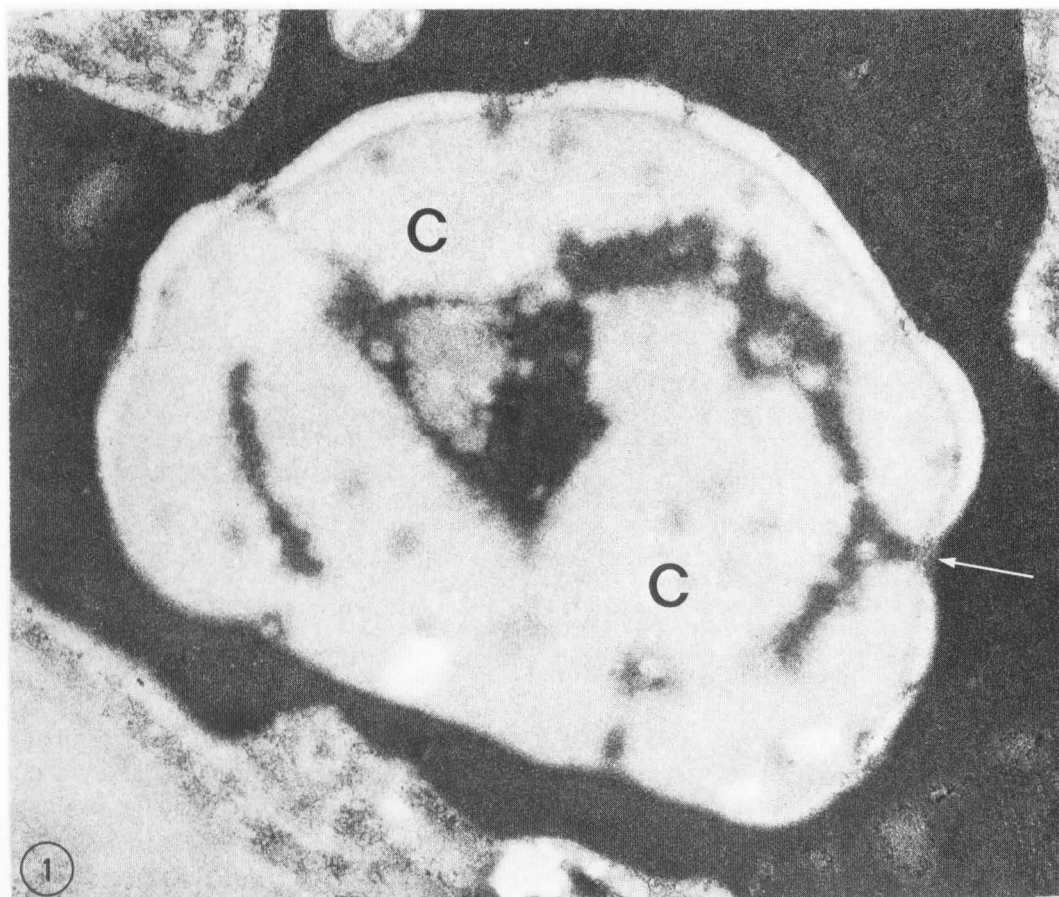


Fig. 1. Electron micrograph of a hen erythrocyte nucleus in a leg muscle capillary as revealed after EDTA-treatment (for details of method, see text). The 'bleached' chromatin areas (C) are penetrated

by deeply stained interchromatinic channels, some of which are directed toward nuclear pores (e.g. at white arrow). $\times 50\ 000$.

RNA and DNA added to aliquots processed in parallel and that of total DNA after each of the individual preparative steps we have found that the minimal RNA content per hen erythrocyte nucleus, and roughly also per total erythrocyte, is from 0.02 to 0.04 pg, corresponding to about 1–2% of the DNA present (for chromatin preparations see also [3, 40]; published figures for DNA contents per hen or chicken erythrocyte nucleus vary from 1.7 to 3.5 pg [5, 17, 63, 67]).

Localization of RNA containing regions

When Bernhard's EDTA staining procedure [6] was applied to hen erythrocytes (fig. 1) the only regions retaining noticeable amounts of uranyl stain were the interchromatinic spaces or 'channels' and the few pore complexes (cf [21]) at which these channels frequently seem to terminate. This staining pattern would be compatible with the localization of the nuclear RNA in the interchromatinic regions. The

Table 1. Incorporation of [^3H]uridine into hen erythrocyte RNA under different assay conditions

Time (min)	In vivo [^3H]Uridine injected into wing vein		In vitro [^3H]Uridine added to freshly prepared blood			In vitro [^3H]Uridine added to erythrocytes which had been washed and suspended in MEM		
	Conc. of precursor (10^6 cpm/ml blood)	Radioactivity incor- porated into TCA- insoluble material (10^3 cpm/ 10^8 cells)	Conc. of precursor (10^6 cpm/ml blood)	Radioactivity incor- porated into TCA- insoluble material (10^3 cpm/ 10^8 cells)	Incorporation relative to that in vivo	Conc. of precursor (10^6 cpm/ml medium)	Radioactivity incor- porated into TCA- insoluble material (10^3 cpm/ 10^8 cells)	Incorporation relative to that in vivo
0	-	-						
30	3.43	5.15	410	135	0.2	110 106 410	0 1 580 6 000	- 10 10
60	2.58	7.20				108	2 270	8
90	2.32	6.72				110	2 580	8
120	2.16	7.11				108	3 170	9
150	2.36	6.47				110	2 230	7
180	2.21	6.26				107	3 000	10

relatively high RNA content of these nuclear zones that contain the 'ribonucleo-protein network' (sensu 14) seems to be well established in other cell types (for review see [14, 60]).

Incorporation of [^3H]uridine into nuclear RNA of mature hen erythrocytes

In accordance with earlier reports [36, 39, 55] we found that radioactive precursors are incorporated into TCA-precipitable RNA of mature hen erythrocytes. The incorporation of [^3H]uridine was increasing for up to 1 h, in some experiments even up to 2 h. It was noted in vivo after intravenous injection of the precursor as well as in vitro after addition of the precursor to freshly prepared blood or after incubating purified and washed erythrocytes in Eagle's MEM containing [^3H]uridine (table 1). When the amounts of incorporation were normalized with respect to the precursor concentration

present it was found that the efficiency was the lowest in the in vitro assay using blood and was highest in the artificial medium (table 1). Absolute rates of incorporation, however, cannot be calculated before determinations of the corresponding intracellular nucleotide pools have been made.

The incorporated radioactivity was stable against 10% TCA and pronase but 99.4% were rendered TCA-soluble after incubation in RNase (100 $\mu\text{g}/\text{ml}$, 30 min, 37°C). 99.7% of the incorporated radioactivity was also hydrolysed during 20 h incubation in 0.3 N KOH at 37°C. When total nucleic acids were extracted from cells incubated in MEM and [^3H]uridine and were centrifuged in Cs_2SO_4 gradients, with and without DMSO present, DNA and RNA were clearly separated (cf [61, 65]). The DNA was identified by its UV-absorbance but very little RNA could be detected (fig. 2). The amount of RNA contained in the com-

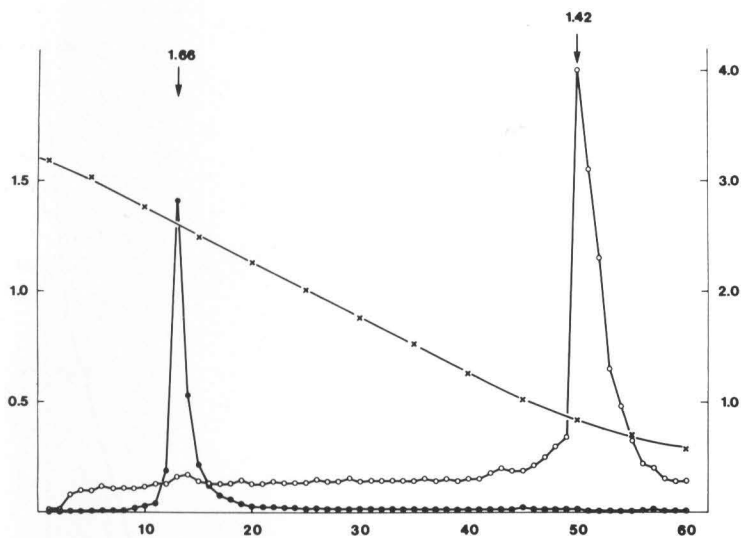


Fig. 2. Abscissa: fraction no.; ordinate: (left) $\text{cpm} \times 10^{-2}$; (right) A_{260} .

Fractionation of nucleic acids extracted from mature hen erythrocytes labelled for 2 h in vivo with 3 mCi $[^3\text{H}]$ uridine in a Cs_2SO_4 gradient. All the radioactive material (●—●) banded at a position char-

acteristic of RNA (1.66 g cm^{-3}). Optical determination at 260 nm (O—O) demonstrates the presence of only very little RNA in this band, in contrast to the large amount of DNA banded with a mean peak density of 1.42 g cm^{-3} . The crosses indicate the slope of the gradient formed.

ponent with a mean density of 1.66 g cm^{-3} was estimated to represent ca 1.5% of the total nucleic acid, in agreement with the corresponding estimation from biochemical determinations mentioned above. The radioactivity incorporated from the $[^3\text{H}]$ uridine, however, was exclusively recovered in the position of the RNA (fig. 2). This also demonstrates that the RNA synthesized was not contained in molecules or fragments stably associated with the hen DNA (see also [31]). When labelled extracted RNA was solubilized and re-precipitated in the presence of 2 M LiCl the major part (up to 90%) of the radioactivity was recovered in a sedimentable form, indicative of its high-molecular weight state. The maximal specific labelling obtained in this purified high-molecular weight RNA after incubation in MEM was approx. $160\,000 \text{ dpm}/\mu\text{g}$ RNA.

Characterization of labelled chicken erythrocyte RNA by gel electrophoresis

When the labelled RNA was applied to agarose gels and subjected to electrophoresis, together with non-radioactive reference RNAs with defined molecular sizes, the bulk of the radioactivity migrated in a position corresponding to molecular weights higher than that of tobacco mosaic virus RNA (fig. 3a). The distribution of the radioactive hen erythrocyte RNA indicated a pronounced heterogeneity in molecular sizes; distinct peaks were not recognized. Gel electrophoresis of this RNA under denaturing conditions, i.e. in 80% formamide containing gels (fig. 3b), revealed a considerable degree of secondary structure as evidenced by the finding that more than three quarters of the RNA migrated faster than the TMV-RNA (fig. 3b). Again, heterogeneity was notable with minor

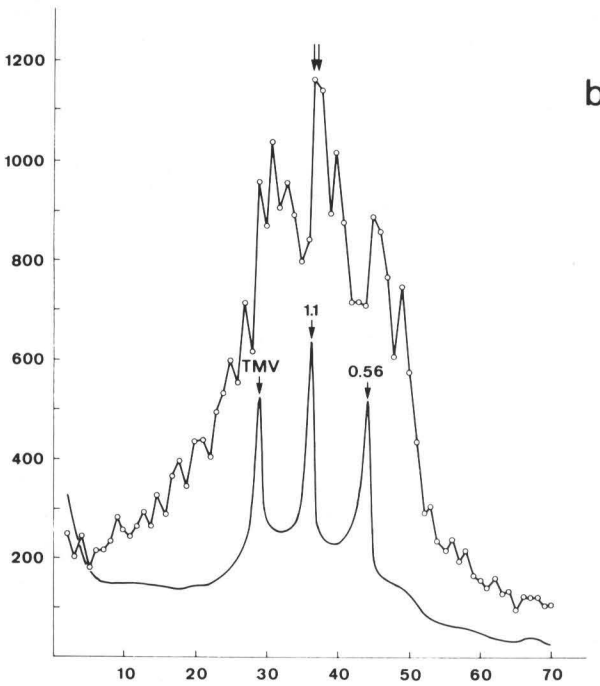
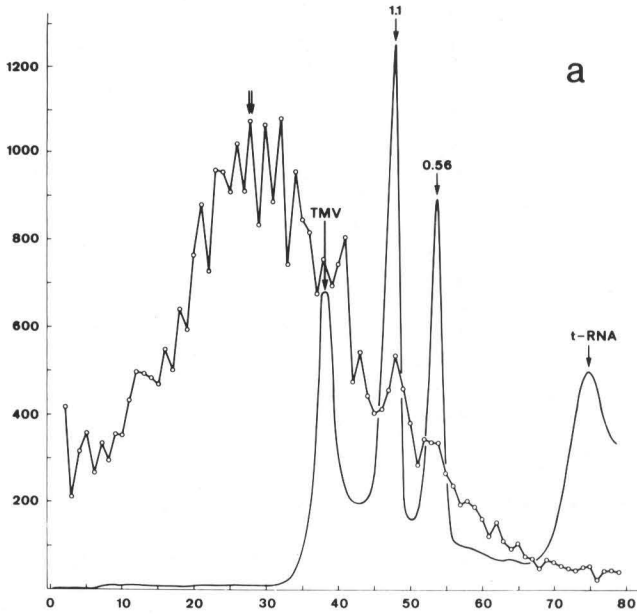


Fig. 3. Abscissa: slice no.; ordinate: cpm.

Separation of [³H]labelled RNA from mature hen erythrocytes on (a) 1% agarose gel; (b) 1% agarose gel containing 80% formamide. Erythrocytes were incubated in vitro for 30 min with 0.25 mCi/ml [³H]uridine in Eagle's essential medium. Unlabelled rRNA isolated from *E. coli* (0.56 and 1.1×10^6 D

molecular weight) and TMV-RNA were used as markers. The double arrows are denoting the most prominent peak. Note the shift of the labelled RNA to positions corresponding to low molecular weights in (b) when compared with gels containing no formamide (a).

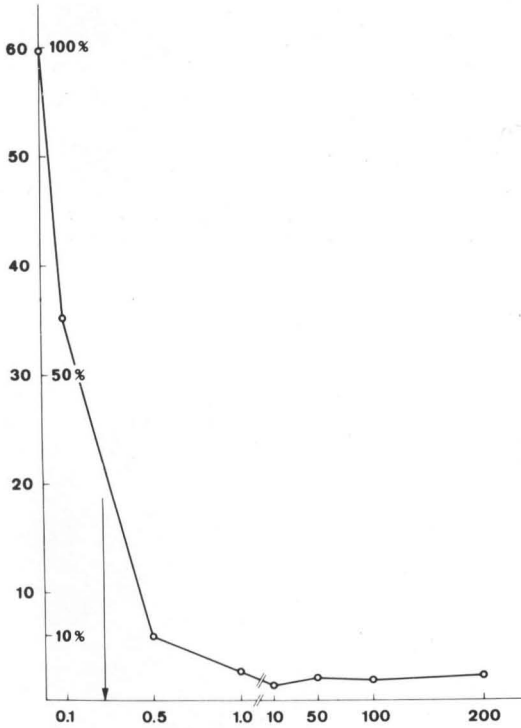


Fig. 4. Abscissa: $\mu\text{g} \times \text{ml}^{-1}$ actinomycin D (AMD); ordinate: $\text{cpm} \times 10^{-3}$ and % cpm.

Inhibition of incorporation of radioactive uridine into RNA at different concentrations of AMD. Mature blood erythrocytes were incubated *in vitro* for 30 min with 0.1 mCi/ml [^3H]uridine in Eagle's minimal essential medium. The arrow indicates the $1/e$ concentration (see text).

peaks appearing at positions corresponding to molecular weights of 1.8, 1.0, and $0.5 \times 10^6 \text{D}$. The electrophoretic pattern of RNA samples precipitated with LiCl was unaltered. The molecular weight distributions shown are in some contrast to those reported by MacLean & Madgwick [36] who reported from similar preparations the appearance of six distinct peaks, three being coincident with the positions of mouse liver rRNAs and *E. coli* tRNA. It should be noted that no peak appeared at a position corresponding to the molecular weight of $2.2 \times 10^5 \text{D}$ determined for the globin chain messenger RNAs [44]. When RNA from cells labelled *in vivo* was puri-

fied, precipitated with LiCl, and analysed by gel electrophoresis an almost identical pattern was found, however, with a maximum of only 42 net cpm/gel slice and a specific radioactivity in the total high-molecular weight RNA sample of as little as 900 dpm/ μg . Quite similar molecular weight distributions of [^3H]uridine labelled RNA as described here in the mature erythrocytes (fig. 3a, b) was obtained with RNA isolated from total cells and isolated nuclei [67] from red blood cell populations that were enriched in erythroblasts by phenyl-hydrazine treatment. The pattern shows a close resemblance to those of nuclear RNAs from avian erythroblasts (cf [38, 53]) and is different from the very low molecular weight RNA which some authors have reported in chicken erythrocyte and erythroblast chromatin [2, 12].

Sensitivity of uridine incorporation to inhibitors

Actinomycin D (AMD) is a template-intercalating, potent inhibitor of transcription [47] which has been shown to penetrate into hen erythrocytes [9]. The effect of AMD on the incorporation of [^3H]uridine into the hen erythrocyte RNA is illustrated in fig. 4. Concentrations higher than $1 \mu\text{g}/\text{ml}$ were almost completely inhibitory. An inhibitory action of $10 \mu\text{g}/\text{ml}$ AMD has also been described in the studies by Madgwick et al. [39] and MacLean & Madgwick [36]. The ' e^{-1} concentration' was about $0.25 \mu\text{g}/\text{ml}$ AMD, a value that is close to the sensitivity reported in L cells to be characteristic for mRNA formation but differs from the sensitivity of the synthesis of rRNA precursors on the one hand and of those for 5s RNA and tRNA on the other hand [45]. Drugs which primarily affect the synthesis of pre-mRNAs by binding to the RNA polymer-

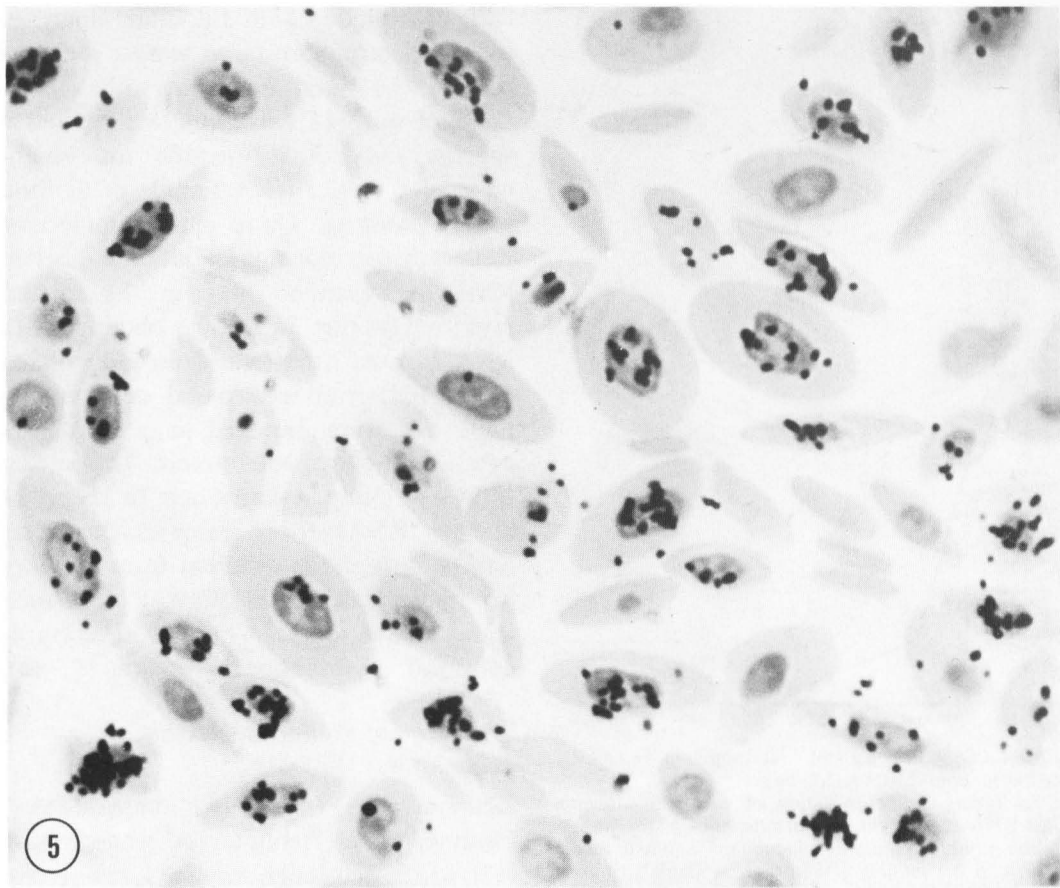


Fig. 5. Light microscopic autoradiograph of a $1\ \mu\text{m}$ section through mature hen erythrocytes from blood after 1 h incubation in vitro with $[^3\text{H}]$ uridine (for de-

tails see text). Most cells are labelled and the majority of the silver grains is located over the nuclei. $\times 2000$.

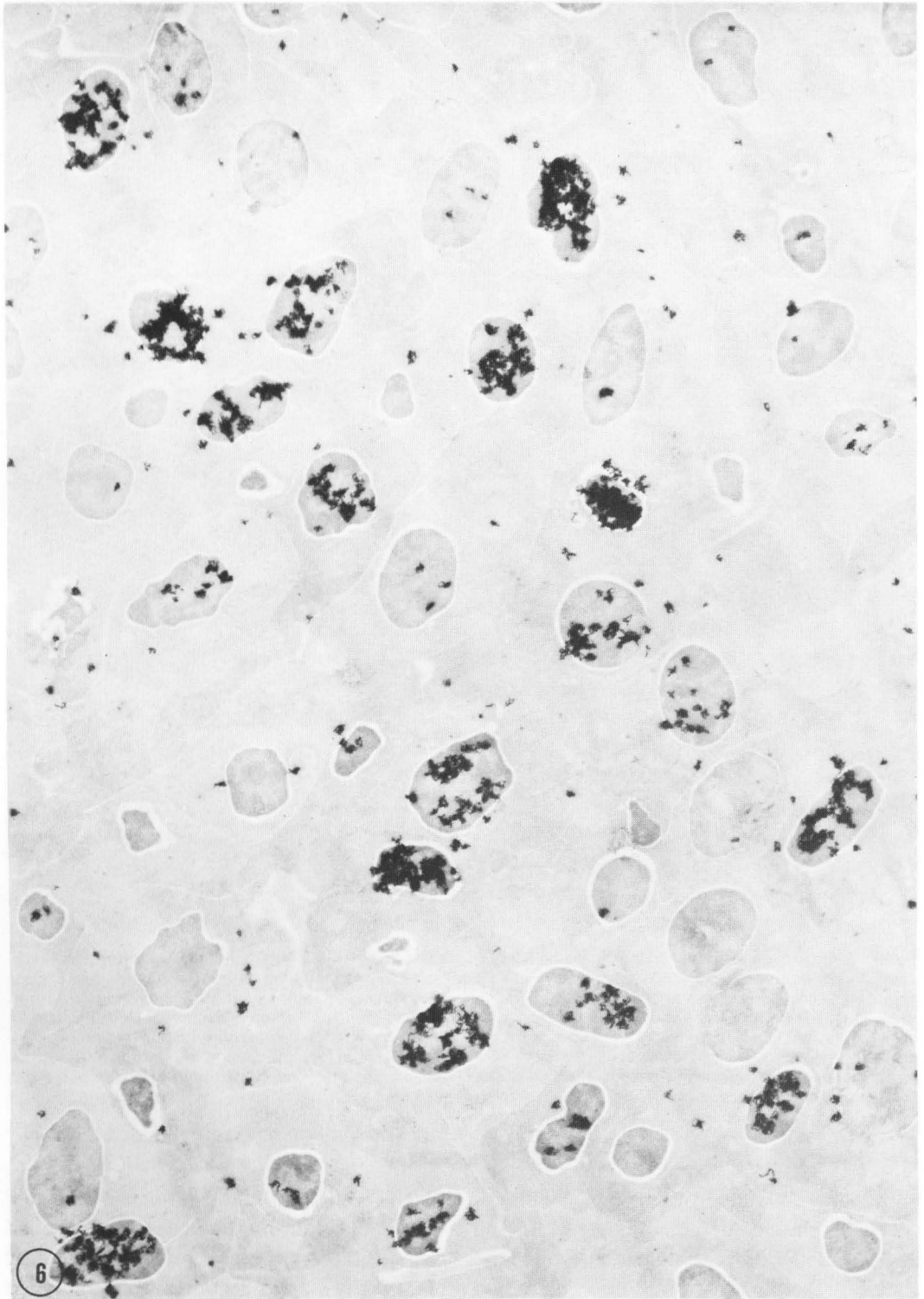
ase B such as α -amanitin and its methyl-derivative [50, 52] reduced the incorporation by about 50% at $100\ \mu\text{g}/\text{ml}$ and resulted in a nearly complete cessation at $400\ \mu\text{g}/\text{ml}$. The effects of both drugs suggest that the observed incorporation of $[^3\text{H}]$ uridine into hen erythrocyte RNA is a template-directed process with characteristics of the transcription of genes coding for precursors of mRNA.

Localization of the $[^3\text{H}]$ labelled RNA

The radioactivity incorporated in erythrocytes was almost exclusively located in

nuclei as demonstrable by light (fig. 5) and electron (fig. 6) microscopic autoradiography, in accord with the observations of Madgwick et al. [39]. The percentage of labelled cells as determined in the two in vitro assays described was somewhat variable from 70 to 95. With

Fig. 6. Electron microscopic autoradiograph of an ultrathin section of tightly pelleted mature hen erythrocytes. The cells had been incubated in vitro for 1 h with $0.25\ \text{mCi}/\text{ml}$ $[^3\text{H}]$ uridine in Eagle's minimum essential medium. The majority of the silver grains is accumulated over the nuclei. Cells with different degrees of labelling occur side by side. $\times 1800$.



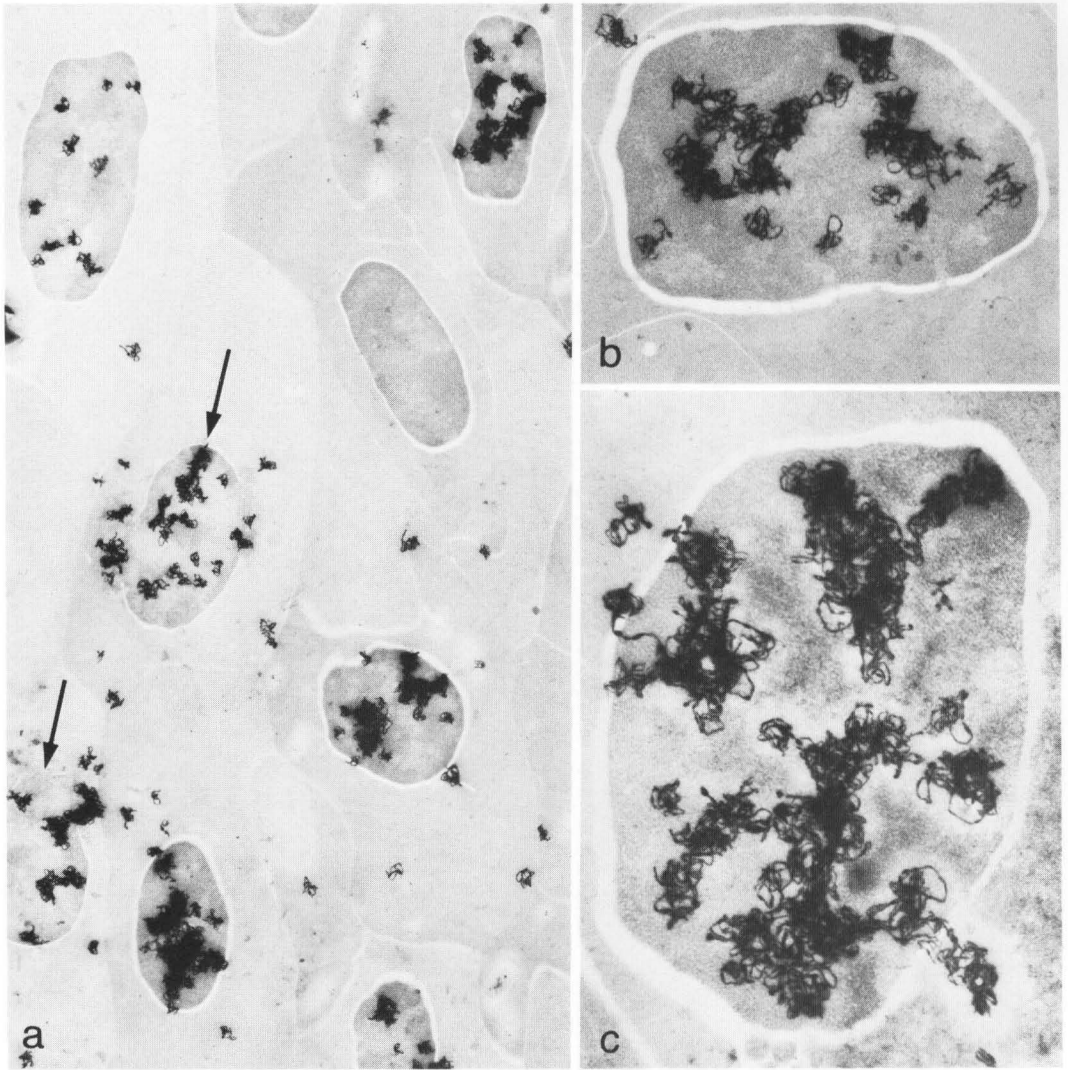


Fig. 7. Electron microscopic autoradiograph of mature hen erythrocytes incubated *in vitro* for 3 h with [^3H]uridine (conditions as described in fig. 6) and pelleted. Most silver grains are located over nuclear areas and cytoplasmic labelling is hardly above background, indicating that considerable amounts of labelled substance have not been transported into the cytoplasm during the incubation period (*a*), (*b*). Cells with strongly labelled, weakly labelled and unlabelled nuclei oc-

cur side by side (*a*). Labelling of the cytoplasm sometimes appears at sites where the nuclear envelope is partly disintegrated; i.e. at artificially induced lesions (arrows in (*a*)). In some nuclei the labelling seems to be restricted to the interchromatinic regions (*a*), (*b*). Occasionally, an enrichment of the silver grains at the periphery of the condensed chromatin areas can also be noted (*b*), (*c*).
 (*a*) $\times 4400$; (*b*) $\times 10600$; (*c*) $\times 18500$.

both light and electron microscopy there was correlation detectable between the degree of chromatin dispersion and the specific labelling. We have no explanation

for the consistent finding of heavily labelled erythrocytes besides completely unlabelled ones (fig. 7 *a*). The extent of cytoplasmic labelling was not significant, even when

the incubation was prolonged for several hours (see fig. 7). We noted, however, that in the course of the incubation an increasing number of nuclei showed breakdown of the nuclear envelope, concomitant with the artificial penetration of chromatin clumps into the cytoplasm (fig. 7*a*; see also [24, 66]); perhaps this does explain the observation reported in [39] of cytoplasmic labelling after 48 h incubation in artificial medium. Frequently, the electron microscopic autoradiographs suggested a non-homogeneous intranuclear labelling. The consistent pattern observed was that interchromatinic regions were heavier labelled and that label appeared to be excluded from the more peripheral blocks of condensed chromatin (e.g. fig. 7).

DISCUSSION

The present results demonstrate that hen erythrocytes not only contain some RNA but also are capable of transcribing some, probably very small, parts of the genome (see also [36, 39]). Both the RNA present and the newly synthesized RNA appears to be confined to the nucleus, nucleocytoplasmic transfer of the synthesized RNA is obviously impaired (fig. 3*b*). This low RNA content and the low level of RNA synthesis, compared with that present in earlier stages of erythropoiesis, might easily have escaped detection in the studies of previous authors (for references see p. 82) and is also compatible with the low level of nucleoside phosphorylation reported by Williams [64]. The synthesized RNA is contained in high molecular weight components and reveals a size distribution typical for heterogeneous nuclear RNA (hn-RNA). This concept would fit with the demonstrated specific sensitivity to AMD and α -amanitin as well as with the

findings of an RNA-polymerase activity in avian erythrocytes that has polymerase B-like characteristics [27, 40, 50, 52]; see, however, [1]. The characteristics of this RNA and its synthesis would be compatible with the idea that the transcriptional activity found in mature erythrocytes is qualitatively, though not quantitatively, related to the transcription of a very small but specific part of the genome described in earlier stages of erythropoiesis as studied in anaemic and embryonic red blood cells [4, 39, 42, 53, 54]. Therefore, one might hypothesize that this RNA represents a mixture of precursors of mRNAs, possibly with an enrichment in those containing the sequences of the globin messages (see above) that are not processed into functional mRNA, and are not transported into the cytoplasm. In this view the RNA synthesis in the hen erythrocyte nucleus would represent a residual activity left over from that present in the reticulocyte and late erythroblast. An alternative which appears less likely to us would be that in these mature cells a totally different type of high molecular weight RNA is synthesized that might function in the process of final cell maturation, perhaps in repressing the expression of other genes as this has been proposed by some authors [10, 13]; see, however, [31]. As to the question whether this RNA is still in transcriptional complexes or otherwise associated with the chromatin a decisive answer cannot be given. The demonstration of a low but significant transcriptional activity in hen and pigeon erythrocyte nuclei and chromatin [25, 62] and preliminary results of the kinetics of incorporation into the RNA synthesized from the labelled precursor pools suggest that the precursors are incorporated into preexisting, slowly growing chains rather

than into newly initiated transcripts. Thus the situation could serve as an indication that the transcriptional complexes remain stably associated with the chromatin fibers even at greatly reduced chain elongation rates, in contrast to the demonstrated release of nascent chains from the template-DNP after complete inhibition of RNA synthesis [51]. On the other hand, the gel-electrophoretic analyses indicate that not all the RNA is contained in nascent chains. Thus, a large part of the nuclear RNA in the mature hen erythrocyte might occur in the form of molecules released from the template, is stored in fibrillar RNP aggregates in the interchromatinic spaces, and is excluded from translocation into cytoplasm by an unknown post-transcriptional control mechanism.

From the present results as well as from the earlier studies quoted it is to be concluded that the reactivation of transcriptional activity as has demonstrated after cell fusion (e.g. [58]) does not start from zero but from a certain reduced 'smouldering' activity with a conserved transcriptional machinery, at least as far as non-nucleolar activity is concerned. It would also be interesting to examine the existence of such residual activity in late stages of spermiogenesis in which low levels of RNA and RNA synthesis as well as active RNA polymerase have been noted [7, 35, 46].

Note added in proof. Recently we found that a significant proportion (18–30%) of the RNA labelled in vitro is bound to poly-(U)-sepharose columns, thus indicating the presence of poly-A regions. Also we have confirmed the autoradiographic localization in the nuclei after labelling in vivo.

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