CHANGES IN THE CYTOCHEMICAL PROPERTIES OF ERYTHROCYTE NUCLEI REACTIVATED BY CELL FUSION

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SUMMARY

When the nucleus of a chick erythrocyte is introduced into the cytoplasm of a HeLa cell it resumes the synthesis of RNA and DNA. This reactivation of the red cell nucleus is associated with an increase in volume and with changes in nuclear composition. These changes have now been studied by quantitative cytochemical techniques. During the process of reactivation the dry mass of the erythrocyte nucleus shows a marked increase which takes place largely before the replication of the DNA begins. Within 24 h of cell fusion, some erythrocyte nuclei already contain an increased amount of DNA, and 48 h after fusion many of them contain twice the normal diploid amount, thus indicating that they have replicated their DNA completely. The physical properties of the nuclear deoxyribonucleoprotein complex also change. The ability of the nuclear chromatin to bind acridine orange increases 4- to 5-fold well before the synthesis of DNA begins; and changes in the melting profile of the deoxyribonucleoprotein suggest that its structure is loosened. This view is also supported by the observation that the reactivity of the erythrocyte nuclei to the Feulgen stain is altered during the early stages of reactivation.

INTRODUCTION

It has been shown that when, under the influence of inactivated Sendai virus, the nucleus of a hen erythrocyte is introduced into the cytoplasm of a HeLa cell, the previously dormant erythrocyte nucleus resumes the synthesis of RNA and DNA (Harris, 1965). This reactivation of the red cell nucleus is associated with a marked increase in its volume and with the dispersion of the previously condensed chromatin within it (Harris, 1967). These changes have now been examined by quantitative cytochemical techniques, and the findings are reported in the present paper.

MATERIALS AND METHODS

Origin of the cells

The HeLa cells were obtained from stock suspension cultures. The technique of suspension culture and the media used have been described (Harris & Watts, 1962). In previous experiments on the behaviour of erythrocyte nuclei in heterokaryons, mature hen erythrocytes from the peripheral blood of adult birds were used. It was subsequently discovered that erythrocytes from embryonic blood fused with other cells much more readily than adult erythrocytes, and most of the measurements
described in the present paper were made on erythrocytes obtained from the blood of 12- to 15-day embryos. The allantoic vessels were cut and allowed to bleed into the allantoic fluid. The erythrocytes were then separated from the allantoic fluid by centrifugation and washed twice in phosphate-buffered saline (Dulbecco & Vogt, 1954).

Formation of heterokaryons

Heterokaryons were made by treating a mixture of the two cell types with Sendai virus inactivated by ultraviolet light (Harris, Watkins, Ford & Schoefl, 1966). A suspension containing $5 \times 10^6$ HeLa cells and $4 \times 10^7$ erythrocytes was treated with 250 haemagglutinating units of inactivated virus. The yield of heterokaryons varied somewhat from experiment to experiment, but in the best preparations up to 60% of all cells in the resulting population contained at least one erythrocyte nucleus (Fig. 10).

Preparation of heterokaryon cultures for cytochemical measurements

The heterokaryons were maintained as described by Harris et al. (1966) on haemocytometer coverglasses or on specially designed quartz slides. The preparations were rinsed in phosphate-buffered saline before being fixed in a mixture of ethanol and acetone in equal parts. The fixed preparations were flown from Oxford to Stockholm, where the cytochemical measurements were made.

Preparation of controls

Three control preparations were also examined: erythrocytes alone, erythrocytes treated with virus by the procedure used for making heterokaryons, and a mixture of HeLa cells and erythrocytes without added virus. The erythrocytes, either before or after treatment with virus, were allowed to settle out on the slides or coverslips. After the lapse of 2 h, during which the erythrocytes adhered to the glass, the preparations were rinsed with phosphate-buffered saline and fixed in ethanol–acetone. HeLa cells from the suspension cultures were grown on the slides or coverslips overnight. The erythrocytes were then deposited on to these HeLa cell cultures in fresh medium, and the preparations were again rinsed and fixed 2 h later.

Preparation of isolated nuclei

Nuclei were isolated from the heterokaryons by a modification of the technique of Fisher & Harris (1962). A greater shear stress was applied than that used originally, and enucleation was complete within 4 min. The isolated nuclei were spun out of the enucleation medium and resuspended in a salt solution designed to have an electrolyte composition similar to that of cytoplasmic water, as calculated from the data of Langendorf et al. (1961). The nuclei were allowed to settle on to the slides or coverslips and, at room temperature, adhered to the glass within 15 min. These preparations with adherent nuclei were also fixed in ethanol–acetone. Nuclei obtained from HeLa cells by this technique have been shown by electron microscopy to be free from adherent cytoplasm (Crawley & Harris, 1963). The nuclei isolated from heterokaryons were also free from adherent cytoplasm as judged by ultraviolet microscopy and fluorescence microscopy after acridine-orange staining. The erythrocyte nuclei could
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Easily be identified by their smaller size and by the absence of well-developed nucleoli. In one experiment the heterokaryons were exposed to tritiated thymidine at a concentration of 2 μCi/ml for 2 h before being enucleated. (Thymidine-6[3H], at a specific activity of 13c/mm, was obtained from the Radiochemical Centre, Amersham.)

Nuclei were also isolated from erythrocytes by the same technique, and by a technique devised by N. R. Ringertz & L. Bolund, in which the erythrocytes were lysed by the detergent 'Nonidet P40' (Shell Company) in the presence of small amounts of serum protein. Details of this technique will be published later.

Cytochemical procedures

Feulgen reaction. This was carried out as described by Gledhill, Gledhill, Rigler & Ringertz (1966). The amount of Feulgen stain in the nuclei was determined by scanning and integrating microspectrophotometry at 546 μm. The instrument described by Lomakka (1965) was used.

Ultraviolet absorption. The ultraviolet absorption of the nuclei was measured at 265, 280 and 315 μm with the same instrument. The absorption at 315 μm was insignificant and no correction was therefore made for non-specific light loss.

Acridine-orange binding. The capacity of the nuclear chromatin to bind acridine-orange was analysed by microfluorimetry at 530 and 590 μm (λexc = 365 μm) after the preparations had been stained by the method of Rigler (1966). The instrument used for microfluorimetry (Caspersson, Lomakka & Rigler, 1965) was equipped with an adjustable diaphragm which made it possible to limit the field of measurement to individual nuclei within intact heterokaryons.

Melting profile of deoxyribonucleoprotein

The effect of heat on the stability of the nuclear deoxyribonucleoprotein complex was studied by the technique of Rigler, Killander, Bolund & Ringertz (1968). This technique involves microfluorimetric measurements on individual nuclei which have been heated to various temperatures in a medium containing formaldehyde and then stained with acridine-orange. The formaldehyde prevents renaturation of the deoxyribonucleoprotein when it is cooled. The decrease in the amount of DNA present in the double stranded form and the increase in the amount present in the single stranded form were analysed by microfluorimetric measurements of the green (double stranded) and red (single stranded) acridine-orange fluorescence at 530 and 590 μm respectively (Rigler, 1966). Slides bearing HeLa and erythrocyte nuclei which had been isolated from heterokaryons or nuclei which had been isolated from erythrocytes were incubated for 20 min at 22, 60, 70, 75, 80, 85, 90, 95 or 100 °C in 0.15 M sodium chloride, 0.015 M sodium citrate and 4 % (v/v) formaldehyde at pH 7.0. The slides were then rapidly transferred to an ice-cold solution containing 0.15 M sodium chloride and 0.015 M sodium citrate at pH 7.0. About 1 h later the slides were dehydrated in 30, 50, 70, 90, 96 and finally 100 % ethanol. The preparations were stored in absolute ethanol at 4 °C until they could be stained. They were then rehydrated, stained with acridine-orange and immediately analysed by microfluorimetry. The details of the technique for determining the melting profile of the chromatin in individual nuclei and the methods
for calculating the degree of single strandedness of the DNA from measurements of ultraviolet absorption and acridine-orange fluorescence are given in Rigler et al. (1968).

**Ethidium bromide binding.** The ability of the nuclei to bind ethidium bromide was studied by microfluorimetry at 590 m\(\mu\). The nuclei were stained with ethidium bromide by a procedure which was identical to that used by Rigler (1966) for staining with acridine-orange: the ethidium bromide stain contained 1 mg/ml of the dye. (The ethidium bromide was obtained from Boots Pure Drug Co., Nottingham, U.K.)

**Determination of dry mass**

The dry mass of the erythrocyte nuclei was measured by microinterferometry. A newly developed, rapid scanning and integrating microinterferometer was used (Caspersson & Lomakka, 1966). With this instrument the field of measurement can be very closely delimited to accommodate objects of widely varying shape. This permitted measurements on individual erythrocyte nuclei within intact heterokaryons. Optical path differences were recorded over HeLa-erythrocyte heterokaryons, over nuclei isolated from these heterokaryons, and over nuclei isolated from erythrocytes.

**Autoradiography**

Autoradiographs were made by the 'stripping film' technique (Pelc, 1947) using Kodak AR10 emulsion.

**Measurement of nuclear volume**

The volume of the nuclei was calculated from measurements of their maximum cross-sectional area. A phase-contrast microscope, equipped with a drawing device, was used to plot the outlines of individual erythrocyte nuclei within heterokaryons. Areas within these outlines were then determined by planimetry. Nuclear volume was calculated on the assumption that the nuclei were spherical, although this assumption appeared not to be valid for the largest erythrocyte nuclei. This point is discussed in more detail later.

**RESULTS**

**Morphological changes in erythrocyte nuclei within heterokaryons**

A few hours after the red cell nuclei are introduced into HeLa cell cytoplasm, they enlarge and the condensed chromatin characteristic of these nuclei in intact erythrocytes undergoes progressive dispersion (Harris, 1967). These changes were examined in the present study by ultraviolet microscopy. Figure 11 shows an ultraviolet photomicrograph at 265 m\(\mu\) of a nucleus within an erythrocyte ghost; Fig. 12 shows a reactivated erythrocyte nucleus isolated after 41 h in a heterokaryon; and Fig. 13 shows a HeLa nucleus isolated after 41 h in a heterokaryon. It will be seen that the chromatin of the reactivated erythrocyte nucleus has become very much more dispersed. Some very small dense aggregates are still present and a structure which may be a rudimentary nucleolus. Most of the chromatin of the erythrocyte nucleus now has the same texture as the euchromatic regions of the HeLa nucleus. Essentially similar appearances are seen in intact heterokaryons.
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Fig. 1. Tracings obtained from heterokaryons by scanning microinterferometry. 
A, tracing from a central scanning line which passes over the cytoplasm of a heterokaryon (CP) and over a HeLa nucleus. The nucleolus produces a prominent peak. 
B, tracing from a scanning line running parallel to A at a distance of approximately 2 μ. This scanning line runs over the same HeLa nucleus as A, and also over an erythrocyte nucleus (EN). 
C, tracings over central portions of a HeLa nucleus isolated from a heterokaryon, a slightly enlarged erythrocyte nucleus isolated from a heterokaryon, and a nucleus isolated from an erythrocyte.

Changes in dry mass and volume during the process of reactivation

Microinterferometric measurements were made on nuclei in intact heterokaryons and on nuclei isolated from heterokaryons. Fig. 1A shows a microinterferometric tracing of the optical path difference along a scanning line running through a heterokaryon. This line passes over the central portion of a HeLa nucleus which contains a large nucleolus. Fig. 1B shows a parallel scanning line at a distance of approximately 2 μ, which passes over an erythrocyte nucleus as well as the HeLa nucleus. These tracings suggest that the heterokaryons are indeed very flat, since the nuclei and especially the HeLa nucleolus can easily be identified as prominent peaks on the tracings. Fig. 1C shows tracings made from a HeLa nucleus isolated from a heterokaryon, a slightly enlarged erythrocyte nucleus isolated from a heterokaryon, and a
normal nucleus isolated from an erythrocyte. The dry mass of the nuclei within the heterokaryons, as calculated from tracings over the intact cells, was greater than that calculated from tracings over isolated nuclei. This discrepancy might be due either to the contribution made by the thin layer of over- and underlying cytoplasm in the intact heterokaryons, or to loss of protein from the nuclei during the isolation procedure, or to both factors. The relative importance of these two sources of error is discussed later.

Figure 2 shows the dry mass and Feulgen-DNA content of a random sample of erythrocyte nuclei within heterokaryons 16 h after cell fusion. The mean dry mass of these nuclei is 5.8 ± 2.4 units (n = 28), compared with 2.6 ± 0.4 units (n = 25) for a random sample of nuclei isolated from erythrocytes. (All cytochemical values are given as mean values ± standard deviation, in arbitrary units. The number of nuclei measured (n) is given in parentheses.) There is a good deal of variation between individual nuclei, some having virtually the same dry mass as the nuclei isolated from erythrocytes, others having a dry mass 4–5 times higher. At this stage virtually all the erythrocyte nuclei in the heterokaryons are still in the G₁ phase, that is, they have not yet begun to replicate their DNA. Forty-seven h after cell fusion the erythrocyte nuclei in heterokaryons have a mean dry mass of 11.9 ± 4.9 units (n = 78) (Fig. 3).
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This represents a greater than 4-fold increase in mean dry mass relative to nuclei isolated from erythrocytes. Erythrocyte nuclei which have completed the replication of their DNA (G₂ phase nuclei) also show great variation in dry mass. The largest nuclei seen in heterokaryons 47 h after cell fusion show an 8-fold increase in dry mass relative to nuclei isolated from erythrocytes.

Fig. 3. Correlation between the Feulgen-DNA value and the dry mass of erythrocyte nuclei in heterokaryons 47 h after cell fusion. Nuclei in G₁, S and G₂ phases show wide variations in dry mass. Nuclei with Feulgen values below the G₁ level are probably being degraded. The small rectangle represents the range of values found in nuclei isolated from adult hen erythrocytes by the ‘Nonidet’ method.

The reactivation of the erythrocyte nucleus is associated with a marked increase in its volume (Harris, 1967). Figure 4 shows the correlation between the volume of erythrocyte nuclei and their dry mass, measured in intact heterokaryons 47 h after cell fusion. The relationship between volume and dry mass is roughly linear in the lower range, but at higher values the volume appears to increase more rapidly than the dry mass. This discrepancy might be accounted for by a change in nuclear geometry with increase in size: the larger nuclei may tend to become more flattened than the smaller ones.

This increase in dry mass was also observed in erythrocyte nuclei isolated from heterokaryons. Forty-one h after cell fusion the mean dry mass of such nuclei was found to be 5.2 ± 1.8 units (n = 67). The largest nuclei showed a 3- to 4-fold increase in dry mass relative to that of nuclei isolated from erythrocytes (Fig. 5).
Synthesis of DNA

Sixteen h after cell fusion virtually all erythrocyte nuclei in heterokaryons gave approximately diploid Feulgen-DNA values and were thus presumably in the $G_1$ phase of the cell cycle (Figs. 2, 6). The mean Feulgen-DNA value for these nuclei, $53 \pm 9$ units ($n = 28$), was 16% higher than the mean value for nuclei isolated from erythrocytes, $46 \pm 5$ units ($n = 20$). This difference is due to a change in the reactivity of the nuclei to the Feulgen stain and does not represent an increase in DNA content. This effect will be discussed in more detail later.

Twenty-four h after cell fusion many of the erythrocyte nuclei in heterokaryons...
had entered the phase of DNA synthesis (S phase) and gave Feulgen–DNA values above the \( G_1 \) level. Forty-seven h after fusion, the mean Feulgen–DNA value had increased to 68 ± 31 units (\( n = 78 \)). From the plots shown in Figs. 3 and 6 it can be concluded that many of the erythrocyte nuclei had completed the replication of their DNA and were in the \( G_2 \) phase of the cell cycle. Some erythrocyte nuclei gave Feulgen–DNA values intermediate between the \( G_1 \) and the \( G_2 \) values, and were presumably in the \( S \) phase of the cell cycle. This was confirmed autoradiographically by the fact that about 60% of the erythrocyte nuclei isolated from heterokaryons

![Graph showing Feulgen-DNA values vs. dry mass](image)

**Fig. 5.** Feulgen–DNA values of erythrocyte nuclei isolated from heterokaryons 41 h after cell fusion, plotted against their dry mass. The small rectangle represents the range of values found in nuclei isolated from the same batch of erythrocytes. The nuclei were isolated by the method of Fisher & Harris (1962) in both cases.

which had been exposed to tritiated thymidine showed some degree of labelling. Some erythrocyte nuclei in heterokaryons gave Feulgen–DNA values lower than the \( G_1 \) level. Parallel measurements on erythrocyte nuclei isolated from heterokaryons revealed a small number with abnormally low \( E_{360}/\text{dry mass} \) ratios as well as low Feulgen–DNA values. It is likely that these represent degenerating erythrocyte nuclei which have lost some of their DNA.

In the present experiments, nuclei isolated from erythrocytes were used as a standard for the Feulgen–DNA determinations and also as a measure of the normal \( G_1 \) Feulgen value. However, erythrocyte nuclei undergoing reactivation in heterokaryons (Fig. 6) and nuclei isolated from such heterokaryons (Fig. 7) gave higher \( G_1 \) values than nuclei isolated from erythrocytes. This effect was studied more closely by
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comparing Feulgen values and ultraviolet absorption in the same group of $G_1$ phase nuclei. Thirty-nine-h cultures of heterokaryons were exposed to tritiated thymidine for 2 h and then enucleated. The ultraviolet absorption of the isolated erythrocyte nuclei was measured at 265 m\(\mu\); the nuclei were then stained by the Feulgen technique and their Feulgen–DNA values determined by microspectrophotometry at 546 m\(\mu\). Autoradiographs were then made of the preparations. The mean ultraviolet absorption of a sample of unlabelled $G_1$ phase nuclei isolated from the heterokaryons was found to be $111.1 \pm 15.6$ units ($n = 12$); for nuclei isolated from erythrocytes this value was $105.3 \pm 4.2$ units ($n = 40$). For the same two groups of nuclei, the Feulgen–DNA values were $60.9 \pm 13.5$ units ($n = 12$) and $45.2 \pm 4.6$ units ($n = 40$) respectively. The ratio of the Feulgen value to the ultraviolet absorption was thus about 30% higher in the nuclei isolated from the heterokaryons. Since these nuclei contain RNA (Harris, unpublished), which contributes to the ultraviolet absorption, the size of the discrepancy in the Feulgen values is probably even greater than this.
The affinity of the chromatin for acridine-orange and ethidium bromide

At an early stage in the reactivation process, and before replication of DNA begins, the ability of the chromatin of the erythrocyte nuclei to bind the intercalating dyes acridine-orange and ethidium bromide increases dramatically. In Fig. 8A and B the ability of erythrocyte nuclei in heterokaryons to bind acridine-orange 14.5 h after cell fusion is compared with that of nuclei in intact erythrocytes. The erythrocyte nuclei in heterokaryons show a 4-fold increase in dye-binding ability compared with the nuclei in erythrocytes: \(2.4 \pm 0.8\) units \((n = 59)\) compared with \(0.6 \pm 0.1\) units \((n = 10)\). This high acridine-orange fluorescence per unit amount of DNA is also seen in erythrocytes in heterokaryons 24, 27, 41 and 48 h after cell fusion. In these cases the mean fluorescence values are still higher, since, by this time, replication of DNA is taking place and many nuclei contain more than the \(G_1\) amount.

Figure 8C shows the acridine-orange fluorescence of nuclei isolated from heterokaryons 41 h after cell fusion. The reactivated \(G_1\) phase nuclei isolated from 41-h heterokaryons show the same acridine-orange fluorescence as \(G_1\) phase nuclei in 14-h heterokaryons. In both cases the acridine-orange fluorescence is approximately four
times higher than that of nuclei in erythrocytes. Some of the smallest nuclei isolated from heterokaryons 41 h after cell fusion have a very condensed chromatin structure; these show the lowest level of acridine-orange fluorescence (cross-hatched areas in Fig. 8c). On the other hand, the acridine-orange fluorescence of the $G_2$ phase erythrocyte nuclei appears to be twice as intense as that of the reactivated $G_1$ phase nuclei.

![Graph showing acridine-orange fluorescence at 530 nm of nuclei in intact erythrocytes (A), $G_1$ phase erythrocyte nuclei in heterokaryons 14.5 h after cell fusion (B), and erythrocyte nuclei isolated from heterokaryons 41 h after cell fusion (C). The reactivation of the erythrocyte nuclei in heterokaryons is associated with a great increase in the binding of the acridine-orange to DNA. The cross-hatched area in C indicates a group of small, very dense, nuclei. The question mark indicates a group of nuclei which could not with certainty be identified as erythrocyte nuclei.]

The technique used for isolating the nuclei from erythrocytes was the same as that used for the enucleation of heterokaryons (Fisher & Harris, 1962). Nuclei isolated from erythrocytes by this technique show a more intense acridine-orange fluorescence (0.5–1.3 units) than the same nuclei in situ (0.4–0.7 units). Fluorescence values of 0.3–0.6 units were usually obtained for nuclei isolated from adult hen erythrocytes by the ‘Nonidet’ technique.

It is conceivable that the increased acridine-orange fluorescence of erythrocyte nuclei in heterokaryons might have been caused by their prior treatment with in-
activated virus, or simply by contact with HeLa cells. The nuclei of erythrocytes which had merely been mixed with HeLa cells gave an acridine-orange fluorescence value of 0.8 ± 0.1 units (n = 15). The value for nuclei in erythrocytes which had been treated with inactivated virus was 1.0 ± 0.2 units (n = 20). These values are only slightly higher than those obtained with intact erythrocytes alone: 0.6 ± 0.1 units (n = 10). This slight increase may well have been due to the fact that when the erythrocytes were mixed with the HeLa cells or treated with virus they were either washed or shaken in salt solutions which did not contain serum proteins. The evidence for this view will be discussed later.

Fig. 9. Two experiments showing melting profiles of nuclei isolated from erythrocytes and from heterokaryons 41 h (A) and 46 h (B) after cell fusion. Note the difference between nuclei isolated from adult (A) and embryonic (B) erythrocytes, on the one hand, and reactivated erythrocyte nuclei and HeLa nuclei, on the other. ×, nuclei isolated from erythrocytes; Δ, erythrocyte nuclei isolated from heterokaryons; ○, HeLa nuclei, nucleoplasm only; ●, HeLa nuclei including nucleoli.

In order to examine whether the increased ability of reactivated erythrocyte nuclei to bind intercalating dyes was limited to acridine-orange, the affinity of the nuclei for ethidium bromide was also measured. A random sample of erythrocyte nuclei isolated from heterokaryons 46 h after cell fusion showed an ethidium fluorescence at 590 mμ (λ<sub>excit</sub> = 365 mμ) of 1.75 ± 0.67 units (n = 30). The mean value for G<sub>1</sub> phase nuclei was about 1.3 units; for G<sub>2</sub> phase nuclei about 2.6 units. Nuclei isolated from erythrocytes by the method of Fisher & Harris (1962) gave a mean ethidium fluorescence value of 0.65 ± 0.04 units (n = 10). Nuclei isolated by the 'Nonidet' method in the presence of serum proteins gave fluorescence values of 0.18 ± 0.03 units (n = 10).
Melting profiles of nuclear deoxyribonucleoprotein complexes

The susceptibility of the nuclear deoxyribonucleoprotein to denaturation by heat was studied by microfluorimetry of cells stained with acridine-orange. Separation of the DNA strands on heating (the melting profile) was reflected by a transition from green to red fluorescence. This was measured by comparing the fluorescence at 590 m\(\mu\) with that at 530 m\(\mu\); the ratio of these two values is termed \(\alpha\). Figure 9 shows that the melting profile of erythrocyte nuclei isolated from heterokaryons 41 h after cell fusion was strikingly different from that of nuclei isolated by the same technique from embryonic erythrocytes (Fig. 9B) or from adult erythrocytes (Fig. 9A). In erythrocyte nuclei reactivated within heterokaryons \(\alpha\) begins to increase at a lower temperature and reaches a higher value than in nuclei within erythrocytes. As shown in Fig. 9A the chromatin of reactivated erythrocyte nuclei has a melting profile which is indistinguishable from that of HeLa nuclei. In this experiment a decrease was observed in \(\alpha\) between 90° and 100°C, both in the reactivated erythrocyte nuclei and in the HeLa nuclei. This may have been due to partial extraction of the DNA at high temperatures. The effect is not seen in nuclei isolated from erythrocytes (Fig. 9A, B); nor was it observed in a second experiment on nuclei isolated from heterokaryons (Fig. 9B).

DISCUSSION

The present investigation shows that the increase in nuclear volume which takes place when erythrocyte nuclei are reactivated in heterokaryons is accompanied by a great increase in dry mass. Part of this increase occurs before the synthesis of DNA begins, that is, during the first 16-20 h after cell fusion. Twenty-four h after cell fusion many of the erythrocyte nuclei give Feulgen-DNA values greater than those normal for the \(G_1\) phase, and at 41 and 47 h many erythrocyte nuclei give \(G_2\) Feulgen values. This clearly shows that the incorporation of tritiated thymidine previously described by Harris (1965) reflects net synthesis of DNA and that many of the erythrocyte nuclei are capable of replicating their DNA completely. During the phase of DNA replication, the dry mass of the nuclei increases further. Measurements made on erythrocyte nuclei within heterokaryons suggest that in the largest \(G_2\) phase nuclei the dry mass has increased 8-fold. Measurements on erythrocyte nuclei isolated from heterokaryons 41 h after cell fusion indicate a 4-fold increase in dry mass in the largest nuclei.

Both sets of measurements are subject to systematic errors. Over- and underlying cytoplasm probably make a contribution to the dry mass values obtained by scanning erythrocyte nuclei within heterokaryons, although the microinterferometric tracings (Fig. 1) suggest that this source of error is likely to be small. Nuclei isolated from heterokaryons, on the other hand, are likely to give low dry mass values since nuclear proteins are lost during the isolation procedure. HeLa nuclei may lose as much as 30% of their dry mass on isolation (Fisher & Harris, 1962) and reactivated erythrocyte nuclei, which, like HeLa nuclei, have a high protein-to-DNA ratio and a loose chromatin structure, might also be expected to lose a good deal of protein on isolation. Nuclei
in intact erythrocytes have a low protein-to-DNA ratio, a low content of non-chromatin protein and a dense chromatin structure; it is therefore possible that they might be less susceptible to losses of protein during isolation. In the light of these considerations, it appears probable that the true increase in dry mass which the largest erythrocyte nuclei undergo during the first 48 h of the reactivation process might be closer to the figure recorded in intact heterokaryons than to the figure recorded in isolated nuclei. In any case, the increase seems likely to be at least 5- to 6-fold.

At an early stage in the reactivation process, before replication of DNA begins, there is a marked increase in the ability of the chromatin to bind the intercalating dyes acridine-orange and ethidium bromide. This change also takes place in the chromatin of small lymphocytes when these cells are activated by the addition of phytohaemagglutinin (Killander & Rigler, 1965). The structural basis for the heightened affinity of the chromatin for this type of dye is at present obscure. It seems possible that the increase in the number of dye-binding sites reflects an increase in the number of accessible negatively charged phosphate groups; and this, in turn, may result from the opening up of the previously condensed deoxyribonucleoprotein complex.

Analysis of the melting profile of the chromatin in erythrocyte nuclei reveals that, during the process of reactivation, there is a marked increase in the susceptibility of the DNA to thermal denaturation. The chromatin of reactivated erythrocyte nuclei melts at a lower temperature and more completely than that of nuclei isolated from erythrocytes; and the melting profile of reactivated erythrocyte nuclei is indistinguishable from that of HeLa nuclei. A similar change in melting profile also takes place in the chromatin of lymphocytes activated by phytohaemagglutinin (Rigler & Killander, 1968). The initial stages of the reactivation process are also characterized by an increase in the reactivity of the chromatin to the Feulgen stain. Under standard conditions, the reactivated erythrocyte nuclei give a Feulgen value which is 15-40% greater than that of normal erythrocyte nuclei with the same DNA content as determined by ultraviolet microspectrophotometry. This finding no doubt also reflects the opening up and dispersion of the normally highly condensed deoxyribonucleoprotein complex. Gledhill et al. (1966) have shown that the condensation and inactivation of the chromatin during spermiogenesis in the bull is associated with a marked decrease in the ability of the DNA to bind acridine orange and a decrease in the reactivity of the nucleus to the Feulgen stain. These changes appear to be the reverse of those which take place during the reactivation of the erythrocyte nucleus.

In some experiments carried out in parallel with those described in the present paper, Ringertz & Bolund (1968) have found that an increase in acridine-orange and ethidium binding, an altered melting profile and a change in reactivity to the Feulgen stain can be induced in isolated erythrocyte nuclei and in intact erythrocytes by washing in serum-free salt solutions. Similar effects can also be produced by treating the nuclei prior to fixation with EDTA. All these changes in the chromatin can occur at low temperatures and they are not significantly affected by a number of enzyme inhibitors. They can, however, be prevented by divalent metal ions. This suggests the possibility that the removal of divalent cations from the chromatin might form an essential part of the mechanism by which the dormant nucleus is reactivated.
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Fig. 12. An ultraviolet photomicrograph of a reactivated erythrocyte nucleus isolated after 41 h in a heterokaryon.

Fig. 13. An ultraviolet photomicrograph of a HeLa nucleus isolated after 41 h in a heterokaryon.
Fig. 10. A population of heterokaryons containing HeLa nuclei and chick erythrocyte nuclei which have not yet begun to enlarge.

Fig. 11. An ultraviolet photomicrograph taken at 265 μ of a nucleus within an erythrocyte ghost.