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SYNTHESIS OF AN ENZYME DETERMINED BY AN ERYTHROCYTE NUCLEUS IN A HYBRID CELL

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SUMMARY

When a chick erythrocyte nucleus is introduced into the cytoplasm of a mutant mouse cell lacking the enzyme inosinic acid pyrophosphorylase, synthesis of the enzyme is induced. However, this synthesis does not begin until the erythrocyte nucleus develops a nucleolus. The kinetics of synthesis of the enzyme are essentially similar to those previously described for the synthesis of surface antigens specified by the chick nucleus. The results of both sets of experiments indicate that the nucleolus plays a critical role in the transfer of information from the genes to the cytoplasm of the cell.

INTRODUCTION

In a previous study (Harris, Sidebottom, Grace & Bramwell, 1969) it was shown that a chick erythrocyte nucleus introduced into the cytoplasm of a mouse cell could eventually determine the synthesis of chick-specific surface antigens in that cell. The erythrocyte nucleus resumed the synthesis of RNA within a few hours of its introduction into the foreign cytoplasm, but chick-specific antigens did not appear on the surface of the cell until the erythrocyte nucleus had developed a visible nucleolus, a process which, in the case of the adult erythrocyte nucleus, required several days. Experiments in which the synthetic activity of the mouse nucleus in the hybrid cell was eliminated by a microbeam of ultraviolet light showed that the failure of the erythrocyte nucleus to determine the synthesis of chick-specific surface antigens before it had developed a nucleolus was due to the inability of the nucleus, at this stage, to transfer the RNA made on its chromosomes to the cytoplasm of the cell. When the erythrocyte nucleus developed a nucleolus, however, the RNA which it made was transferred to the cytoplasm of the cell, and the synthesis of chick-specific surface antigens began.

The present paper describes similar experiments in which the synthesis of a soluble enzyme was examined. In these, as in previous experiments, Littlefield's A9 cell line was used (Littlefield, 1964). These cells were derived from the L strain of mouse fibroblast (Earle, 1943) by progressive selection for azaguanine-resistance. The cells lack the enzyme inosinic acid pyrophosphorylase (IMP: pyrophosphate phosphoribosyl transferase E.C. 2.4.2.8), and are therefore unable to incorporate hypoxanthine into nucleic acids. When, however, a chick erythrocyte nucleus was reactivated

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within the cytoplasm of an A9 cell, inosinic acid pyrophosphorylase activity gradually appeared, and the hybrid cell acquired the ability to incorporate hypoxanthine into nucleic acids. As had previously been found for the species-specific surface antigens, synthesis of the enzyme closely followed the appearance of nucleoli in the erythrocyte nuclei; those hybrid cells in which the erythrocyte nuclei did not develop nucleoli failed to synthesize the enzyme. These experiments strengthen the view that the nucleolus plays a critical role in the transfer of information from the genes to the cytoplasm of the cell.

MATERIALS AND METHODS

Heterokaryons were made from X-irradiated A9 cells and 11- to 14-day chick embryo erythrocytes, as described by Harris *et al.* (1969). The techniques used for cultivating and studying the heterokaryons on coverslips were as described in Harris, Watkins, Ford & Schoefl (1966). Twenty-four hours after cell fusion, and at daily intervals thereafter, several coverslips bearing heterokaryons were exposed for 4 h to tritiated hypoxanthine at a concentration of 2 μC/ml. ([^{3}H]Hypoxanthine, generally labelled, at a specific activity of 634 mC/mM, was obtained from The Radiochemical Centre, Amersham, Bucks.) The preparations were then fixed, extracted and subjected to autoradiography as previously described. The autoradiographs were developed after exposure for periods which gave grain densities suitable for counting.

Direct assay of the enzyme in cell homogenates was carried out as follows. For each assay, about 16×10^6 cells were harvested, washed twice by centrifugation in 0·14 M NaCl at 4 °C and suspended in 10 mM tris-HCl (pH 7·4) at 4 °C. The cells were then frozen and thawed rapidly three times, and the resulting suspension of broken cells spun at 110000 *g* for 1 h at 4 °C. The supernatant was dialysed against 10 mM tris-HCl (pH 7·4) at 4 °C for 16 h and stored at −20 °C. All assays were made within 3 days, during which the enzyme activity remained unchanged. The protein content of the cell extracts was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). Since tris-HCl interfered slightly with this estimation, 10 mM tris-HCl (pH 7·4) was incorporated into the standards.

The enzyme assay was a modification of Littlefield's procedure (Littlefield, 1963). Inosinic acid formed from [^{14}C]hypoxanthine by the enzyme was separated from unutilized substrate by adsorption on to DEAE paper: subsequent washing of the paper removed all the hypoxanthine, but left the inosinic acid. This technique permitted the detection of lower levels of enzyme activity than methods previously described.

The incubation mixture, in a final volume of 45 μl, contained the following: 8 mμmoles of hypoxanthine-8-[^{14}C] (obtained from The Radiochemical Centre, Amersham, Bucks., at a specific activity of 19·5 mC/mM), 50 mμmoles of 5-phospho-ribosyl pyrophosphate (obtained from Calbiochem, Los Angeles, California, U.S.A., as the magnesium salt), 500 mμmoles of $MgCl_2$, 5000 mμmoles of tris-HCl (pH 7·4). The protein concentration was between 0·1 and 1·0 mg/ml. The incubation temperature was 21 ± 1 °C. The reaction was started by the addition of 5-phospho-ribosyl

pyrophosphate and stopped by the addition of 2 μmoles of EDTA (pH 7·4) at 4 °C. 42·2 μl of the incubation mixture were applied to a 2 cm DE 81 filter disk (Whatman), which was then allowed to dry. The filter disk was applied to a second disk and washed on a Buchner filter with 50 ml of 4mM $(NH_4)HCO_3$ followed by 500 ml of distilled

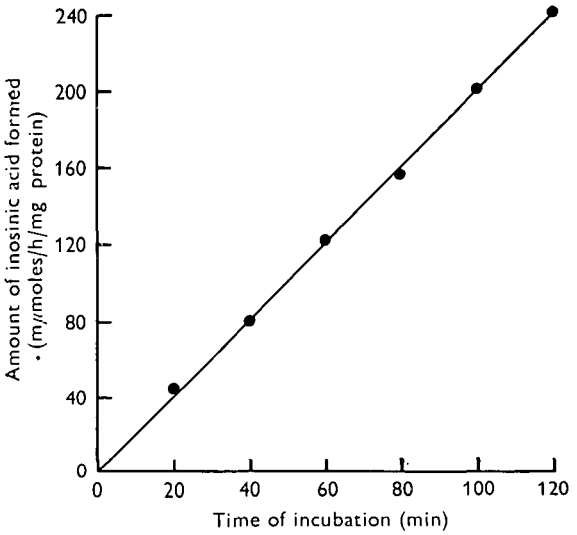

Fig. 1. Calibration of the assay for inosinic acid pyrophosphorylase activity in a homogenate of Ehrlich ascites tumour cells. The formation of inosinic acid is linear with time.

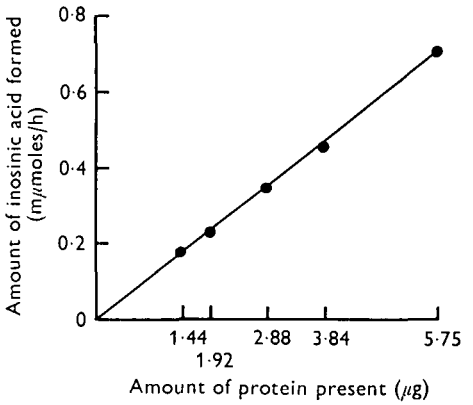

Fig. 2. Calibration of the assay for inosinic acid pyrophosphorylase activity in a homogenate of Ehrlich ascites tumour cells. The relationship between the amount of inosinic acid formed and the concentration of protein is linear.

water at a rate of 100 ml/min. The disks were dried and their radioactive content measured in a Nuclear-Chicago liquid-scintillation system 725 (Nuclear-Chicago Corporation, Des Plaines, Illinois, U.S.A.). Essentially no radioactivity was bound to the disk when 5-phospho-ribosyl pyrophosphate was omitted from the incubation mixture. Figure 1 shows that the rate of the enzymic reaction was constant, and Fig. 2 shows that

this rate was proportional to the enzyme concentration. The reaction rate was not altered by increased concentrations of hypoxanthine-8-[^{14}C], 5-phospho-ribosyl pyrophosphate, $MgCl_2$ or tris-HCl. Separation of inosinic acid and hypoxanthine from other nucleotides and bases by a modification of the method of Katz & Comb (1963) confirmed that inosinic acid was the only radioactive reaction product produced by the cell extract from [^{14}C]hypoxanthine. Assay of the extract for nucleotidase activity showed that this was negligible.

OBSERVATIONS

Incorporation of hypoxanthine into A9 *cells*

Autoradiographs of X-irradiated A9 cells which had been exposed to tritiated hypoxanthine showed a level of labelling which was slightly, but significantly, greater than background. Occasional cells showed heavier labelling than the majority. The

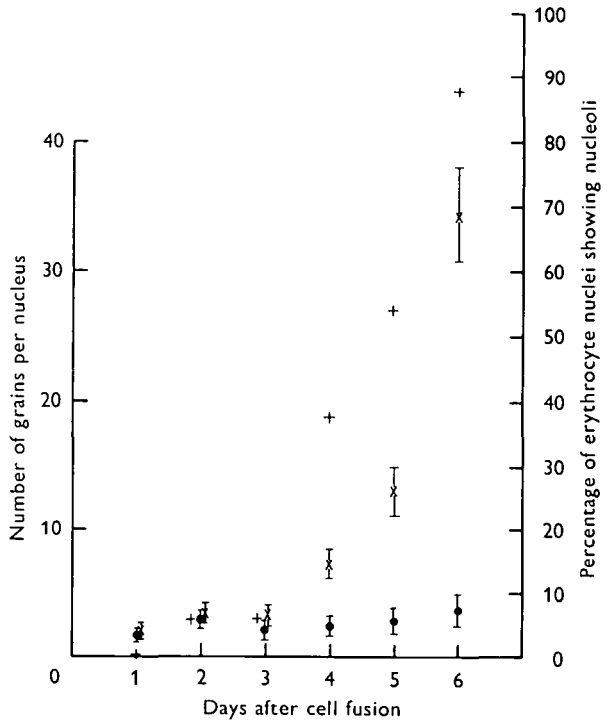

Fig. 3. The development of inosinic acid pyrophosphorylase activity in A9-chick erythrocyte dikaryons, as measured by their ability to incorporate tritiated hypoxanthine into nucleic acid. The incorporation of hypoxanthine in the dikaryons is initially only marginally greater than that in A9 cells, but when the erythrocyte nuclei develop nucleoli, this incorporation increases markedly. ●, A9 cells; ×, dikaryons; +, erythrocyte nuclei showing nucleoli.

origin of this low level of labelling in the A9 cells is obscure. Direct assay of a homogenate of the cells failed to detect any inosinic acid pyrophosphorylase activity. It is possible that some degradation of hypoxanthine may occur in the cultures,

liberating tritium which can be incorporated into nucleic acids in trace amounts by other pathways. It is also possible, since the autoradiographic technique is more sensitive than the direct enzyme assay, that trace amounts of the enzyme are present in the cells, but fail to be detected in the direct assay. Since the X-irradiated nuclei enlarge on cultivation, autoradiographs show a slight increase in the number of grains per nucleus. This effect is shown in Fig. 3. If, however, the incorporated tritium is scored as the number of grains per unit area of the nucleus, the degree of labelling is found to remain constant. This indicates that the ability of X-irradiated A9 cells to incorporate tritiated hypoxanthine into nucleic acids does not increase on cultivation.

Incorporation of hypoxanthine into A9*-chick erythrocyte heterokaryons*

Direct assay of a homogenate of the erythrocytes revealed that they contained inosinic acid pyrophosphorylase: approximately 1·2 mμmoles of inosinic acid/h/mg protein were formed from hypoxanthine. During the process of cell fusion, however, haemolysis of the erythrocytes (Schneeberger & Harris, 1966) released most of this enzyme into the medium, so that only small amounts were incorporated into the heterokaryons.

The experiment recorded in Fig. 3 shows that the ability of A9-chick erythrocyte

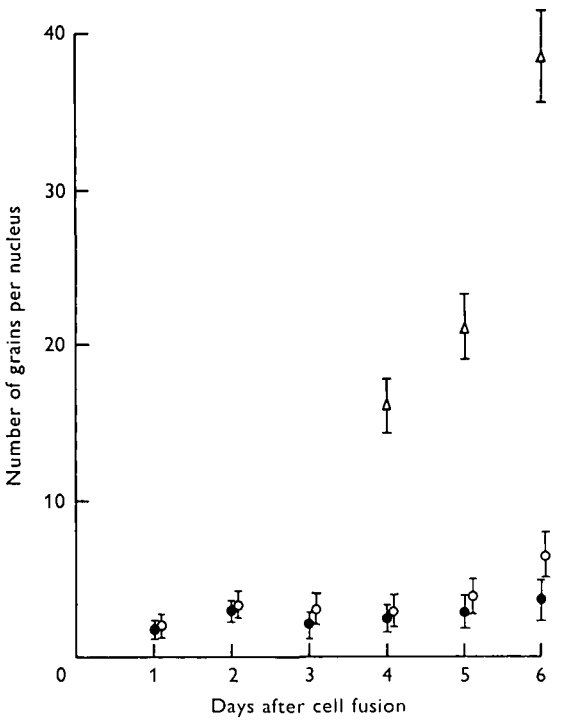

Fig. 4. A comparison between A9-chick erythrocyte dikaryons in which the erythrocyte nuclei have developed nucleoli and those in which they have not. The former show marked increase in their ability to incorporate hypoxanthine; the latter are not much different from A9 cells. ●, A9 cells; △, erythrocyte nuclei showing nucleoli; ○, erythrocyte nuclei not showing nucleoli.

dikaryons to incorporate hypoxanthine was initially only marginally greater than that of A9 cells, and it changed little for 3 days after cell fusion. During this period the erythrocyte nuclei underwent enlargement and resumed the synthesis of RNA, but very few of them at this stage showed discrete nucleoli under the light microscope. On the 4th day, however, there was a sharp increase in the proportion of erythrocyte nuclei showing nucleoli and in the ability of the heterokaryons to incorporate hypoxanthine. On the 5th and 6th days the number of erythrocyte nuclei showing nucleoli and the ability of the heterokaryons to incorporate hypoxanthine continued to rise *pari passu*. In Fig. 4 dikaryons in which the erythrocyte nuclei had developed nucleoli are compared with those in which the erythrocyte nuclei had not yet done so. It will be seen that from the 4th day onward those dikaryons in which the erythrocyte nuclei had developed nucleoli showed a progressive increase in their ability to incorporate hypoxanthine, whereas, in the same sample, those in which the erythrocyte nuclei had not yet developed nucleoli differed little from A9 cells alone. Towards the end of the experiment there appeared to be a slight increase in labelling in some dikaryons in which the erythrocyte nuclei did not yet show visible nucleoli. This observation presents no difficulty, since some development of the nucleolar apparatus occurs before the nucleolus becomes visible as a discrete structure under the light microscope.

The values plotted in Figs. 3 and 4 represent grain counts over the A9 nuclei in the dikaryons. Essentially similar results were obtained for counts over the erythrocyte nuclei. Before the development of nucleoli, the erythrocyte nuclei failed to incorporate hypoxanthine into nucleic acids in more than trivial amounts; when they developed nucleoli, their ability to incorporate hypoxanthine rose sharply and continued to increase with time.

Figures 6–8 show autoradiographs of cells from a 4-day culture exposed for 4 h to tritiated hypoxanthine. Figure 6 shows an X-irradiated A9 cell. Figure 7 shows an A9-chick erythrocyte dikaryon in which the erythrocyte nucleus has enlarged but has not yet developed a discrete nucleolus. The labelling of this cell is not significantly different from that of the A9 cell. Figure 8 shows a dikaryon in which the erythrocyte nucleus has developed a discrete nucleolus. Both the A9 nucleus and the erythrocyte nucleus are now heavily labelled.

Figure 5 shows the development of inosinic acid pyrophosphorylase activity in the heterokaryons as measured by direct assay of the cell homogenate. In this experiment a low level of enzyme was detected in the heterokaryons directly after cell fusion. The amount of enzyme present fell during the first 2 days of cultivation and none could be detected on the 3rd day. On the 4th day, however, when appreciable numbers of the erythrocyte nuclei began to show nucleoli, the level of enzyme rose sharply and continued to rise as increasing numbers of erythrocyte nuclei developed nucleoli.

These experiments establish that the chick erythrocyte nucleus does induce the synthesis of inosinic acid pyrophosphorylase in the hybrid cell, but they do not prove that the enzyme formed is chick inosinic acid pyrophosphorylase. While this is very likely, the activity of the chick erythrocyte nucleus might also induce the resumption of synthesis of the mouse enzyme. The nature of the genetic defect in the A9 cell is not known, and it cannot be assumed that the defect necessarily involves a mutation

in the structural gene for inosinic acid pyrophosphorylase. Attempts to distinguish between mouse and chick inosinic acid pyrophosphorylase by kinetic measurements and inactivation studies have not so far been successful; other approaches are now being tried. It is, in any case, clear that the erythrocyte nucleus does not determine the synthesis of the enzyme, whether chick, mouse or both, until the nucleus develops a nucleolus.

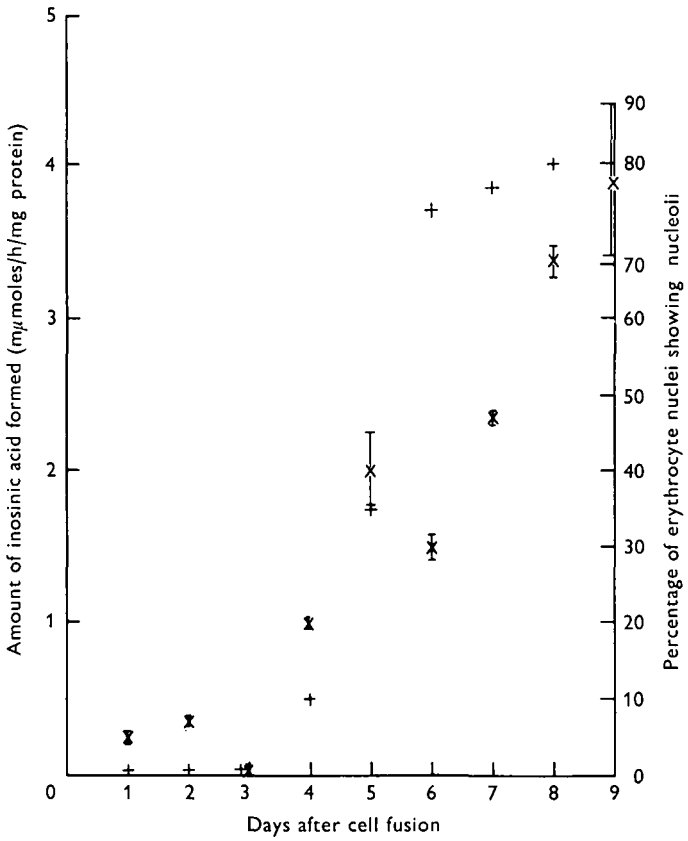

Fig. 5. The development of inosinic acid pyrophosphorylase activity in A9-chick erythrocyte heterokaryons as measured by direct assay of the cell homogenate. There is a marked increase in enzyme activity when the erythrocyte nuclei in the heterokaryon develop nucleoli. ×, Inosinic acid pyrophosphorylase activity; +, erythrocyte nuclei showing nucleoli.

The effect of gene dosage on the synthesis of the enzyme

Measurements were made to see whether the appearance of inosinic acid pyrophosphorylase in the heterokaryon was influenced by the number of erythrocyte nuclei in the cell. It was found that, irrespective of the number of erythrocyte nuclei present, the ability of a heterokaryon to incorporate hypoxanthine remained negligible so long as the erythrocyte nuclei had not developed nucleoli (Fig. 9). Nucleoli commonly began to appear in all the erythrocyte nuclei in a heterokaryon at about the same time, and, when this occurred, there was, once again, a sharp increase in the ability of the

cell to incorporate hypoxanthine (Fig. 10). Cells containing larger numbers of erythrocyte nuclei thus behaved in essentially the same way as dikaryons. Grain counts were made over the A9 nuclei of heterokaryons in samples taken on the 4th, 5th and 6th days after cell fusion: cells containing from 1 to 8 erythrocyte nuclei and variable numbers of A9 nuclei were examined. At no time was any statistically significant difference found, in any one sample, between the heterokaryons which contained only 1 erythrocyte nucleus and those which contained higher numbers.

In order to eliminate any influence which might result from variation in the number of A9 nuclei in the cell, a comparison was also made between heterokaryons containing 1 A9 nucleus and 1 erythrocyte nucleus and those containing 1 A9 nucleus and 2 erythrocyte nuclei. It was found that, after the first 3 days, labelling of the A9 nucleus in the heterokaryons which contained 2 erythrocyte nuclei was consistently about 30% lower than that in the heterokaryons which contained only 1 erythrocyte nucleus. This paradoxical effect is probably due to the fact that as the number of erythrocyte nuclei introduced into a single A9 cell is increased, so the speed with which these erythrocyte nuclei become reactivated and develop nucleoli is reduced. At any one time the erythrocyte nuclei in A9-erythrocyte dikaryons were, on average, larger and had more prominent nucleoli than the erythrocyte nuclei in cells containing 1 A9 nucleus but more than 1 erythrocyte nucleus. This disparity persisted to some extent even in samples taken on the 10th day after cell fusion, when virtually all erythrocyte nuclei were active and contained well developed nucleoli.

It thus appears that the rate of synthesis of the inosinic acid pyrophosphorylase in the heterokaryon is not noticeably affected by increasing the number of erythrocyte nuclei introduced into the cell. Any effect which might be produced by increased gene dosage in this situation appears to be counteracted by more complex regulatory phenomena.

DISCUSSION

The present experiments and those previously described show that a chick erythrocyte nucleus reactivated in a mouse cell can determine the synthesis not only of a surface antigen, but also of a soluble enzyme. The kinetics of synthesis of the soluble enzyme are essentially similar to those previously described for the surface antigen. In both cases there is a lag period which is determined by the time required for the development of a nucleolus within the erythrocyte nucleus: both antigen and enzyme are formed when the nucleolus appears. Since the antigen and the enzyme are neither structurally nor functionally related, their simultaneous appearance makes it probable that similar kinetics will be observed for other proteins. It is, in any case, unlikely that a substantial synthesis of any chick protein will be found before the development of the nucleolus in the erythrocyte nucleus, since it has been shown that, at this stage, very little, if any, of the RNA made on the chromosomes of the erythrocyte nucleus is transferred to the cytoplasm of the cell (Harris *et al.* 1969; Sidebottom & Harris, 1969). The fact that the surface antigen appears in the hybrid cell at about the same time as the soluble enzyme also suggests that any interval

which might elapse between the synthesis of the antigen and its appearance on the surface of the cell is likely to be short relative to the time scale over which these experiments are carried out.

It was concluded from the experiments on the synthesis of the surface antigen that transcription of the genes and synthesis of the corresponding proteins were not mandatorily, or even closely, coupled in mammalian cells (Harris *et al.* 1969). The observations on the synthesis of the enzyme strengthen this conclusion. In heterokaryons in which a chick erythrocyte nucleus is reactivated, autoradiographs show that the RNA synthesized during the 2 or 3 days which precede the development of the nucleolus is made all over the nucleus; and sedimentation studies on this RNA indicate that it is of high molecular weight (Harris *et al.* 1969). It can therefore be assumed that, during this period, many chick genes must be transcribed. But it is not until nucleoli develop that any detectable amount of RNA is transferred from the chick nuclei to the cytoplasm of the cell and any detectable amount of antigen or enzyme is synthesized.

These findings argue against any model for the transfer of information from nucleus to cytoplasm which postulates that the RNA carrying the information for protein synthesis diffuses from the nucleus into the cytoplasm and there attaches to pre-existing ribosomes; and they also argue against any model which postulates the passage of cytoplasmic ribosomes into the nucleus in order to release this RNA from the genes or serve as a vehicle for its transport to the cytoplasm. The chick erythrocyte nucleus in the heterokaryon does not begin to transfer detectable amounts of RNA to the cytoplasm of the cell, and does not initiate the synthesis of any antigen or enzyme, until it develops its own nucleolus and begins to synthesize its own 28s RNA (and presumably its own ribosomes). The pre-existing ribosomes in the cytoplasm of the hybrid cell cannot encompass the transfer of genetic information from the anucleolate erythrocyte nucleus.

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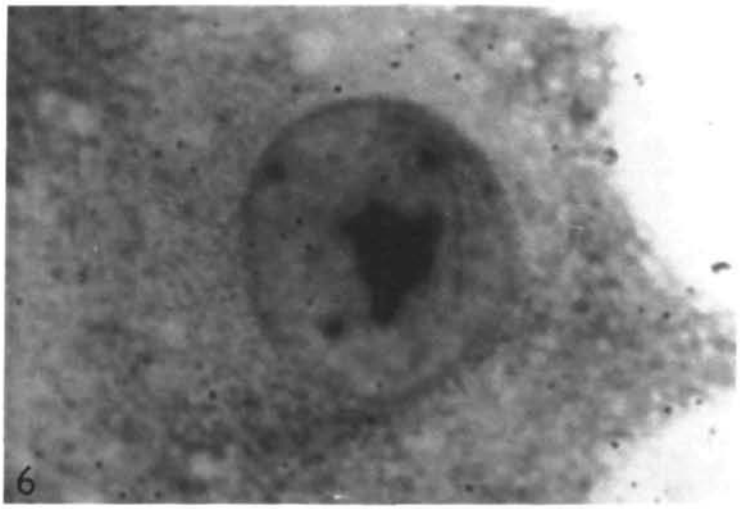
Fig. 6. Autoradiograph of an X-irradiated A9 cell from a 4-day culture exposed for 4 h to tritiated hypoxanthine. There is very little incorporation of the label.

Fig. 7. Autoradiograph of an A9-chick erythrocyte dikaryon from the same preparation as the cell shown in Fig. 6. Although the erythrocyte nucleus has been reactivated, it has not yet developed a nucleolus, and the labelling of the cell is not much different from that seen in A9 cells alone.

Fig. 8. Autoradiograph of an A9-chick erythrocyte dikaryon from the same preparation as the cells shown in Figs. 6 and 7. In this cell, however, the erythrocyte nucleus shows early development of the nucleolus, and both the A9 and the erythrocyte nucleus are clearly labelled. The cell has acquired the ability to incorporate tritiated hypoxanthine.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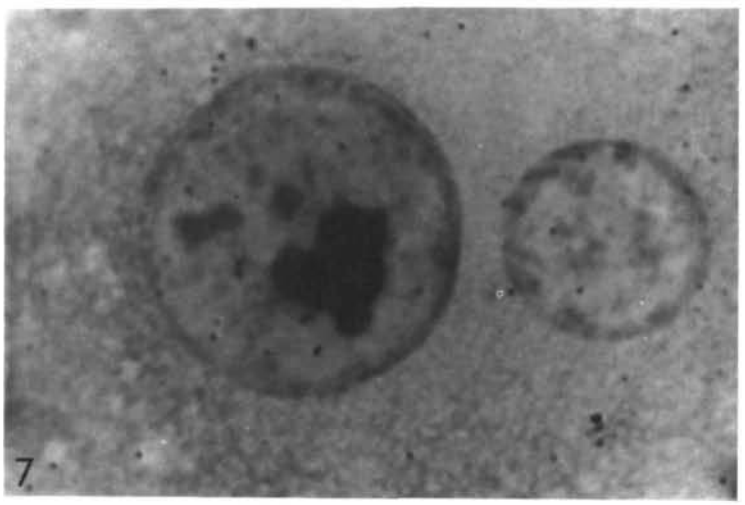
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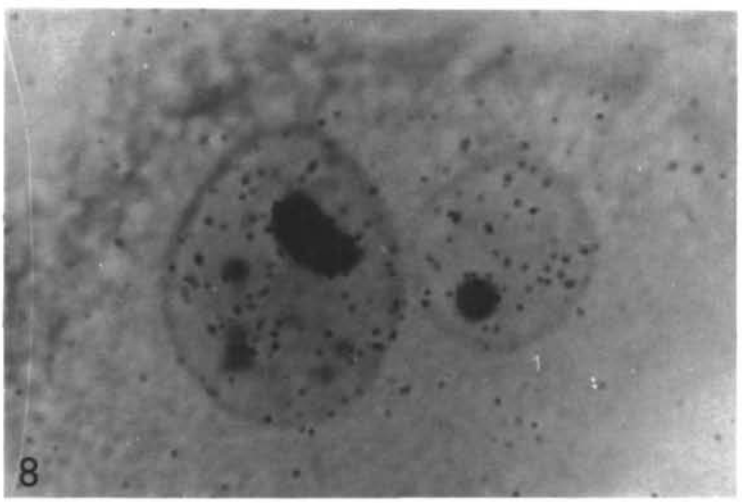
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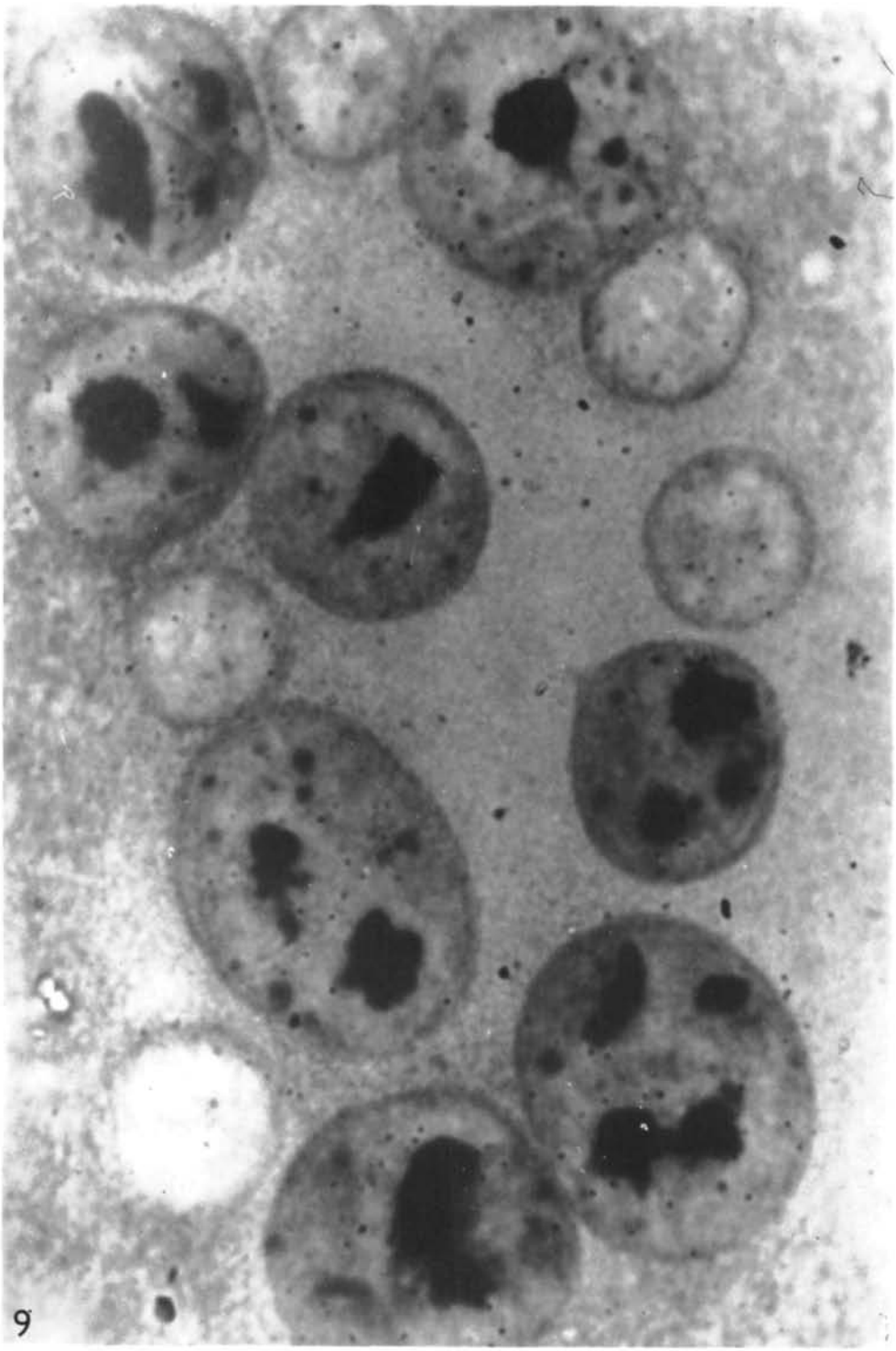

Fig. 9. A heterokaryon containing several A9 and chick erythrocyte nuclei, from a 4-day culture exposed for 4 h to tritiated hypoxanthine. The erythrocyte nuclei have been reactivated but have not yet developed nucleoli. There is little incorporation of the label.

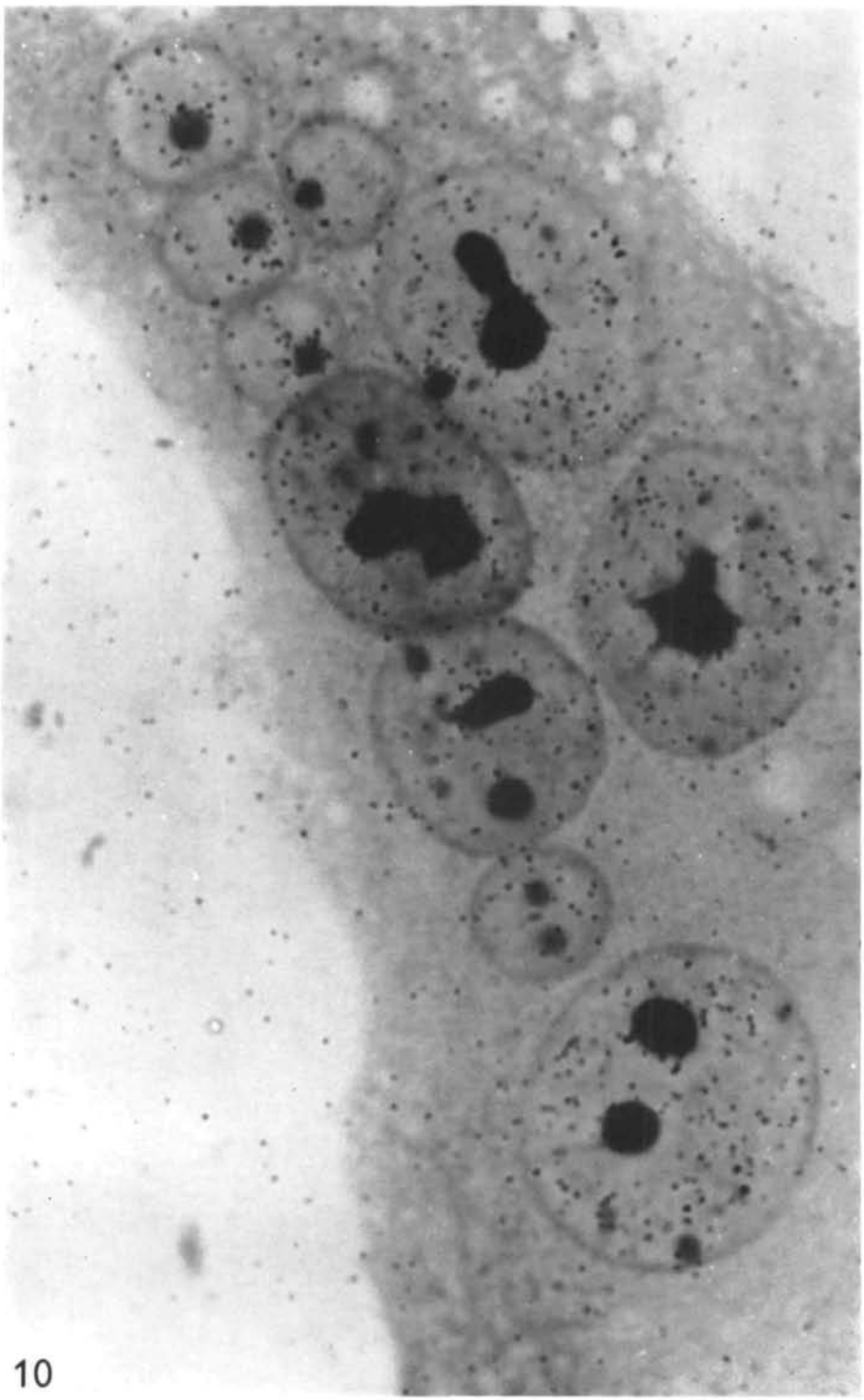

Fig. 10. A heterokaryon containing several A9 and chick erythrocyte nuclei, from the same preparation as the cell shown in Fig. 9. In this case, however, the erythrocyte nuclei show early development of nucleoli, and all the nuclei are clearly labelled.