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ARTIFICIAL HETEROKARYONS OF ANIMAL CELLS FROM DIFFERENT SPECIES

H. HARRIS, J. F. WATKINS, C. E. FORD* AND G. I. SCHOEFL†
The Sir William Dunn School of Pathology, University of Oxford

SUMMARY

A virus, inactivated by ultraviolet light, was used to fuse together cells from different species of vertebrate, and the resulting heterokaryons were examined by autoradiographic and cytological techniques. Heterokaryons could be made with both differentiated and undifferentiated cells: HeLa and Ehrlich ascites cells were studied as examples of undifferentiated cells; rabbit macrophages, rat lymphocytes and hen erythrocytes as examples of differentiated cells. These last three cells were chosen because in them, in varying degrees, the process of differentiation has resulted in suppression of the synthesis of DNA or of both DNA and RNA. This suppression was in all cases found to be reversible: the dormant nuclei could be induced to resume the synthesis of RNA or DNA or both when the differentiated cells were fused with a cell which normally synthesizes RNA and DNA. Observations on heterokaryons in which differentiated cells were fused with HeLa cells and with each other permitted certain general conclusions to be drawn about the regulation of nucleic acid synthesis in the heterokaryon. It was found that if either one of the parent cells normally synthesized RNA, RNA synthesis took place in both types of nuclei in the heterokaryon. If either of the parent cells normally synthesized DNA, DNA synthesis took place in both types of nuclei in the heterokaryon. If neither of the parent cells synthesized DNA, no DNA synthesis took place in the heterokaryon. In all cases where a cell which synthesized a particular nucleic acid was fused with one which did not, the active cell initiated the synthesis of this nucleic acid in the inactive partner. In no case did the inactive cell suppress synthesis in the active partner.

The nuclei of heterokaryons in which DNA synthesis took place underwent mitosis, and those nuclei which entered mitosis synchronously usually fused together. This process resulted in the progressive formation of mononucleate hybrid cells, which might thus contain within a single nucleus chromosomal complements derived from different species. These mononucleate hybrid cells were also capable of RNA and DNA synthesis, and many of them in turn underwent mitosis. At metaphase these cells showed, in various combinations, the chromosomal complements of the two parent cells. Mononucleate hybrid cells formed by the fusion of a large number of single cells did not appear to be capable of continued multiplication; but mononucleate cells containing one chromosomal set from each parent cell were still found to be undergoing mitosis many days after cell fusion.

INTRODUCTION

The idea of using viruses to produce artificial heterokaryons of animal cells has its origins in experiments which go back for more than a century. Multinucleate cells in vertebrates were apparently first described by Müller (1838), who observed them in tumours. Robin (1849) noted their presence in bone-marrow, Rokitansky (1855) in tuberculous tissue and Virchow (1858) in a variety of normal tissues and both inflam-

* Medical Research Council, Radiobiological Research Unit, Harwell.
† Recipient of N.I.H. Postdoctoral Fellowship No. 2–F2–HE–8900–03.

matory and neoplastic lesions. By the time Langhans (1868, 1870) wrote his classical papers on multinucleate cells, an extensive literature about them already existed. The view that some of these cells were produced by fusion of mononucleate cells derived from the work of de Bary (1859), who observed that the life-cycle of certain myxomycetes involved the fusion of single cells to form multinucleated plasmodia. Lange (1875) appears to have been the first to describe a process of this sort in vertebrates. Lange observed the coalescence of blood-borne amoeboid cells in the frog; and similar observations were made a little later by Cienkowski (1876), Buck (1878) and Geddes (1880) in invertebrates. Metchnikoff (1884) considered that the fusion of phagocytic cells to form plasmodia was a characteristic cellular defence mechanism in both vertebrates and invertebrates.

Following the introduction of tissue culture methods by Harrison (1907), numerous observations were made on cell fusion in cultures of animal tissues. The first of these appears to have been that of Lambert (1912); fifteen years later, Lewis (1927) was able to list twenty-one references to observations of this kind. Fell & Hughes (1949) observed that when binucleate cells in mouse tissue cultures entered mitosis only one spindle was usually formed and the chromosomes of both nuclei joined a single metaphase plate; cell division then gave rise to mononucleate daughter cells with abnormally large nuclei apparently containing twice the normal number of chromosomes. Barski, Sorieul & Cornefert (1960, 1961) noticed that when two different lines of mouse cells were grown together, hybrid mononucleate cells eventually appeared which contained the chromosomal complements of both parent cells. Similar observations were made by Ephrussi, Scaletta, Stenchever & Yoshida (1964) on a number of other mouse-cell lines.

It is very probable that some of the inflammatory lesions in which multinucleate cells were observed in the last century were caused by viruses, although, of course, the viral aetiology of these conditions was not recognized until very much later. The earliest reports of multinucleate cells in lesions which can with certainty be identified as of viral origin appear to be those of Luginbühl (1873) and Weigert (1874), who described such cells at the periphery of smallpox pustules. Unna (1896) observed multinucleate cells in the skin lesions of chicken pox, and Warthin (1931) observed them in the tonsils of patients with measles. Warthin's paper prompted Enders & Peebles (1954) to examine the effects of measles virus in tissue culture. These authors found that the virus induced the cells in the tissue culture to fuse together to form multinucleated syncytia. A similar observation was made by Henle, Deinhardt & Girardi (1954) with mumps virus, by Chanock (1956) with the virus of infantile croup and by Marston (1958) with a virus of the para-influenza group. Numerous other examples of this phenomenon have since been described. Okada (1958, 1962) demonstrated that animal tumour cells in suspension could be rapidly fused together to form multinucleate giant cells by high concentrations of HVJ virus, another member of the para-influenza group.

The work described in this paper demonstrates that inactivated viruses may be used to provide a general method for fusing together both differentiated and undifferentiated animal cells. Cells from different species and even different orders of

vertebrate may be fused in this way. The resulting heterokaryons offer interesting possibilities for the study of nucleo-cytoplasmic relationships and lend themselves to experiments which have not hitherto been feasible. Preliminary accounts of part of this work have appeared elsewhere (Harris & Watkins, 1965; Harris 1965; Harris *et al.* 1965).

MATERIALS AND METHODS

Growth and assay of the virus

A strain of 'Sendai' virus, one of the para-influenza I group of myxoviruses (Andrewes, 1964), was chosen for these experiments. Of the viruses known to induce the formation of multinucleate cells, the para-influenza viruses seemed the most promising because of their ability to induce the rapid formation of multinucleate cells in cell suspensions (Okada, 1958, 1962), and because of the wide range of animal hosts susceptible to them (Andrewes, 1964). The virus was obtained from Dr H. G. Pereira of the National Institute for Medical Research, Mill Hill, and was propagated in the following way.

Infected allantoic fluid with a titre of 8000 haemagglutinating units/ml was diluted 1 in 10^4 with phosphate-buffered saline (Dulbecco & Vogt, 1954); 0·1 ml of the preparation was injected into the allantoic cavity of 10- or 11-day-old fertile hens' eggs, which were incubated for 3 days at 37 °C. The eggs were then maintained at 4 °C overnight and the allantoic fluid collected. The pooled allantoic fluid was centrifuged at 400 *g* for 10 min and the haemagglutination titre of the supernatant determined. The supernatant was then centrifuged at 30 000*g* for 10 min and the deposit resuspended in one-tenth of the original volume in Hanks's solution (Hanks, 1948). (Hanks's solution was used throughout the present experiments without glucose, in order to avoid a marked fall in pH when the cells were incubated at very high density.) The haemagglutination titre was again determined and the concentrated virus suspension stored in 1 ml lots at −70 °C. This suspension, suitably diluted in Hanks's solution, was used for the experiments.

Haemagglutination titrations were carried out in Salk-pattern haemagglutination trays. Doubling dilutions of virus, made in 0·5 ml of phosphate-buffered saline, and 0·05 ml of a 3 % (v/v) suspension of guinea-pig erythrocytes in this saline (approximately $2{\cdot}5 \times 10^7$ cells) were added to each cup. The smallest amount of virus which produced complete haemagglutination after 2 h at room temperature was defined as one haemagglutination unit (HAU).

The infectivity of the virus was titrated by a modification of the method of Fulton & Armitage (1951). Serial dilutions of the virus in medium 199 (Morgan, Morton & Parker, 1950) were incubated with fragments of chlorio-allantoic membrane in screw-capped tubes. Three tubes were used for each dilution. The tubes were shaken at 100 excursions/min in a 37 °C water bath for 24 h and the haemagglutination titre of the medium then determined. The ID (infectious dose)$_{50}$ was estimated by the method of 'moving averages' (Thomson, 1947).

Inactivation of the virus with ultraviolet light

Since any investigation of the physiology of the heterokaryons would be greatly complicated if these cells were engaged in the production of virus, the virus used to produce cell fusion was inactivated by ultraviolet light. This procedure was suggested by the experiments of Okada (1962), who showed that the infectivity of the virus was much more easily destroyed by ultraviolet light than its ability to induce cell fusion. One ml of a concentrated suspension of virus (80000 HAU/ml) in a watch glass 7·5 cm in diameter was placed at a distance of 30 cm from a Philips 15-W 18-in. germicidal tube, type 'T.U.V.', and exposed to the ultraviolet light for 3 min. The suspension was mixed by pipetting at the end of the first and second minutes. The calculated intensity of the radiation incident on the surface of the fluid was 3000 ergs/cm^2/sec. Infectivity titrations, carried out as described above, indicated that the infectivity of the virus had been reduced by more than 10^6 by this procedure. The haemagglutination titre of the inactivated virus and its ability to induce cell fusion *in vitro* were not noticeably altered.

Maintenance of stock cell lines

The HeLa cells were obtained from a stock supension culture. These cells were not derived from a clone, but were taken into suspension culture from monolayers in 1959. They have been grown both as monolayers and suspension cultures since that time. The technique of suspension culture and the media used have been previously described (Harris & Watts, 1962). The Ehrlich ascites cells were a tetraploid line maintained by serial passage in the peritoneal cavity of Swiss mice.

Techniques for obtaining macrophages, lymphocytes and nucleated erythrocytes

Macrophages were obtained from the peritoneal cavity of rabbits by the technique of Mackaness (1952). About 50 ml of heavy liquid paraffin (Harrington Bros., London) were injected under sterile conditions into the peritoneal cavity of a rabbit and the resulting exudate washed out 4–6 days later with physiological saline. The cells were spun out of the peritoneal washings, and, in a satisfactory exudate, between 85 and 95% of them were macrophages. Most of the rest were small lymphocytes.

Virtually pure populations of lymphocytes were kindly supplied by Professor J. L. Gowans and Dr P. J. McCullagh. These cells were obtained by cannulation of the thoracic duct of albino rats from a non-inbred colony.

About 5 ml of blood were taken by venesection from the wing vein of an adult domestic fowl into a flask containing 200 international units of heparin. The heparinized blood was freed of leucocytes as far as possible by repeated centrifugation. The blood was introduced into a capillary tube 16 cm long with a bore of 4 mm and sealed at one end. It was centrifuged in this tube for 15 min at approximately 2000 *g*. The tube was then scored about half way up the column of packed red cells and the part of the tube above this mark was broken off and discarded. This completely removed the buffy coat containing the leucocytes and the top half of the packed red cell column. The bottom half of the red cell column was taken up in physiological saline and transferred to a fresh capillary tube in which the centrifugation was repeated. The top half of the packed red cell column was again broken off and the centrifugation procedure repeated on the bottom half. The bottom half of the red cell column produced by the third cycle of centrifugation was used for the experiments. Films were made from a sample of packed red cells prepared in this way and were stained with Leishman's stain. An examination of three such films did not reveal a single leucocyte.

Technique of cell fusion

The initial experiments in this series were carried out with HeLa and Ehrlich ascites cells. The technique used for inducing fusion of these cells was essentially similar to that described by Okada (1962). HeLa cells from a suspension culture were spun down and resuspended at a concentration of 2×10^7 cells per ml in Hanks's solution. Ehrlich ascites cells withdrawn from the peritoneal cavity were washed once by centrifugation in this solution and then resuspended in it at a concentration of 2×10^7 cells per ml; 0·5 ml of each cell suspension was pipetted into a chilled inverted T-tube together with 1 ml of a suitably diluted suspension of virus (between 80000 and 800 HAU, as required). The cells clumped immediately, and the size of the clumps was roughly proportional to the amount of virus added. The T-tube was kept at 4 °C for 15 min and then shaken in a water bath at 37 °C for 20 min at a rate of 100 excursions/min. During this time the cells in the clumps underwent various degrees of fusion. It was later found that the period of agitation at 37 °C, as carried out by Okada (1962), was not necessary for the cell fusion to occur. Once the cell suspension and the virus had been mixed together and clumping of the cells had occurred, the preparation could be transferred directly to culture medium and maintained at 37 °C. Fusion of the cells in the aggregates took place no less readily in preparations treated in this way than in those which had been previously shaken.

Macrophages fused readily with each other and with other cells; and heterokaryons containing macrophage nuclei were produced in the same way as HeLa–Ehrlich ascites heterokaryons. Lymphocytes, however, fused with other cells much less readily, and with each other only rarely. In order to obtain adequate numbers of lymphocytes in heterokaryons, it was

usually necessary to have about 5 times as many lymphocytes as other cells in the suspension which was to be treated with the virus.

Nucleated erythrocytes underwent lysis in the presence of high concentrations of the virus and released their nuclei into the medium. These nuclei adhered to the surfaces of the other cells in the preparation, and some of them were incorporated into the cytoplasm of these cells. Nuclei which were not incorporated in this way rapidly underwent degeneration, but most of the incorporated nuclei remained intact. The process by which erythrocyte nuclei enter the cytoplasm of other cells under the influence of the virus appears to be different from phagocytosis. Phagocytosis normally results in the dissolution of ingested nuclei, but, as shown later (see Observations), erythrocyte nuclei incorporated into the cytoplasm of other cells under the influence of the virus may establish a normal structural relationship with the recipient cytoplasm and synthesize both RNA and DNA. It is possible that in rare cases heterokaryons containing erythrocyte nuclei are formed by fusion of intact erythrocytes with other cells, since intact erythrocytes partially fused with other cells have been seen in stained preparations; but partially incorporated free nuclei are far more common.

Heterokaryons containing erythrocyte nuclei are much more readily produced with macrophages than with HeLa cells. Suitable preparations of HeLa–erythrocyte heterokaryons were made by treating a mixture of 2×10^7 HeLa cells and 0·2 ml of packed red cells with 16000 HAU of ultraviolet-inactivated virus; suitable preparations of macrophage–erythrocyte heterokaryons were made with 2×10^7 macrophages, 0·1 ml of packed red cells and 8000 HAU of inactivated virus.

The total number of cells in the mixed suspension, the ratio of the two cell types and the concentration of virus all influenced the number and the composition of the heterokaryons produced. Satisfactory conditions for each combination of cells were arrived at by trial and error. Examples of the results obtained under different sets of conditions are given under Observations.

Growth of hybrid cultures

The fused cells were grown either on coverslips or, when larger numbers were required, in 20 oz. medical flat bottles. For coverslip preparations, 0·5–1 ml of the virus-treated cell suspension and 5 ml of culture medium were pipetted into a Petri dish 6 cm in diameter containing 12 to 15 coverslips 1 cm in diameter. Except in experiments involving the use of tritiated leucine, the culture medium consisted of 20% calf serum and 1% tryptose broth in medium 199, to which penicillin at a concentration of 100 international units/ml and streptomycin at a concentration of 100 μg/ml were added. When the incorporation of tritiated leucine was being studied, the medium consisted of 20% calf serum in Eagle's solution (Eagle *et al.* 1956) from which unlabelled leucine had been omitted. The dishes were incubated at 37 °C in a gas mixture of 5% carbon dioxide in air. The coverslips were transferred to fresh medium after 24 h. For cultures in medical flat bottles, the whole of the virus-treated cell suspension from one T-tube was seeded into three bottles each containing approximately 40 ml of culture medium. These preparations were also incubated at 37 °C and the medium in them changed after 24 h.

Cytological examination of the hybrid cultures

For routine examination, coverslips were removed from the Petri dishes, rinsed in phosphate-buffered saline and fixed in methanol. The fixed preparations were then washed in 0·1 M phosphate buffer at pH 6·8 and stained for 10 min with a mixture of 10% Giemsa and 1% May–Grünwald stain in distilled water. After staining, the coverslips were washed in phosphate buffer, water, acetone and xylene, and then mounted in DePeX (G. T. Gurr, London).

For examination of chromosomes two procedures were used, both modifications of the method of Rothfels & Siminovitch (1958). Coverslip preparations were made in the following way. The coverslip with adherent cells was immersed for 1 h at room temperature in a hypotonic solution containing 1 part of Hanks's saline, 9 parts of distilled water, and colcemid (CIBA) at a concentration of 10^{-5} M. The cells were then fixed for 15 min in Carnoy's fixative, dried at room temperature for 30 min and stained with 1% orcein in 50% glacial acetic acid for 3 h. The stained preparation was washed for an hour in two changes of ethanol, rinsed in xylene and mounted in DePeX.

Mitotic cells from the cultures growing in medical flat bottles were obtained by incubating the preparations for 1·5 h with colcemid at a concentration of approximately 10^{-5} M and then shaking the cultures to free cells in mitosis from the glass. The freed cells were sedimented by centrifugation for 3 min at 200*g*, resuspended in hypotonic sodium citrate solution (1 % w/v) at 37 °C and left to stand at room temperature for 10 min. The cells were again sedimented and then fixed by several changes of a mixture of 3 parts ethanol and 1 part glacial acetic acid. Preparations were made by air drying, either from the fixative or from 45 % acetic acid. Similar preparations were made with Ehrlich ascites cells taken directly from carrier mice.

Autoradiography

The coverslips were rinsed in phosphate-buffered saline and fixed in methanol. Heterokaryons in which one of the cell types was a HeLa cell adhered closely to the glass and gave satisfactory autoradiographs when fixed simply by immersion in methanol. Macrophage–lymphocyte and macrophage–erythrocyte heterokaryons, however, were not flat enough when fixed in this way. Coverslips bearing these cells were therefore applied to small agar blocks (3 % (w/v) agar in physiological saline), so that the cells were compressed between the coverslip and the surface of the agar. A hexagonal nut weighing approximately 1·3 g was placed on the upper surface of the coverslip, and the methanol allowed to diffuse across the interface between the coverslip and the agar for an hour. In this way the cells were flattened before fixation and gave autoradiographs with excellent resolution. The fixed preparations were extracted with 0·3 N trichloroacetic acid at 4 °C for 1 h. When DNA was to be removed from the fixed cells the preparations were treated for 30 min at 37 °C with deoxyribonuclease (Worthington Biochemical Corporation, New Jersey) at a concentration of 0·5 mg/ml in a solution of magnesium sulphate at pH 6·0 (Darlington & La Cour, 1962). RNA was removed by a similar treatment with ribonuclease (Worthington Biochemical Corporation) at a concentration of 0·5 mg/ml in 0·1 M phosphate buffer at pH 7·6. When both nucleic acids were to be removed the preparation was heated with 0·3 N trichloroacetic acid at 90 °C for 30 min. Autoradiographs were made by the 'stripping film' technique (Pelc, 1947) using Kodak AR 10 film, and exposed for 2–20 days depending on the time required to produce a satisfactory density of grains for any particular experiment. After development, the autoradiographs were stained through the emulsion with Leishman's stain.

Ciné-micrography

In order to permit the use of phase-contrast optics, special perspex Petri dishes were constructed with recesses machined into the floor and lid to hold circular coverslips of $1\frac{1}{8}$ in. diameter. The coverslips were sealed into these recesses with paraffin wax and when in position were separated by a depth of $\frac{1}{8}$ in. A Reichert inverted photomicroscope permitted the cells adherent to the bottom coverslip to be photographed with phase-contrast optics. Vinten time-lapse equipment (W. Vinten Ltd., London) was used, usually set to take 15 frames/hour.

Radioactive precursors

Uridine-5[^{3}H], specific activity 13 C/mM, thymidine-6[^{3}H], specific activity 13 C/mM and DL-leucine-4,5[^{3}H], specific activity 5·1 C/mM, were obtained from the Radiochemical Centre, Amersham. The uridine and thymidine were used at a concentration of 10 μc/ml of medium; the leucine at a concentration of 20 μc/ml.

Electron microscopy

Suitable concentrations of inactivated virus were added to suspensions of cells, which were maintained at 4 °C for 10 min and shaken at 37 °C for 20 min as described above. The preparations were then centrifuged at 200*g* for 10 min. The supernatant was discarded and the pellet of cells fixed for 1–2 h in 1 or 2 % (w/v) osmium tetroxide, buffered at pH 7·4–7·5 with 0·1 M sodium phosphate or 0·1 M sodium cacodylate. The blocks were stained with potassium permanganate (Parsons, 1961) or uranyl acetate during acetone dehydration, and then embedded in Araldite. In one experiment cells grown on coverslips for 24 h were fixed, dehydrated and

embedded *in situ*. Thin sections were cut with glass knives on an LKB Ultrotome and stained with lead citrate (Reynolds, 1963) or with aqueous uranyl acetate followed by lead citrate. The sections were examined with a Philips EM 200 electron microscope (60 kV, objective aperture 30–50 μ).

OBSERVATIONS

HeLa–Ehrlich heterokaryons

Mechanism of cell fusion. The adsorption of the virus to the surface of the cells caused them to adhere to each other. Sections through pellets obtained by centrifugation of cell suspensions which had been treated with virus 30 min previously showed that the cells had already undergone various degrees of fusion. The surfaces of the virus-treated cells showed many areas densely covered with microvilli (Fig. 3), an appearance similar to that described by Okada (1962) in cells treated with HVJ virus. Where the surfaces of two cells were in contact the microvilli appeared to fuse with one another, thus establishing minute cytoplasmic bridges between the two cells. Extension of this process resulted in the progressive dissolution of the cell membranes over the area of contact between the two cells, so that the two cytoplasms eventually coalesced. Fig. 4 shows a typical area of fusion between two cells. Several cells may fuse together simultaneously or sequentially in this way, and thus give rise to multinucleate cells containing variable numbers of nuclei. Fig. 5 shows a typical multinucleate cell, and Fig. 6 shows a single cell in the process of fusing with a multinucleate cell.

Demonstration of heterokaryosis. Within 4 h of their being introduced into Petri dishes containing coverslips or into medical flat bottles the multinucleate cells had become adherent to the glass. When they were examined 24 h later most of them had flattened out on the glass, and the morphology of their nuclei could be satisfactorily examined in fixed and stained preparations. The nuclei were of two easily distinguishable types. With May–Grünwald–Giemsa or Leishman stain one type of nucleus stained more deeply than the other. The more deeply staining nucleus contained numerous small nucleoli or coarse condensations of chromatin, while the less deeply staining nucleus contained one to three large nucleoli. The two types of nucleus are illustrated in Fig. 7, which shows a tetranucleate cell containing two nuclei of each type. Since the deeply staining pachychromatic nuclei clearly resembled the nuclei of Ehrlich ascites cells, and the less deeply staining nuclei resembled the nuclei of HeLa cells, it was difficult to avoid the conclusion that the multinucleate cells were formed by fusion of the two cell types. This was confirmed by fusing together HeLa cells in which the nuclei had been labelled by a prior period of growth in tritiated thymidine with unlabelled Ehrlich ascites cells. One hundred microcuries of tritiated thymidine were added to a suspension culture of HeLa cells at 10 a.m., 2 p.m., 6 p.m. and 10 p.m. on one day, and again at 7 a.m. and 10 a.m. on the following day. Since the generation time of the cells under these conditions was about 20 h, it seemed probable that these repeated exposures to tritiated thymidine would succeed in labelling virtually all the cells in the culture. Autoradiographs of smears of the cells, made 2 h after the last addition of tritiated thymidine to the medium, confirmed that

this was so. When a mixture of labelled HeLa cells and unlabelled Ehrlich ascites cells was treated with virus, autoradiographs of the resulting multinucleate cells revealed that the deeply staining pachychromatic nuclei were not labelled, whereas the less deeply staining nuclei with large nucleoli were. Fig. 8 illustrates a cell containing three labelled HeLa nuclei and one unlabelled Ehrlich ascites nucleus. When the reciprocal experiment was made with labelled Ehrlich ascites cells and unlabelled HeLa cells, it was found that the deeply staining pachychromatic nuclei were labelled and the others were not. It is thus clear that the multinucleate cells were indeed heterokaryons containing both HeLa and Ehrlich ascites nuclei.

Effect of virus concentration on the character of the heterokaryons produced. Within certain limits the average number of nuclei per heterokaryon and the ratio of multinucleate to mononucleate cells could be varied by changing the concentration of virus used. Table 1 shows the effect of virus concentration over a range of 800–80000 HAU/ml. It will be seen that at lower concentrations of virus the number of nuclei per heterokaryon and the ratio of multinucleate to mononucleate cells fell. At a virus concentration of 800 HAU the multinucleate cells contained an average of about four nuclei per cell and about 80 % of these multinucleate cells were heterokaryons, that is, they contained both HeLa and Ehrlich ascites nuclei. With higher concentrations of virus, and hence a greater number of nuclei per cell, virtually all the multinucleate cells were heterokaryons. Under the present conditions about 10 % of the cell suspension was recovered as multinucleate cells. This recovery was little affected by virus concentration over the range tested. The increase in the ratio of multinucleate

Table 1. *Effect of virus concentration on the character of the heterokaryons produced*

Counts made 24 h after formation of heterokaryons	Concentration of virus used: Virus inactivated by ultraviolet light: 800 HAU	8000 HAU	80000 HAU	Infective virus 8000 HAU
Percentage of multinucleate cells which were heterokaryons	77	99	98	97
	84	98	97	99
	85	97	·	99
Mean	82	98	98	98
No. of nuclei per heterokaryon	4·0	(18·4)	10·0	6·3
	4·3	5·9	8·5	5·1
	4·4	9·4	·	7·4
Mean	4·2	(11·2)	9·3	6·3
Ratio of HeLa nuclei to Ehrlich ascites nuclei	2·8	1·9	3·2	2·3
	1·9	2·1	2·7	2·6
	2·5	2·0	·	2·6
Mean	2·4	2·0	3·0	2·5
Ratio of multinucleate to mononucleate cells	1·0	3·5	8·4	3·1
	0·6	2·0	7·5	9·0
	0·8	3·1	·	8·4
Mean	0·8	2·9	8·0	6·8

to mononucleate cells at higher concentrations of virus must therefore have been mainly due to preferential elimination of mononucleate cells. As pointed out by Okada (1962) the multinucleate cells appeared to be more resistant to the cytotoxic effect of the virus than normal mononucleate cells, a conclusion supported by the observation that infective virus eliminated a greater proportion of mononucleate cells than inactivated virus at the same concentration (Table 1). Table 1 also shows that, although the two cell types were present in the cell suspension in equal numbers, the ratio of HeLa nuclei to Ehrlich ascites nuclei in the heterokaryons was between two and three irrespective of virus concentration. This preponderance of HeLa nuclei was apparently due to the fact that HeLa cells fused more readily than Ehrlich ascites cells. An alternative explanation, which was initially thought to be more probable, was that the cells with surfaces containing a large HeLa contribution might adhere more readily to glass. Since Ehrlich ascites cells adhere poorly to glass, it seemed possible that heterokaryons containing a preponderance of Ehrlich ascites components might also adhere poorly. In this way, adhesion to glass might have selected heterokaryons with predominantly HeLa surface characteristics. However, subsequent experiments in which the proportions of HeLa and Ehrlich ascites nuclei were varied showed that the number of heterokaryons which adhered to glass did not reflect their content of HeLa nuclei. Later work made it clear that different cell types showed wide variation in their ability to fuse together under the influence of the virus.

By varying the proportion of the two cell types in the suspension and the concentration of virus, it was found possible to control, within certain limits, the average number of nuclei per heterokaryon, the proportion of nuclei of each type and the ratio of multinucleate to mononucleate cells.

Fate of the virus. Since each heterokaryon received a large number of virus particles, the possibility existed that the virus inactivated by ultraviolet light might undergo multiplicity reactivation. Virus-specific haemadsorption (Vogel & Shelokov, 1957) was therefore studied in populations of heterokaryons produced by both infective and inactivated virus. The heterokaryons were produced in the usual way with various concentrations of virus, and two coverslips from each preparation were examined daily. The coverslips with adherent cells were washed once in Hanks's solution and placed in the cups of a haemagglutination tray; 0·5 ml of a 3% (v/v) suspension of guinea-pig erythrocytes in medium 199 was added to each cup and the tray left at room temperature for 2 h, during which time the erythrocytes settled evenly over the coverslips. These were then removed, washed 4 times in Hanks's solution, fixed in methanol and stained with May–Grünwald–Giemsa stain. The percentage of heterokaryons showing adsorption of one or more erythrocytes was then determined. The results of the experiment are summarized in Fig. 1. This shows that when infective virus was used to produce the heterokaryons, all of them exhibited the presence of surface haemagglutinin for at least 5 days. When the heterokaryons were made with inactivated virus, however, surface haemagglutinin disappeared from the cells at a rate which depended on the initial virus concentration. Moreover, with infective virus, the haemadsorption was massive and involved the whole of the cell surface; with inactivated virus, haemadsorption, in the decreasing number of cells which

showed it, involved a progressively smaller part of the individual cell surface. At the lowest concentration of virus (800 HAU/ml) less than 0·05% of the heterokaryons exhibited haemadsorption at 24 h. On subsequent days occasional heterokaryons (between 0·5 and 1%) did show haemadsorption, but it is clear that at least 99% of the cells did not produce any viral haemagglutinin during the 5-day period. The loss of haemagglutinin from the surface of the heterokaryons produced by inactivated virus presumably reflects the gradual destruction or elution of the initial inoculum.

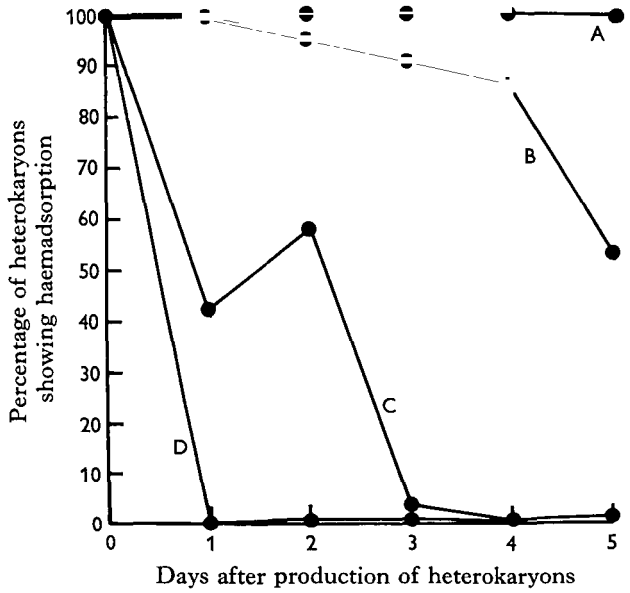

Fig. 1. Rate of loss of surface haemagglutinin in heterokaryons produced by infective virus and virus inactivated by ultraviolet light. A, infective virus at a concentration of 8000 HAU/ml. B, C, D, inactivated virus at concentrations of 80000, 8000 and 800 HAU/ml, respectively.

In order to determine whether heterokaryons produced by inactivated virus released any infectious virus into the medium, ten coverslips bearing such heterokaryons were transferred to fresh medium after 24 h cultivation and were then incubated in this medium for a further 48 h. At the end of this period ten-fold dilutions of the medium were made with Hanks's solution and 0·1 ml of each dilution inoculated into the allantoic cavity of two 10- or 11-day-old fertile hens' eggs. After incubation for 48 h at 37 °C the allantoic fluids were collected and their haemagglutinin content titrated. The results of this experiment are set out in Table 2, in which the calculated amount of infectious virus produced per heterokaryon is shown. (One unit of infectious virus is defined as the amount which produced 2 HAU/ml of allantoic fluid in 48 h.) It will be seen that, when the heterokaryons were made with inactivated virus at a concentration of 800 or 8000 HAU/ml, less than one heterokaryon in a thousand produced a single unit of infectious virus. With 80000 HAU of inactivated virus a yield of 1–10 units of infectious virus per heterokaryon was obtained. This does not, however, necessarily mean that multiplicity reactivation had occurred. Small amounts of virus

Table 2. *Production of infectious virus by heterokaryons*

Concentration of virus used to produce heterokaryons	Haemagglutinin titre (HAU/ml allantoic fluid) produced in 48 h by 0·1 ml of culture medium diluted as shown						Units of infectious virus produced per hetero-karyon
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
Virus inactivated by ultraviolet light							
800 HAU/ml	< 2	< 2	0	0	0	0	$< 10^{-3}$
	< 2	< 2	0	0	0	0	
8000 HAU/ml	< 2	< 2	< 2	0	0	0	$< 10^{-3}$
	< 2	< 2	< 2	0	0	0	
80000 HAU/ml	2048	512	512	< 2	< 2	< 2	1–10
	2048	—	512	64	< 2	< 2	
Infective virus							
8000 HAU/ml	512	2048	< 2	< 2	< 2	< 2	1–10
	512	256	256	256	< 2	< 2	

probably escaped inactivation by ultraviolet light, and these might have been responsible for the yield of infectious virus. When the heterokaryons were made with 8000 HAU/ml of virus which had not been inactivated, the yield of infectious virus was also only 1–10 units per heterokaryon. This indicates either that auto-interference had occurred or that the virus, even when it is not inactivated, grows poorly in this system. It is, in any event, clear that with moderate doses of inactivated virus at least 99% of the heterokaryons produce neither infectious virus nor viral haemagglutinin. In experiments in which the production of even trace amounts of infective virus is undesirable, anti-viral antiserum may be incorporated in the growth medium.

Synthesis of RNA. In order to study synthesis of RNA in the heterokaryons, cells maintained on coverslips were exposed to tritiated uridine for periods of 2–6 h and then fixed. Autoradiographs were made of the fixed preparations after the DNA and acid-soluble material had been extracted. These autoradiographs demonstrated that both sets of nuclei in the heterokaryons were synthesizing RNA. Cells exposed to the tritiated uridine for 2 h showed labelling over all the nuclei in the heterokaryons, with the heavy nucleolar labelling characteristic of animal cells exposed for short periods of time to a radioactive RNA precursor. Over a period of 5 days of cultivation, the intensity of nuclear labelling in the heterokaryons did not differ greatly from that seen in neighbouring mononuclear cells. Longer exposure to the tritiated uridine resulted in progressively heavier labelling of the cytoplasmic RNA. Fig. 9 shows a heterokaryon containing 3 HeLa and 2 Ehrlich ascites nuclei, from a 24-h culture exposed for 2 h to tritiated uridine. All the nuclei are heavily labelled and there is some labelling over the cytoplasm. The pattern of RNA synthesis in the hybrid cells is thus essentially similar to that seen in normal cells, and both Ehrlich and HeLa nuclei contribute to the process. In the heterokaryon, therefore, some of the genes of both mouse and man are being transcribed.

Synthesis of protein. Synthesis of protein was studied autoradiographically by observing the pattern of incorporation of tritiated leucine into hot acid-insoluble material. Heterokaryons cultivated on coverslips for periods of up to 5 days were exposed to tritiated leucine for 2–6 h and then fixed. The fixed preparations were treated with 0·3 N trichloroacetic acid at 90 °C for 30 min. Autoradiographs of heterokaryons treated in this way showed generalized labelling over both nuclei and cytoplasm, thus indicating that leucine had been incorporated into protein in these cells. Fig. 10 shows a heterokaryon containing 2 HeLa and 2 Ehrlich ascites nuclei from a culture exposed for 2 h to tritiated leucine. There is generalized labelling over the whole cell. The pattern of leucine incorporation in heterokaryons showed no features which might distinguish it from the pattern of leucine incorporation in normal cells.

Synthesis of DNA. Heterokaryons made with 8000 HAU of inactivated virus and containing an average of about 8 nuclei per cell were cultivated on coverslips for 7 days. Twenty-four hours after cell fusion, samples of these cultures were exposed for 2 h to tritiated thymidine and fixed. Autoradiographs were made of the fixed preparations. These autoradiographs showed that about 70% of the heterokaryons contained labelled nuclei. Of these labelled heterokaryons about 5% showed labelling over all the nuclei in the cell; in the rest labelled and unlabelled nuclei were present in varying proportions. In any one cell some nuclei of each type might be labelled and others not. Fig. 11 shows a heterokaryon containing 9 HeLa nuclei and 6 Ehrlich ascites nuclei, from a 24-h culture exposed for 2 h to tritiated thymidine. All the nuclei are labelled. Fig. 12 shows another heterokaryon from the same preparation. This cell contains 5 HeLa nuclei and 6 Ehrlich ascites nuclei. Only 2 Ehrlich ascites nuclei are labelled. The percentage of heterokaryons showing any form of nuclear labelling after 2-h exposure to tritiated thymidine fell from 70 at 24 h to 55 at 48 h, 44 at 4 days and 31 at 7 days, but throughout this period only a small proportion of the labelled cells showed synchronized DNA synthesis in their nuclei. In cultures exposed to tritiated thymidine 24 h after cell fusion about 80% of the Ehrlich ascites nuclei in labelled heterokaryons, but only about 30% of the HeLa nuclei, were found to be synthesizing DNA; and this ratio showed little change on subsequent days.

While it is thus clear that even after some days residence in a common cytoplasm some nuclei in these heterokaryons may be synthesizing DNA at any one time and others not, cells like that shown in Fig. 11 demonstrate that in some cases the cytoplasm none the less does succeed in establishing synchrony of DNA synthesis. If the nuclei in a heterokaryon synthesized DNA independently of each other, the probability of 15 nuclei being simultaneously in the phase of DNA synthesis as a result of chance would be negligible. It must therefore be supposed that in many of the heterokaryons in which all the nuclei are synthesizing DNA at the same time some cellular mechanism has operated to impose this synchrony. The very high proportion of Ehrlich ascites nuclei found to be synthesizing DNA in heterokaryons 24 h after cell fusion also indicates the operation of some form of synchronization. In a random population of Ehrlich ascites cells growing exponentially, about 50% of the nuclei would be synthesizing DNA at any one time; but in the heterokaryons this figure may be as high as 80%. The reason for the disparity between the Ehrlich ascites and

the HeLa nuclei in this respect is not clear. One possibility is that HeLa nuclei in the heterokaryon are slower to enter the phase of DNA synthesis than Ehrlich ascites nuclei; but a similar result would be obtained if the HeLa nuclei passed through this phase more rapidly than Ehrlich ascites nuclei and were then held up in the post-synthetic phase. Why synchrony of DNA synthesis should be achieved in some heterokaryons and not in others is at present obscure.

Mitosis and nuclear fusion. In heterokaryons made with high concentrations of virus and consequently containing large numbers of nuclei, mitosis may be markedly suppressed. Indeed, in the initial experiments with HeLa–Ehrlich heterokaryons mitosis was not observed in the multinucleate cells. Later work revealed, however, that the nuclei in the heterokaryons regularly entered mitosis if cultural conditions were favourable and the initial cytotoxic effect produced by the virus was not too great. In most heterokaryons only some of the nuclei in the cell entered mitosis together, while others remained in interphase, a situation apparently first observed in multinucleate animal cells by Flemming (1880) and recently described by Stubblefield (1964) in multinucleate cells induced by colcemid. In a small proportion of the heterokaryons, however, the nuclei entered mitosis synchronously. In cells containing a large number of nuclei the onset of nuclear mitosis was not accompanied by the formation of a spindle or division of the cell. The nuclei which had entered mitosis were usually reconstituted as a single unit, whose size depended on the number of nuclei involved. In cells in which all the nuclei entered mitosis together, post-mitotic reconstitution usually resulted in a single large nucleus often irregular in shape. Nuclear mitoses in the absence of spindle formation thus resulted in progressive nuclear fusion without cell division. After 48-h cultivation under favourable conditions heterokaryons could be seen showing all degrees of nuclear fusion, from cells which contained one fused nucleus together with a number of normal nuclei to cells in which all the constituent nuclei had fused together to form a single large mass. Fused nuclei could easily be recognized by their size and by the fact that they contained a much larger number of nucleoli than normal. Fig. 13 shows a cell in which all the original nuclei have fused together to produce a single large nucleus. In cells originally containing a small number of nuclei, however, synchronous nuclear mitosis is in some cases associated with the formation of a spindle and cell division. This has been clearly seen in a number of binucleate cells, but it may also occur in some cells containing more than two nuclei. In those binucleate cells in which cell division has been observed, the course of mitosis was apparently similar to that described by Fell & Hughes (1949) in binucleate mouse cells in tissue culture; only one spindle appeared to be formed, and cell division resulted in two daughter cells each with a single nucleus which was larger than normal and which apparently contained the chromosomal complements of both the original nuclei.

The fact that progressive nuclear fusion could occur in the absence of cell division suggested initially that this fusion took place between interphase nuclei. Interphase nuclei showing various degrees of fusion can indeed be seen in the heterokaryons, but there is now little doubt that the main mechanism of nuclear fusion is coalescence of nuclei during mitosis. Two additional pieces of evidence support this conclusion:

(1) There is an inverse relationship between the concentration of virus used to produce the heterokaryons and the rate of nuclear fusion. This relationship is illustrated in Fig. 2. It will be seen that with infective virus or high concentrations of inactivated virus, both the rate and the extent of nuclear fusion are markedly reduced: these concentrations of virus largely suppress mitotic activity in the heterokaryons. (2) In heterokaryons made with certain combinations of differentiated cells, no synthesis of DNA or mitosis takes place. In such heterokaryons, which will be discussed in detail in a later section, progressive nuclear fusion does not occur.

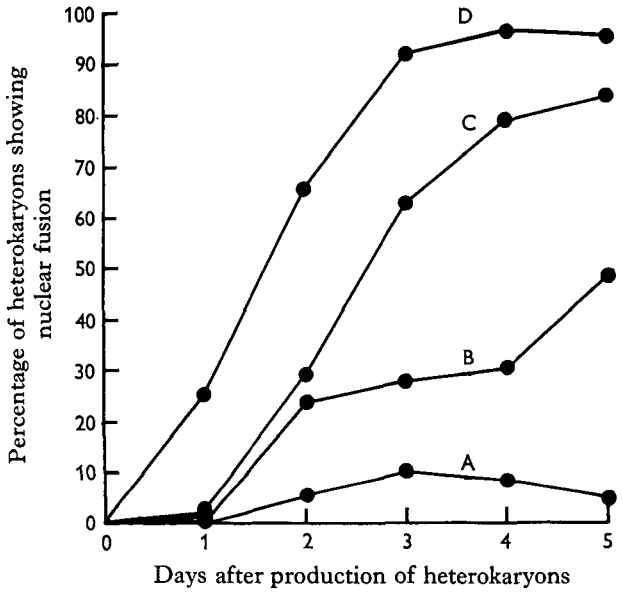

Fig. 2. Relationship between the proportion of heterokaryons showing nuclear fusion and the concentration of virus used to produce the heterokaryons. A, infective virus at a concentration of 8000 HAU/ml. B, C, D, inactivated virus at concentrations of 80000, 8000 and 800 HAU/ml, respectively.

For brevity, the term synkaryon has been adopted to describe a multinucleate cell in which the nuclei have fused together to form a single nucleus: homosynkaryon, where the original nuclei were of the same kind, and heterosynkaryon, where they were of different kinds.

Behaviour of mononucleate hybrid cells (synkaryons). Synkaryons synthesize protein, RNA and DNA, and are capable of undergoing mitosis. Some 48-h cultures of heterokaryons containing numerous synkaryons were exposed for 2 h to tritiated uridine; others at the same stage were exposed for 4 h to tritiated leucine and others for 2 h to tritiated thymidine. Autoradiographs of the preparations showed nuclear labelling in all synkaryons exposed to tritiated uridine, and generalized labelling in all synkaryons exposed to tritiated leucine. About 25% of the synkaryons were labelled by 2-h exposure to tritiated thymidine. Observations on living cultures revealed that many of the synkaryons entered mitosis, and this was confirmed in fixed stained preparations by the presence of synkaryons showing nuclei in prophase. Fig. 14 shows, in the same

field, two synkaryons in prophase and one dikaryon with both nuclei in synchronous prophase. In some cases synkaryons were seen to complete mitosis and give rise to two mononucleate daughter cells, each containing a large nucleus with numerous nucleoli resembling the nucleus of the parent synkaryon. In other cases, however, especially in very large synkaryons resulting from the fusion of many individual HeLa and Ehrlich ascites nuclei, the mitosis was not completed. The large mitotic cell remained rounded up for several hours, then lost its attachment to the glass, and eventually disintegrated. It should perhaps be pointed out that, in the great majority of cases, any mitosis in a synkaryon is already at least a second mitosis for the original fused cell, since the nuclear fusion is itself produced by mitosis in the heterokaryon. The subsequent history of HeLa–Erhlich heterosynkaryons was studied by chromosome analysis and will be discussed in the next section.

Analysis of chromosomes in hybrid cells. The chromosome count in 37 out of a sample of 40 Ehrlich ascites cells fell within the range 73–80 with a weak mode (9 cells) at 76. The counts in the remaining 3 cells were 62 (possibly a broken cell), 151 and 154. Thirty-four of the cells examined contained a long metacentric marker chromosome like that described by previous workers (Ising, 1958; Hauschka & Levan, 1958). Many cells also contained up to three additional marker chromosomes. Two of these were very similar, perhaps identical, long acrocentric chromosomes with prominent subterminal (distal) secondary constrictions. The third was a long acrocentric chromosome with a closely proximal secondary constriction (Fig. 15). Otherwise, the chromosomes were typical of the mouse, that is, they were all acrocentric with minute (or undetected) short arms.

Counts made on 53 HeLa cells revealed a sharp mode at 57 (36 cells), an 'inner' range of 54–59 and an extreme range of 48–82. This mode was much lower than any reported by previous workers (German, Evans, Cortner & Westfall, 1964). The chromosome set as a whole was clearly human in character. Three decisive marker chromosomes were identified, although detailed examination would almost certainly reveal others in the D, E, F and G groups. One of these marker chromosomes had an arm ratio of approximately 4:1, as in the B group, but was as long as, or even longer than, the presumptive no. 1 and no. 2 chromosomes. The other two markers correspond to the C group in length but have arm ratios of 5:1 or 6:1, one being somewhat longer than the other and having a relatively longer short arm. A cell with the modal number is illustrated in Fig. 16, and the markers are indicated. This cell contains 13 acrocentric chromosomes, of which 9 and 2, respectively, are not obviously distinct from the normal D and G groups, and 2 are intermediate in length between them. These are the only chromosomes of the HeLa karyotype that could be confused with any of the Ehrlich ascites chromosomes (other than the metacentric marker already mentioned).

Examination of a culture in which 2×10^7 HeLa cells and 2×10^7 Ehrlich ascites cells had been fused together 72 h previously with 8000 HAU of inactivated virus revealed that most of the mitotic cells at this stage were HeLa cells. Hybrid cells in mitosis (Figs. 17, 18) were also present and were easily identified by their large number of chromosomes and the high proportion of acrocentrics among them. Only one

example of an Ehrlich ascites cell in mitosis was found. The rarity of Erhlich ascites cells in mitosis is to be expected, in view of the fact that this strain of Ehrlich ascites cells does not grow *in vitro*. An unselected sample of 28 mitotic hybrid cells was examined, and counts ranging from 111 to approximately 412 chromosomes were recorded. Twenty-three of the counts are believed to be substantially correct and are set out in Table 3 in comparison with the expected counts from various combinations of Ehrlich ascites and HeLa nuclei, minimum, modal and maximum values being calculated from the modes and 'inner' ranges already given. On this basis 8 of the cells fall in the 1 Ehrlich/1 HeLa class, 5 in the 1 Ehrlich/2 HeLa class, 2 in the 1 Ehrlich/3 HeLa class, and 2 in the 2 Ehrlich/2 HeLa class. The remaining six cells could be products of fusion between cells with numbers of chromosomes lying outside the 'inner' range or, more probably, artifactual counts due to breakage of the rather fragile cells and loss of chromosomes during cytological processing. Of the 5 cells excluded from Table 3, one had 112 chromosomes but was quite clearly broken, 3 were estimated to contain 150, 300 and 300 chromosomes, respectively, 1 was the cell with 412 chromosomes already mentioned (Fig. 18). Two of the 28 cells exhibited large numbers of structural changes in the chromosomes, particularly 'double minutes', but other changes at both the chromatid and chromosome levels were also identified (Fig. 18). A detailed analysis was made of one of the cells (Fig. 17). Of a total of 181 chromosomes, 72 (including one marker) were identified as of Ehrlich ascites origin, 100 (including 2 markers) as from HeLa, and 9 could not be assigned to either. These are very close to the numbers expected from the fusion of one modal Ehrlich cell and two modal HeLa cells.

The foregoing analysis clearly confirms that hybrid cells produced by the fusion of HeLa and Ehrlich ascites cells and containing the two types of nuclei in various proportions are capable of undergoing mitosis. The unlikely possibility that these hybrid mitotic figures could have arisen by chance association of HeLa and Ehrlich ascites cells which have dried out together to give a single group of chromosomes can be ruled out on the grounds of the evenness of distribution of the chromosomes in many spreads and the rarity of free Ehrlich ascites cells in mitosis. This last consideration also eliminates the possibility that the hybrid mitotic figures could have arisen by coalescence of HeLa and Ehrlich ascites cells simultaneously in mitosis. The even-

Table 3. *Counts of the chromosomes in 23 Hela–Ehrlich hybrid cells*

Nuclear combination	Expected count			Actual counts
	Min.	Mode	Max.	
.	.	.	.	111, 116, 118
1E+1H	125	133	139	126, 128, 129, 131, 132, 132, 134, 137,
.	.	.	.	153, 171, 172
1E+2H	177	190	198	178, 181, 186, 189, 193
2E+1H	198	211	219	
1E+3H	229	247	257	230, 241
2E+2H	250	266	278	271, 275
3E+1H	271	287	299	

ness of distribution and thorough mixing of the chromosomes in most spreads strongly suggest that most of the hybrid mitotic figures seen at this stage arise from mitosis in synkaryons rather than from synchronous mitosis in multinucleate cells.

The question remains whether these hybrid cells can multiply continuously to give rise to a strain of mixed parentage. Populations of heterokaryons made with high doses of virus, and consequently containing large numbers of nuclei per cell, show no net growth: the number of hybrid cells remains more or less stationary for about 5 days and then gradually falls. It is therefore clear that most of these multinucleate cells do not give rise to viable progeny. This conclusion is borne out by the observation that mitosis in very large synkaryons is generally abortive, and by the fact that giant mitotic figures containing several chromosomal sets, like that shown in Fig. 18, disappear from the cultures within 5 or 6 days. In cultures examined 10 days after cell fusion, the only hybrid mitoses seen were those in which the number of chromosomes approximated to two chromosomal sets. Table 3 shows that even at 72 h, hybrids containing only one chromosomal set from each parent cell were already the commonest type of hybrid undergoing mitosis. It thus appears that, under the usual cultural conditions, only hybrids containing initially a small number of nuclei are capable of continued multiplication. This observation might explain why the hybrid cells discovered by Barski *et al.* (1960, 1961), which arose spontaneously in mixed cultures of mouse cell lines, and the great majority of similar 'spontaneous' hybrids subsequently isolated by Ephrussi *et al.* (1964) contained only one set of chromosomes from each parent. The possibility must, moreover, be considered that these 'spontaneous' hybrids are also produced by the action of viruses, especially in the light of recent experiments which indicate that para-influenza and other myxoviruses may be carried by cell lines in a latent form (Henle, 1964).

Further examination of the multiplication of HeLa–Ehrlich hybrids has been frustrated by the fact that these hybrids are eventually overgrown in the cultures by HeLa cells, and cultural conditions which might select for the hybrids have not so far been found. It is, however, of interest to note that since the initial account of the present experiments was published a rapidly growing strain of rat–mouse hybrid cell has been isolated from mixed cultures of rat and mouse cells by the use of selective media (Ephrussi & Weiss, 1965), and clones of interspecific hybrid cells have been grown from heterokaryons produced by fusing together a wide variety of cells from different species by means of inactivated Newcastle disease virus (Bayreuther, personal communication). It is therefore clear that hybrids derived from cells of different species are in many cases capable of prolonged multiplication. It will be of interest to observe what limitations there may be in this respect.

Dissociation of heterokaryons. During the course of some cinematographic studies on HeLa–Ehrlich heterokaryons an observation was made which seems worth reporting at this stage, because it offers interesting possibilities for genetical experiment. Among other material, a record was obtained of the behaviour of a dikaryon which had been produced 6 h previously by the fusion of an Ehrlich ascites cell and a HeLa cell. The Ehrlich nucleus and the HeLa nucleus were easily distinguishable, and the intimate mixing of the two cytoplasms was attested by the fact that organelles carried by

protoplasmic streaming moved freely throughout the cytoplasm of the cell. Over a period of 2 h the two nuclei moved towards the opposite poles of the cell, a constriction developed across its middle, and the two halves, each containing a single nucleus, finally separated. Two mononucleate cells were thus produced, one containing a HeLa nucleus and the other an Ehrlich ascites nucleus, but both with mixed HeLa–Ehrlich ascites cytoplasm. Four frames from the sequence illustrating the course of events are shown in Fig. 19, *a–d*. This phenomenon is essentially similar to that described by Wright & Lederberg (1957) in heterokaryons of yeast cells and should, in principle, lend itself to the same sort of genetic analysis. Three cases of dissociation of dikaryons have now been observed, so that the event cannot be of great rarity. There is some evidence to suggest that dissociation may be favoured by poor cultural conditions. Whether heterokaryons containing more than two nuclei can dissociate in this way is not known.

Heterokaryons made by fusing HeLa cells with differentiated somatic cells

Three types of differentiated cell were chosen for these experiments: macrophages from the peritoneal cavity of the rabbit; lymphocytes from the thoracic duct of the rat; and nucleated erythrocytes from the blood of the domestic fowl. These three cell types were chosen for the following reasons: (1) They represent, together with the HeLa cell, four different species and two different orders of vertebrate. (2) They are highly specialized cells. Indeed, in some respects, erythrocytes exhibit the most extreme form of differentiation seen in vertebrate cells. (3) The lymphocyte is an immunologically competent cell, that is to say, it is a cell capable of mounting an immune response to a foreign antigen. (4) The three cells show marked differences in their ability to synthesize nucleic acids. The macrophages which accumulate in the peritoneal cavity of the rabbit after intraperitoneal injection of liquid paraffin all synthesize RNA, but they do not synthesize DNA or undergo mitosis, either in the peritoneal cavity or *in vitro* (Watts & Harris, 1959). Synthesis of DNA has not so far been induced experimentally in these cells, although a family of macrophages which do synthesize DNA and undergo mitosis can be induced to accumulate in the peritoneal cavity of the mouse after immunological stimulation (Forbes & Mackaness, 1963). The small lymphocytes from the thoracic duct of the rat synthesize very variable amounts of RNA, but, like the rabbit macrophage, they do not synthesize DNA (Gowans & Knight, 1964). DNA synthesis leading to cell multiplication can, however, be induced in these cells when they are exposed to certain antigenic stimuli (Gowans, 1962). The mature nucleated erythrocytes of the hen do not synthesize DNA and synthesize RNA only in trivial amounts, if at all (Schweiger, Bremer & Schweiger, 1963; Cameron & Prescott, 1963). No condition has been described in which erythrocytes resume DNA synthesis or substantial RNA synthesis. It would be of great interest to know, especially in the case of the nucleated erythrocyte, whether the suppression of nucleic acid synthesis produced by differentiation is irreversible, or whether the dormant nuclei can be induced to resume the synthesis of RNA or DNA or both, when they are placed in the cytoplasm of a cell in which synthesis of both nucleic acids takes place in the normal way.

HeLa–rabbit macrophage heterokaryons. When 2×10^7 HeLa cells and 10^7 macrophages were treated with 8000 HAU of inactivated virus, preparations examined 24 h after cell fusion showed that approximately 30% of the multinucleate cells adherent to the coverslips contained both HeLa and macrophage nuclei. The rest contained either HeLa nuclei alone or macrophage nuclei alone. Fig. 20 shows a heterokaryon containing 1 HeLa nucleus and a number of macrophage nuclei. When 24 h cultures of these heterokaryons were exposed for 30 min to tritiated uridine, autoradiographs of preparations from which the DNA had been extracted showed that virtually all the nuclei, both HeLa and macrophage, were synthesizing RNA. Longer exposures to tritiated uridine resulted, as usual, in labelling of cytoplasmic RNA. Fig. 21 shows a heterokaryon containing 1 HeLa and 1 macrophage nucleus, from a culture exposed to tritiated uridine for 4 h. Both nuclei are heavily labelled, and there is also labelling over the cytoplasm. When 24-h cultures were exposed for 2 h to tritiated thymidine, about three-quarters of the HeLa–macrophage heterokaryons showed some form of nuclear labelling. In the majority of these labelled heterokaryons, as in the HeLa–Ehrlich heterokaryons, only some of the nuclei were labelled. At 24 h between 60 and 80% of the macrophage nuclei in labelled heterokaryons were synthesizing DNA, and about 30% of the HeLa nuclei. Fig. 22 shows an autoradiograph of a heterokaryon containing 1 HeLa and 1 macrophage nucleus, from a 24-h culture exposed for 2 h to tritiated thymidine. In this cell both nuclei are synthesizing DNA. None of the multinucleate cells which contained only macrophage nuclei was labelled under these conditions. This result indicates that macrophages which do not normally synthesize DNA, and which do not synthesize DNA when they are fused with each other, can be induced to do so when they are fused with cells in which DNA synthesis normally takes place. The problems posed by asynchronous DNA synthesis in HeLa–macrophage heterokaryons appear to be essentially similar to those discussed in the previous section with reference to asynchrony of DNA synthesis in HeLa–Ehrlich heterokaryons.

The nuclei of HeLa–macrophage heterokaryons undergo both synchronous and asynchronous mitosis in the same way as HeLa–Ehrlich heterokaryons, and progressive nuclear fusion results. Numerous synkaryons are thus formed which contain chromosomal complements from both the HeLa cell and the rabbit macrophage. These synkaryons are capable of RNA and DNA synthesis and some of them undergo mitosis. HeLa–macrophage synkaryons cannot easily be distinguished by their gross appearance or by their behaviour from HeLa–Ehrlich synkaryons. The characteristic locomotion of the macrophage is not seen in the HeLa–macrophage synkaryons, nor do these cells show any morphological features which might suggest a partly macrophage parentage. Although the question must, of course, be examined in terms of physiological functions such as chemotaxis and phagocytosis, it is difficult to avoid the impression that macrophages lose many of their specific features after fusion with HeLa cells, and the resulting synkaryons appear to behave essentially as large, undifferentiated tissue culture cells.

Multinucleate cells containing only macrophage nuclei fail to show any mitotic activity; and although occasional bridges are seen between the nuclei in such cells, progressive nuclear fusion does not occur. This finding supports the conclusion that

nuclear fusion is, in general, the result of mitosis. Multinucleate cells containing only macrophage nuclei degenerate within a few days.

HeLa–lymphocyte heterokaryons. It has been shown that the small lymphocytes in the thoracic duct do not incorporate thymidine into DNA; only the large lymphocytes, which constitute about 5 % of the cells in the thoracic duct, are labelled after exposure to tritiated thymidine (Gowans & Knight, 1964). However, when small lymphocytes are injected into an appropriate foreign host, a proportion of them undergo a transformation which is characterized by enlargement of the cell, involving both nucleus and cytoplasm, the development of prominent nucleoli and the onset of DNA synthesis. These transformed lymphocytes may undergo multiplication, and they or their progeny are thought to be responsible for mounting an immunological response to the foreign antigens to which they have been exposed (Gowans, 1962; Gowans, McGregor, Cowen & Ford, 1962). Between 50 and 80 % of the small lymphocytes in the thoracic duct show labelling of RNA after an exposure of 1 h to tritiated adenosine *in vitro* (Gowans & Knight, 1964). The amount of labelling is very variable, and many cells do not appear to become labelled within an hour, even with very high concentrations of radioactive RNA precursors (Gowans, unpublished).

About 2×10^7 HeLa cells and 10^8 lymphocytes were treated with 8000 HAU of inactivated virus. Preparations examined 24 h later showed that about 20 % of the multinucleate cells on the coverslip contained both HeLa and lymphocyte nuclei. Fig. 23 shows a heterokaryon containing 3 HeLa and 2 lymphocyte nuclei. At 24 h about two-thirds of the lymphocyte nuclei in heterokaryons still had the dimensions and morphological characteristics of typical small lymphocyte nuclei, but about a third of them had undergone a marked increase in size during this period. The chromatin of these larger nuclei was more dispersed than that of normal small lymphocytes, and apparent nucleoli were present in many cases. At 48 h about 80 % of the lymphocyte nuclei still discernible in heterokaryons showed these morphological changes in some degree. Fig. 24 shows a heterokaryon from a 48-h culture. This cell contains 3 HeLa nuclei and 1 lymphocyte nucleus which has undergone the transformation described. This nucleus is now much larger than a normal small lymphocyte nucleus and contains three structures which appear to be small nucleoli. These morphological changes resemble those which take place in the nuclei of small lymphocytes when these cells are injected into an appropriate foreign host (Gowans, 1962).

In order to determine whether lymphocyte nuclei in heterokaryons underwent any change in their ability to synthesize DNA, the cultures were exposed for 2 h to tritiated thymidine 3 h and 24 h after cell fusion. Observations were not possible much before 3 h, since this was the time taken by the heterokaryons to spread out over the glass coverslips. Autoradiographs of the fixed preparations showed that in the 3-h cultures about 10 % of all the lymphocyte nuclei in heterokaryons were labelled, but in the 24-h cultures this figure had risen to 40 %. Since not more than 2–3 % of the cells in the thoracic duct would be labelled after exposure for 2 h to tritiated thymidine *in vitro* (the fraction of the large lymphocytes in the phase of DNA synthesis), it is clear that DNA synthesis is initiated in many of the lymphocyte nuclei shortly after their inclusion in the heterokaryons. During the first 24 h increasing numbers of these

nuclei are recruited into the phase of DNA synthesis. Fig. 25 shows part of a heterokaryon from a 24-h culture exposed for 2 h to tritiated thymidine. This cell contains a number of HeLa nuclei and 1 lymphocyte nucleus. Only the lymphocyte nucleus is synthesizing DNA.

In order to determine the proportion of lymphocyte nuclei synthesizing RNA in heterokaryons, 24-h cultures were exposed to tritiated uridine for periods of 30 min to 2 h. Autoradiographs of fixed preparations from which the DNA had been extracted revealed that after 30 min exposure to tritiated uridine 95 % of all lymphocyte nuclei in heterokaryons were already labelled. Since between a quarter and a half of the small lymphocytes from the thoracic duct cannot be labelled with RNA precursors within an hour *in vitro*, it appears that in the heterokaryon RNA synthesis is initiated or substantially increased in many of the lymphocyte nuclei. Fig. 26 shows a dikaryon in which both the HeLa nucleus and the lymphocyte nucleus are synthesizing RNA.

Enlargement of the nucleus, appearance of nucleoli, stimulation of RNA synthesis and onset of DNA synthesis are features which the small lymphocyte shows when it is in the process of initiating an immune response to a foreign antigen. It was therefore possible that in HeLa–lymphocyte heterokaryons, the lymphocyte components might mount an immunological attack against the HeLa components and thus cause the breakdown of the heterokaryon. However, the behaviour of HeLa–lymphocyte heterokaryons did not appear to be any different from that of HeLa–Ehrlich or HeLa–macrophage heterokaryons. Progressive nuclear fusion occurred, and by the fourth day few discrete lymphocyte nuclei remained. Like HeLa–macrophage synkaryons, synkaryons produced by the fusion of HeLa and lymphocyte nuclei behaved like undifferentiated tissue culture cells and showed no features suggestive of their partly lymphocyte origin. These synkaryons also synthesized RNA and DNA, and some of them underwent mitosis.

In order to examine whether the presence of a lymphocyte component curtailed the survival time of HeLa–lymphocyte synkaryons, a population of multinucleate cells was studied in which the majority contained lymphocyte nuclei. When 10^7 HeLa cells and 10^8 lymphocytes were treated with 16 000 HAU of inactivated virus, the resulting multinucleate cells contained an average of about 8 nuclei, and 70 % of them contained at least one lymphocyte nucleus. Populations of heterokaryons containing such large numbers of nuclei per cell show no net growth; but the survival time of this population, in which 70 % of the cells contained lymphocyte nuclei, did not differ from a comparable population of HeLa–Ehrlich heterokaryons. In both cases the total number of multinucleate cells and synkaryons showed little change for about 5 days and then fell gradually. Many HeLa–lymphocyte synkaryons were still alive after 2 weeks. It thus appears that the life of a heterokaryon is not materially curtailed by the presence within it of a foreign lymphocyte nucleus. The significance of nuclear enlargement and initiation of DNA synthesis in the lymphocyte nuclei in HeLa–lymphocyte heterokaryons will become clearer after the behaviour of rabbit macrophage–rat lymphocyte heterokaryons has been discussed.

HeLa–hen erythrocyte heterokaryons. These heterokaryons differ from all those previously discussed in that the great majority of them contain erythrocyte nuclei but

no erythrocyte cytoplasm. As previously mentioned, the erythrocyte nuclei, in most cases, are incorporated into the HeLa cell after the red cells have been haemolysed by the virus. This means that any effects produced in the erythrocyte nucleus by residence in the heterokaryon are produced by HeLa cytoplasm alone. A mixture containing 2×10^7 HeLa cells and 0·2 ml of packed red cells was treated with 16000 HAU of inactivated virus. Examination of coverslip preparations 20 h later revealed that about 40% of all cells with 4 or more nuclei contained within their cytoplasm at least one erythrocyte nucleus. Fig. 27 shows a heterokaryon containing 2 HeLa nuclei and 7 erythrocyte nuclei.

The ability of intact nucleate erythrocytes to synthesize RNA and DNA was tested by incubating samples of these cells with tritiated uridine and tritiated thymidine. About 0·2 ml of packed red cells was suspended in 5 ml of culture medium containing in one case tritiated uridine and in the other tritiated thymidine. The cell suspensions were incubated at 37 °C for 3 h with gentle agitation, and films of the preparations were then made. Autoradiographs of these films, which in the case of the uridine sample were treated with deoxyribonuclease, were developed after exposure for 7 days. No significant labelling of red-cell nuclei was detected either in the thymidine or in the uridine samples. Fig. 28 shows an autoradiograph of a preparation of erythrocytes which had been incubated for 3 h with tritiated uridine. It is clear that the nuclei of these red cells do not, under the present conditions, incorporate these precursors into either RNA or DNA in amounts which can be detected by autoradiography.

In order to examine whether erythrocyte nuclei within heterokaryons could synthesize RNA or DNA, 24-h cultures containing HeLa–erythrocyte heterokaryons were also incubated with tritiated uridine or tritiated thymidine. In the case of tritiated uridine, the cells were fixed after 20 min incubation. This short period of incubation was chosen because under these conditions only the nuclei of the cells were labelled after 20 min; longer periods of exposure to the radioactive precursor resulted in some cytoplasmic labelling also. Since the small erythrocyte nuclei were embedded in a relatively large volume of HeLa cytoplasm, labelling of these nuclei would be obscured if the HeLa cytoplasmic RNA were also labelled. Autoradiographs of fixed preparations, from which the DNA had been extracted, were developed after 3 days exposure. These autoradiographs showed that approximately 60% of all erythrocyte nuclei within the heterokaryons were labelled. As an estimate of the number of erythrocyte nuclei synthesizing RNA, this figure is probably a low one: radiation from some of the erythrocyte nuclei deeply embedded in HeLa cytoplasm might fail to reach the photographic emulsion, and some nuclei which were not labelled after incubation for 20 min with tritiated uridine might have become labelled on longer incubation with the precursor. However, some of the erythrocyte nuclei in the heterokaryons appeared to be markedly pyknotic, and it is possible that these had been damaged during the fusion procedure. Fig. 29 shows a heterokaryon containing 4 HeLa nuclei and 4 erythrocyte nuclei from a culture exposed for 20 min to tritiated uridine. It will be seen that all the erythrocyte nuclei and the HeLa nuclei are labelled. Since the nuclei of intact red cells showed no labelling after incubation for 3 h with the

same concentration of tritiated uridine, even when the autoradiographs were exposed for 7 days, it appears that in the heterokaryon RNA synthesis has been initiated, or at least very greatly increased, in the majority of the erythrocyte nuclei.

When 24-h cultures of HeLa–erythrocyte heterokaryons were incubated for 2 h with tritiated thymidine, autoradiographs, developed after exposure for 3 days, showed that about 15 % of the erythrocyte nuclei in heterokaryons were synthesizing DNA. As in the case of other heterokaryons, synthesis of DNA did not usually occur synchronously in all the nuclei in the heterokaryon, although this was seen in some cases. Fig. 30 shows a heterokaryon containing 1 HeLa and 1 erythrocyte nucleus, from a 24-h culture which had been exposed for 2 h to tritiated thymidine. The erythrocyte nucleus is labelled. Since 7-day autoradiographs of intact red cells which had been exposed for 3 h to tritiated thymidine showed no labelling, it can be concluded that in the heterokaryon DNA synthesis has also been initiated in the erythrocyte nuclei. The subsequent behaviour of HeLa–erythrocyte heterokaryons did not differ from that of the other heterokaryons which have been described. Mitosis resulted in progressive nuclear fusion, and the synkaryons thus formed were indistinguishable from those produced by fusion of HeLa cells with Ehrlich ascites cells, macrophages or lymphocytes.

Heterokaryons made by fusing differentiated cells with each other

The results described in the preceding section make it clear that the processes of differentiation which result in the suppression of DNA or RNA synthesis in the nuclei of specialized cells are reversible. The nuclei of these cells can be induced to resume RNA synthesis or DNA synthesis or both when they are incorporated into the cytoplasm of a HeLa cell which normally synthesizes both RNA and DNA. It is not, however, clear whether the initiation of DNA synthesis and RNA synthesis in these differentiated nuclei is due to the fact that the HeLa cell has the ability to synthesize DNA and RNA, or whether these nuclei react in this way as a consequence of finding themselves in a foreign cytoplasm. This question was investigated by studying the behaviour of heterokaryons produced by fusing rabbit macrophages with rat lymphocytes and with hen erythrocytes.

Rabbit macrophage–rat lymphocyte heterokaryons. In this case the two parent cells are derived from different species, but neither of them normally synthesizes DNA. All the macrophages and the majority of the lymphocytes normally synthesize RNA. The heterokaryons were made by treating 2×10^7 macrophages and 5×10^7 lymphocytes with 8000 HAU of virus. About 20 % of the resulting multinucleate cells contained both macrophage and lymphocyte nuclei, which could be readily distinguished by their size and by the fact that lymphocyte nuclei stained more deeply with Leishman or Giemsa stain. A striking feature of these heterokaryons was the peripheral distribution of their nuclei. A typical heterokaryon containing 3 macrophage and 2 lymphocyte nuclei is shown in Fig. 31. It will be seen that the morphology of these cells is essentially similar to that of the 'Langhans'-type giant cells found in certain inflammatory lesions, such as tuberculosis, and also thought to be produced by the fusion of macrophages. This characteristic morphology was seen when macrophages

were fused with each other, with lymphocytes or with erythrocytes, but not when macrophages were fused with HeLa cells. In the latter case, as previously described, the appearance and behaviour of the heterokaryon was dominated by HeLa features.

Twenty-four-hour cultures containing rabbit macrophage–rat lymphocyte heterokaryons were incubated for 30 min to 2 h with tritiated uridine. Autoradiographs of fixed preparations from which the DNA had been extracted showed that after 30 min exposure to the radioactive precursor all the nuclei in the heterokaryons were labelled. Both macrophage and lymphocyte nuclei were therefore synthesizing RNA. Fig. 32 shows an autoradiograph of a heterokaryon containing 2 macrophage nuclei and 1 lymphocyte nucleus, from a culture exposed for 30 min to tritiated uridine. All the nuclei are labelled. Longer incubation with tritiated uridine resulted in labelling of the cytoplasmic RNA.

However, when these heterokaryons were incubated for periods up to 4 h with tritiated thymidine, either 24 h after cell fusion or on subsequent days, autoradiographs showed no labelling of any of the nuclei in the heterokaryons at any time. The DNA synthesis which took place in lymphocyte nuclei in heterokaryons with HeLa cells was not therefore simply due to the lymphocyte nuclei finding themselves in a foreign environment. Rat lymphocyte nuclei in heterokaryons with rabbit macrophages were also in a foreign environment, but in this case no synthesis of DNA took place. It thus appears that it is the ability of the HeLa cell to synthesize DNA which determines this synthesis in the lymphocyte nuclei in the HeLa–lymphocyte heterokaryons. The rabbit macrophage, which lacks the ability to synthesize DNA, fails to initiate DNA synthesis in the lymphocyte nuclei in macrophage–lymphocyte heterokaryons. Moreover, the morphological changes which take place in lymphocyte nuclei in HeLa–lymphocyte heterokaryons, namely, enlargement, dispersion of chromatin and appearance of structures resembling nucleoli, do not take place in the lymphocyte nuclei in macrophage–lymphocyte heterokaryons. In the latter case the lymphocyte nuclei remain recognizable as typical small lymphocyte nuclei throughout the life of the heterokaryon. These morphological changes are not therefore a necessary consequence of the lymphocyte nucleus finding itself in a foreign environment, nor are they necessarily indicative of an immunological response on the part of the lymphocyte. They appear to be visible signs of the process by which a dormant, or relatively inactive, nucleus is transformed into one in which the DNA may be replicated and the synthesis of RNA initiated or greatly increased. In the light of recent studies associating condensation of chromatin with inactivity, it seems possible that enlargement of the lymphocyte nucleus and an increase in the state of dispersion of its chromatin may be the actual mechanism by which stimulation of nucleic acid synthesis is effected. The nuclei of macrophage–lymphocyte heterokaryons do not undergo mitosis, nor do they fuse together, thus providing further evidence in support of the conclusion that nuclear fusion is a consequence of mitosis. These heterokaryons degenerate within a few days, and there is no appreciable difference between the survival time of multinucleate cells containing both macrophage and lymphocyte nuclei and those containing macrophage nuclei alone.

Rabbit macrophage–hen erythrocyte heterokaryons. Macrophage–erythrocyte hetero-

karyons were made by treating a mixture of 2×10^7 macrophages and 0·1 ml of packed red cells with 8000 HAU of virus. The erythrocyte nuclei were incorporated into the macrophage cytoplasm more readily than into HeLa cytoplasm, and most multinucleate cells contained both types of nuclei. As in the case of macrophage–lymphocyte heterokaryons, the nuclei of macrophage–erythrocyte heterokaryons were located at the periphery of the cell. Fig. 33 shows a heterokaryon containing 4 macrophage nuclei and 3 erythrocyte nuclei. The distribution of the nuclei is typical of that seen in 'Langhans'-type giant cells. Many of the erythrocyte nuclei incorporated into the macrophage cytoplasm appeared pyknotic and had apparently been damaged during the fusion procedure; others showed no obvious morphological abnormalities. This difference is also observable in electron micrographs. Fig. 35 shows an electron micrograph of a section through a heterokaryon containing an erythrocyte nucleus and a macrophage nucleus. The erythrocyte nucleus shows no evidence of structural damage and has established normal relationships with the cytoplasm of the cell. As in the neighbouring macrophage nucleus, a typical perinuclear cisterna is present, and nucleo-cytoplasmic bridges have been formed. Fig. 36 shows a section through a cell containing a macrophage nucleus and an apparently damaged erythrocyte nucleus. In this case the internal structure of the erythrocyte nucleus is denser and more homogeneous than normal, perhaps reflecting the pyknosis seen with the light microscope in stained preparations. There is no perinuclear cisterna and nucleo-cytoplasmic bridges have not been established. Why some erythrocyte nuclei should be damaged during the fusion procedure, and others not, is not clear, but the difference may be related to the length of time that a particular nucleus is exposed to the suspending medium.

Autoradiographs of macrophage–erythrocyte heterokaryons exposed for 30 min to tritiated uridine showed labelling of all the macrophage nuclei and about 15% of the erythrocyte nuclei, presumably those which were not damaged during the fusion procedure. Fig. 34 shows a heterokaryon containing 4 macrophage nuclei and 1 erythrocyte nucleus from a 24-h culture exposed for 30 min to tritiated uridine. All the macrophage nuclei and the erythrocyte nucleus are labelled. Longer incubation with tritiated uridine labels the cytoplasmic RNA also. When these heterokaryons were incubated with tritiated thymidine, no labelling of any of the nuclei occurred at any time during the life of the cell. Since intact erythrocytes are not labelled by either tritiated uridine or thymidine, it is clear that the macrophage, which itself normally synthesizes RNA, has initiated RNA synthesis in a proportion of the erythrocyte nuclei in the heterokaryons. Neither of the parent cells synthesizes DNA, however, and no DNA synthesis occurs in the heterokaryon. Macrophage–erythrocyte heterokaryons show no mitosis or nuclear fusion and degenerate within a few days.

The observations on the behaviour of differentiated cells in heterokaryons are summarized in Table 4. Certain principles emerge: (1) If either of the parent cells normally synthesizes RNA, then RNA synthesis will take place in both types of nuclei in the heterokaryon, even if one of the parent cells normally does not synthesize RNA. (2) If either of the parent cells normally synthesizes DNA, then DNA synthesis will take place in both types of nuclei in the heterokaryon, even if one of the parent cells normally does not synthesize DNA. (3) If neither of the parent cells synthesizes

Table 4. *Synthesis of RNA and DNA in heterokaryons*

	RNA		DNA	
Cell Type				
HeLa	+		+	
Rabbit macrophage	+		o	
Rat lymphocyte	+		o	
Hen erythrocyte	o		o	
Cell combination in heterokaryon				
HeLa–HeLa	+	+	+	+
HeLa–rabbit macrophage	+	+	+	+
HeLa–rat lymphocyte	+	+	+	+
HeLa–hen erythrocyte	+	+	+	+
Rabbit macrophage–rabbit macrophage	+	+	o	o
Rabbit macrophage–rat lymphocyte	+	+	o	o
Rabbit macrophage–hen erythrocyte	+	+	o	o

o, No synthesis in any nuclei; o o, no synthesis in any nuclei of either type; + synthesis in some or all nuclei; + +, synthesis in some or all nuclei of both types.

DNA, DNA synthesis will not take place in the heterokaryon. It will be noticed that in all cases where a cell which synthesizes a particular nucleic acid is fused with one which does not, the active cell initiates the synthesis of this nucleic acid in the inactive partner. In no case does the inactive cell suppress synthesis in the active partner.

DISCUSSION

Perhaps the most important conclusion to be drawn from these experiments is the fact that cells from different species of vertebrate are compatible with each other when they are fused together into a single unit. This is true for highly specialized cells and even for cells capable of mounting an immune response to a foreign antigen. It thus appears that in the somatic cells of vertebrates there are no intracellular incompatibility systems comparable to those which determine histocompatibility in different individuals. Not only do the cytoplasms of these different cells fuse amicably together, but their nuclei also; and when nuclear fusion has occurred the composite cell carries out its functions in a perfectly integrated way and may in some cases even undergo prolonged multiplication.

This remarkable integration throws an interesting light on the mechanisms of genetic regulation. Whatever cytoplasmic signals are responsible for the control of nucleic acid synthesis in animal cells, these signals cannot be species specific. Hen erythrocyte nuclei, which are normally present in heterokaryons without erythrocyte cytoplasm, are induced to synthesize RNA by the cytoplasm of rabbit macrophages, and to synthesize both RNA and DNA by the cytoplasm of human HeLa cells. If, as has been proposed, the mechanism underlying this regulation is the reversible attachment of specific cytoplasmic repressors to specific regulatory genes, the cytoplasmic repressors of one species must be able to recognize the regulatory genes of another; and if recog-

nition of the relevant genes involves the recognition of base sequence, one must suppose that these genes have identical or very similar base sequences throughout much of the vertebrate sub-phylum. Moreover, in heterokaryons, regulation of nucleic acid synthesis is essentially unilateral: dormant nuclei are induced to resume the synthesis of RNA or DNA, but active nuclei are not repressed. In terms of a model in which the cytoplasmic repressors recognize the regulatory genes of both types of nuclei, this finding is rather surprising; but it could be accommodated by supposing that the active cell contains compounds which inhibit the cytoplasmic repressors of both cell types and remove them from the genes to which they are attached.

It is, however, possible that the regulation is much less specific than this. Synthesis of RNA might cease because the cytoplasm of the cell has lost some essential part of the machinery for carrying out this synthesis: perhaps the concentration of some enzyme or some substrate has fallen below a certain critical level. In this case there is no difficulty in understanding why dormant nuclei are induced to resume the synthesis of nucleic acid, but active nuclei are not repressed. A mechanism of this sort implies that at least some of the genetic material in the inactive cell, for example the erythrocyte, is available for RNA synthesis, but does not produce RNA as a secondary, and, as far as individual genes are concerned, unselective consequence of cytoplasmic failure. If, in the heterokaryon, RNA is synthesized in the first instance on those parts of the genetic material of the dormant nucleus which are accessible, this synthesis must also be essentially unselective, since there is no reason why the parts of the genetic material which remain accessible in the erythrocyte should contain precisely those genes which need to be activated in the heterokaryon.

Failure of cytoplasmic synthetic mechanisms may well be responsible for the total cessation of RNA synthesis which occurs terminally in erythrocytes (Schweiger *et al.* 1963); but the partial suppression of RNA synthesis seen in other differentiated cells does appear to be associated with some restriction of the accessibility of the genetic material. The observations made in the present experiments lend support to the view that this restriction is effected by a change in the chromatin which leads it to assume a more tightly packed configuration. Differentiated nuclei which show severe limitations in their ability to synthesize RNA are small and stain deeply; when fusion with other cells induces these nuclei to resume RNA synthesis or greatly increase it, they enlarge and their chromatin becomes much more dispersed. There is evidence that a process of this sort can involve a single chromosome (Evans, Ford, Lyon & Gray, 1965), a haploid set of chromosomes (Berlowitz, 1965) and, apparently, a whole nucleus; but one may doubt whether such a mechanism would have the discrimination necessary to activate or inactivate single genes.

Is it, indeed, necessary to invoke the activation and inactivation of single genes or small groups of genes to explain the process of differentiation? Those who advocate this point of view apparently overlook the cardinal fact that differentiation can take place, and indeed can go to completion, in the absence of the nucleus (Hämmerling, 1963). All the regulation necessary to effect the complete differentiation of a highly specialized cell can be carried out after its nucleus has been removed; and this regulation involves not only morphological events but also the differential synthesis

of specific enzymes (Spencer & Harris, 1964). This fact does not conflict with observations which suggest that some higher cells do possess mechanisms which activate and inactivate small groups of genes; but it does make it clear that this activation and inactivation is not the immediate determinant of those cytoplasmic events which directly regulate enzyme synthesis and which govern morphological differentiation.

An interesting feature of heterokaryons made by fusing differentiated cells with HeLa cells is that the differentiated cells do not appear to impose the gross characteristics of their differentiation on the hybrid cell, even when several differentiated cells are fused with one HeLa cell. In the cases so far studied the characteristic morphological features of the differentiated cell appear to be rapidly lost. This again could be explained by selective reprogramming of the genetic material of the differentiated cells, but on this view it is difficult to see why the genetic material of the HeLa cell does not undergo reprogramming, especially in cases where the differentiated cells contribute most of the cytoplasm of the hybrid. In the light of what has already been said about the mechanism of differentiation, the possibility should be considered that the 'dedifferentiation' which takes place in the heterokaryon is, in the first instance, a cytoplasmic reaction which the differentiated cell undergoes in response to the new situation; the nuclear changes may be secondary.

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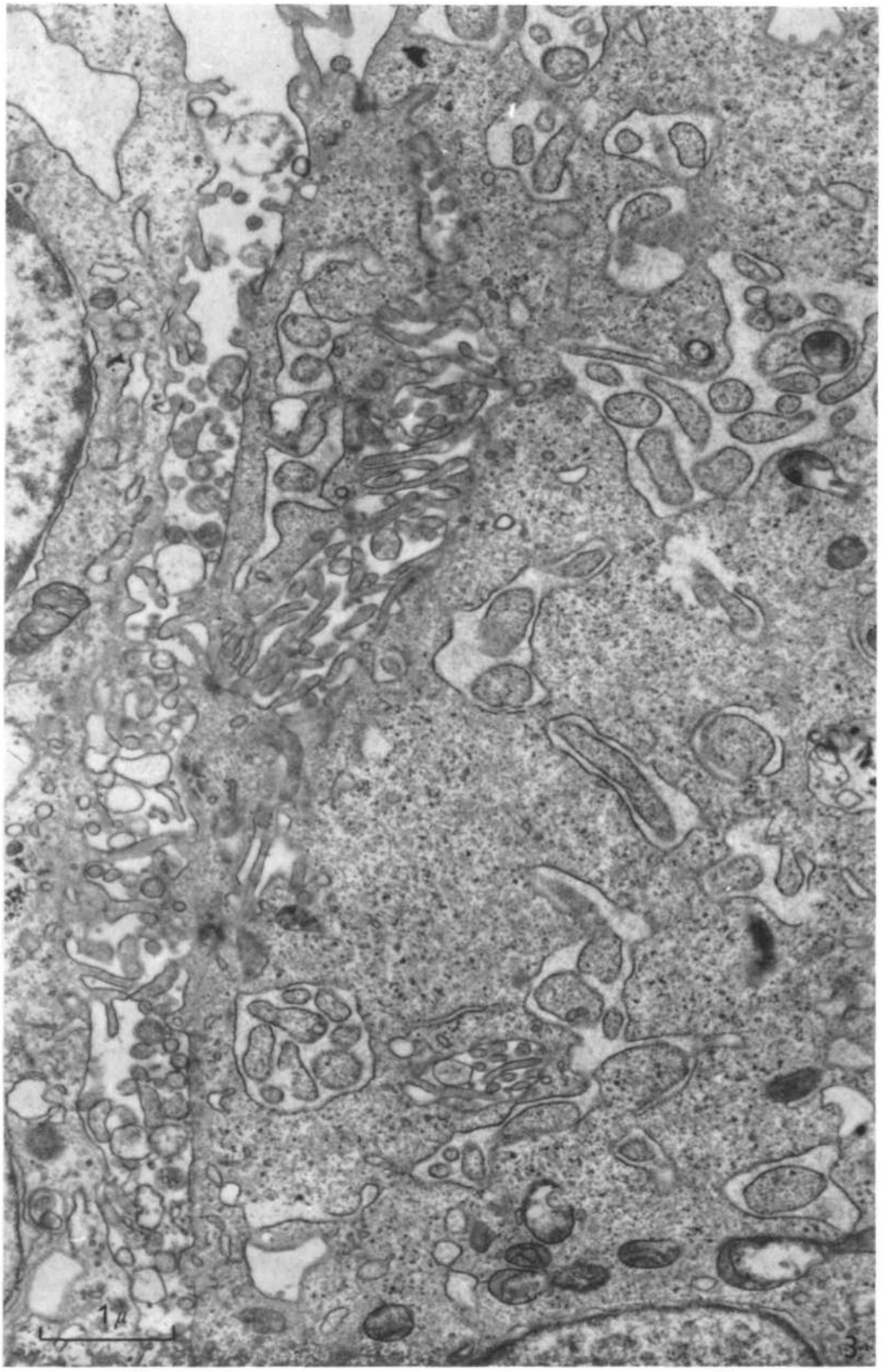


Fig. 3. Electron micrograph of part of the area of contact between two adjacent cells treated 40 min previously with inactivated virus. The cell surfaces are densely covered with microvilli.

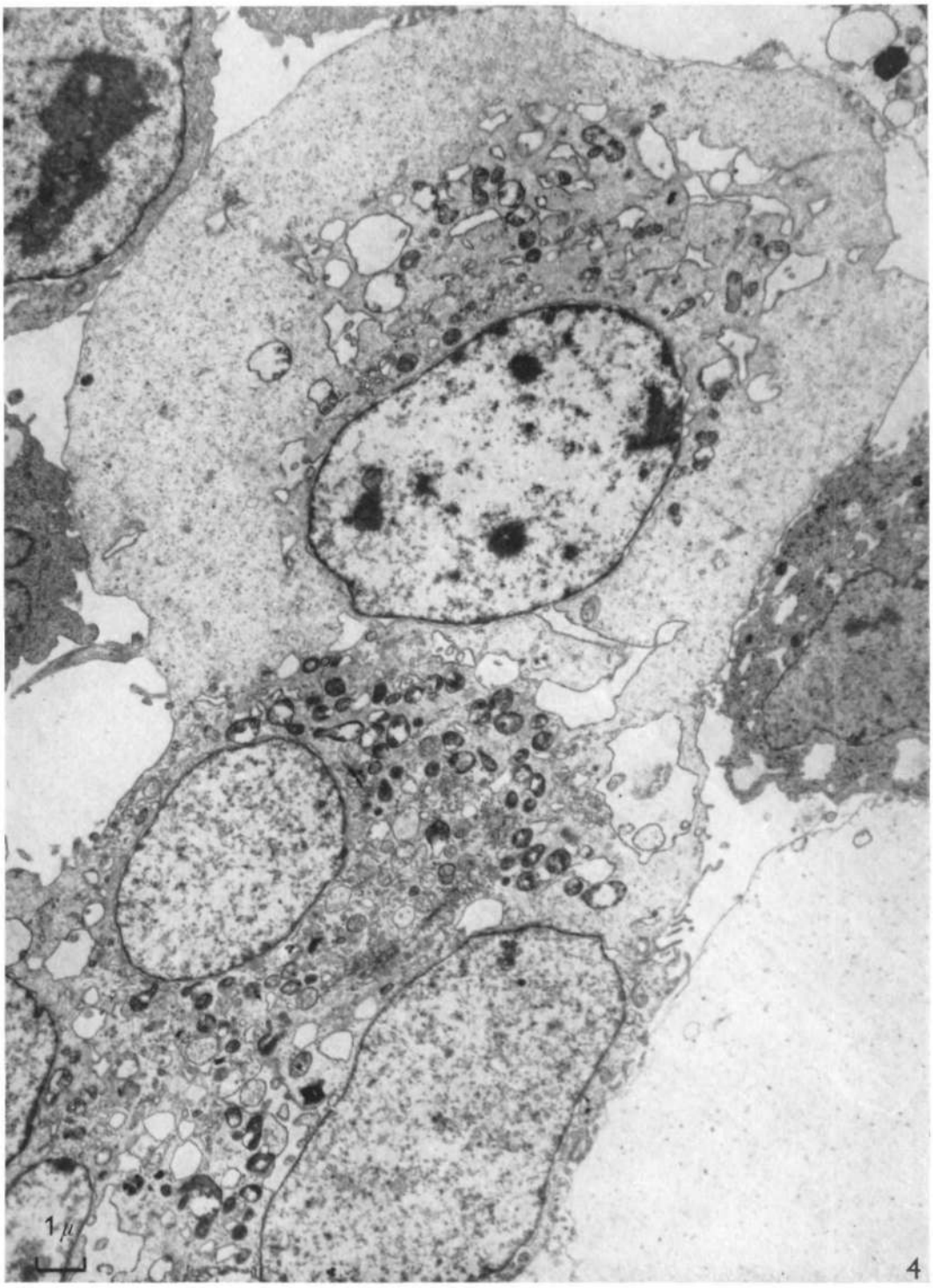


Fig. 4. Electron micrograph of an early stage of fusion between two cells. Note the high concentration of cell organelles along the line of fusion in one of the cells and their relative absence in the other.

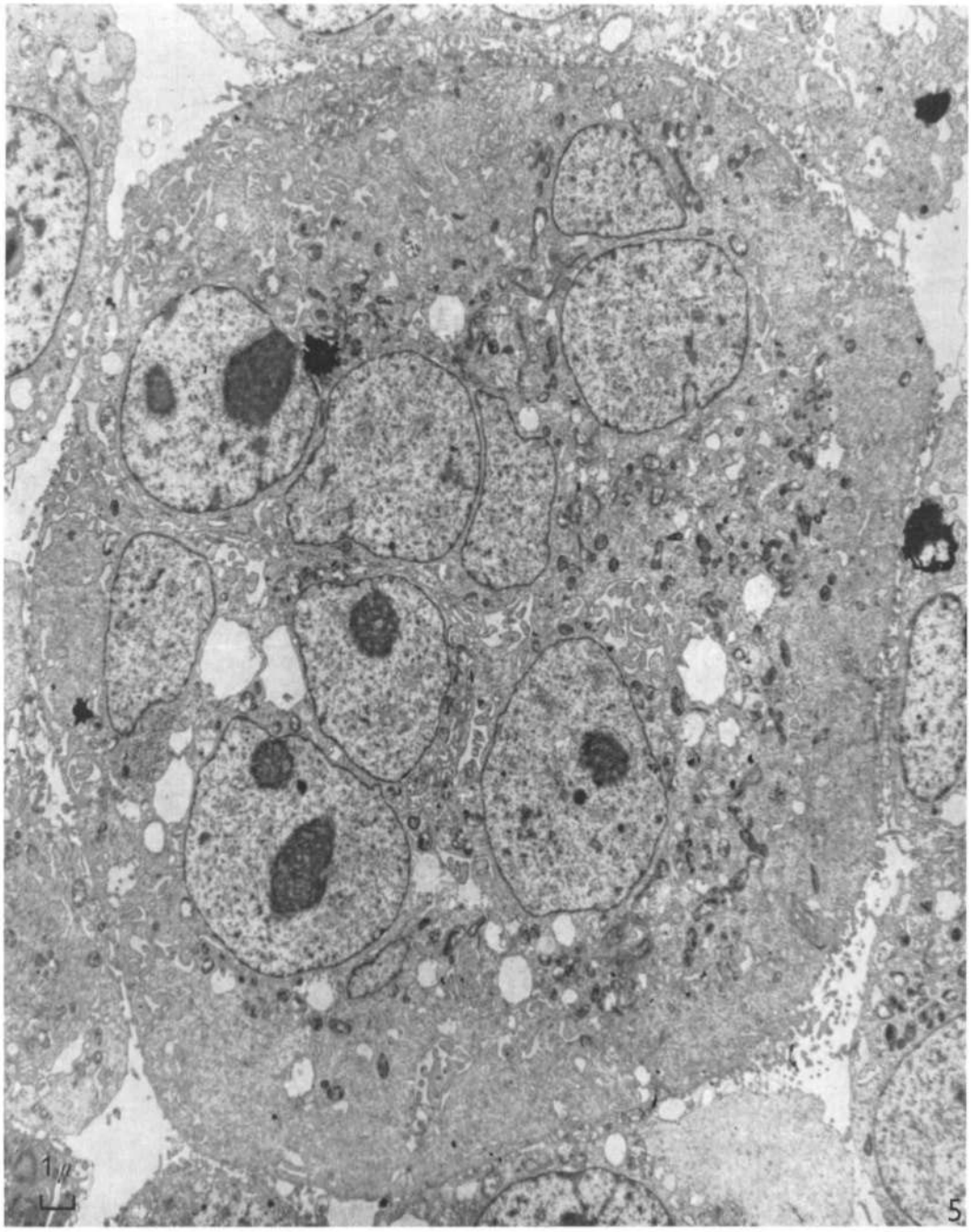


Fig. 5. Low-power electron micrograph of a multinucleate cell produced by the fusion of HeLa and Ehrlich ascites cells.

Fig. 6. Electron micrograph of a single cell in the process of fusing with a large multinucleate cell.

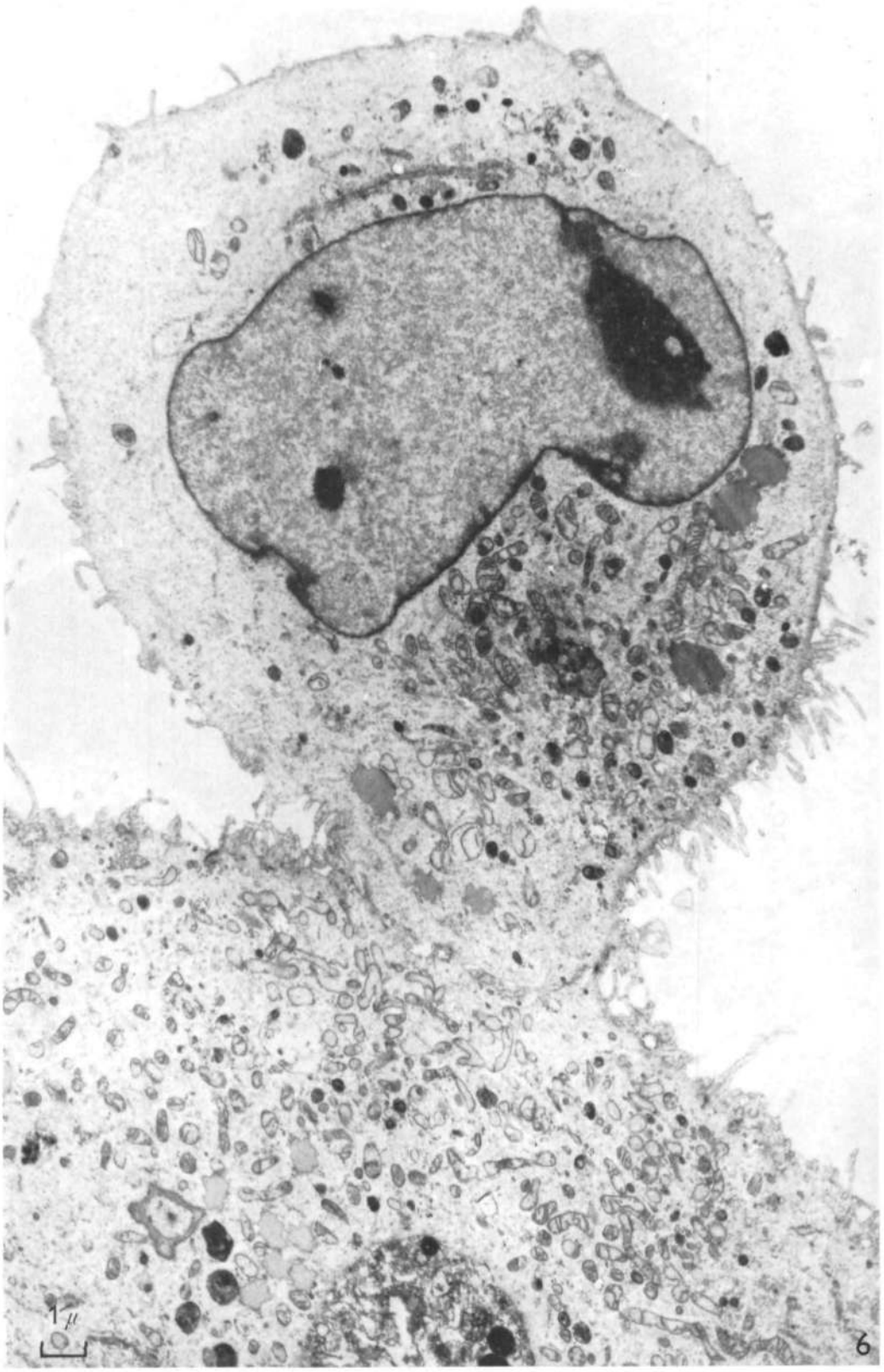
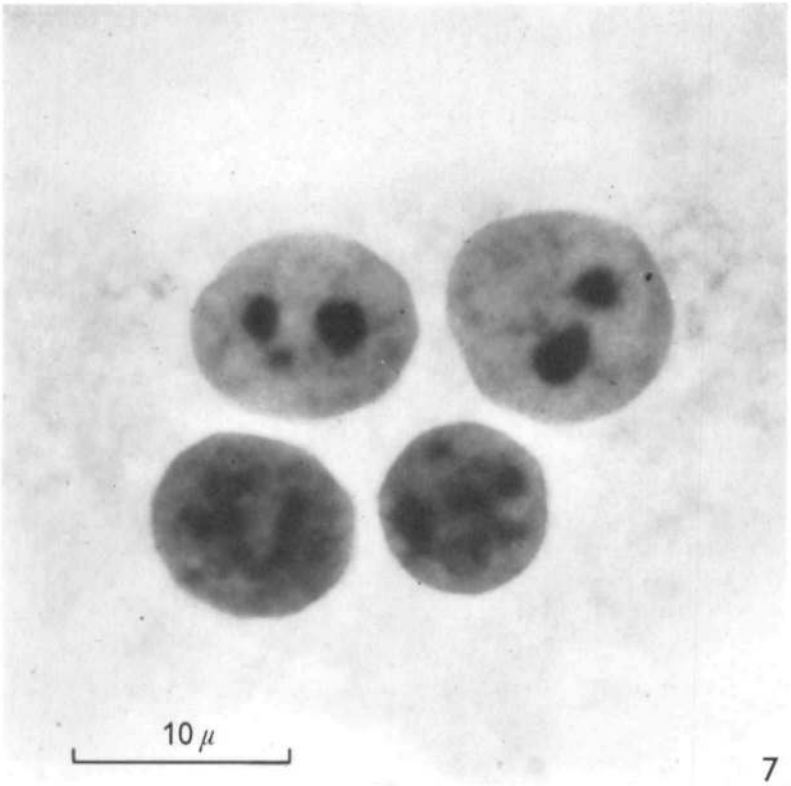

1 μ
6

Fig. 7. A tetranucleate cell in which the 2 upper nuclei are derived from HeLa cells and the 2 lower ones from Ehrlich ascites cells.

Fig. 8. Autoradiograph of a tetranucleate cell containing 3 HeLa nuclei and 1 Ehrlich nucleus. The HeLa cells had been grown in tritiated thymidine before the heterokaryons were produced. The HeLa nuclei are labelled and the Ehrlich ascites nucleus is not.



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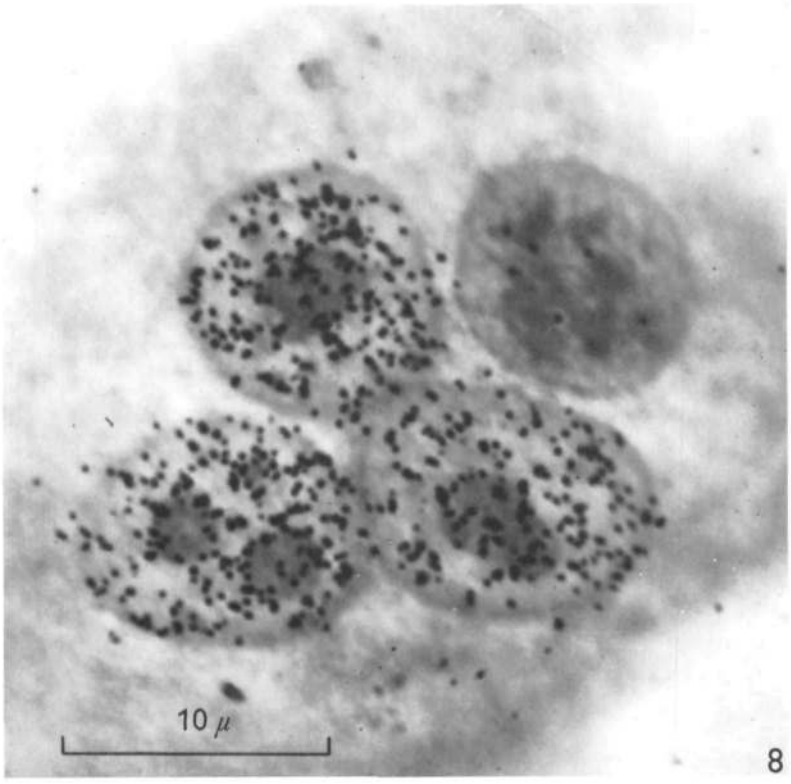


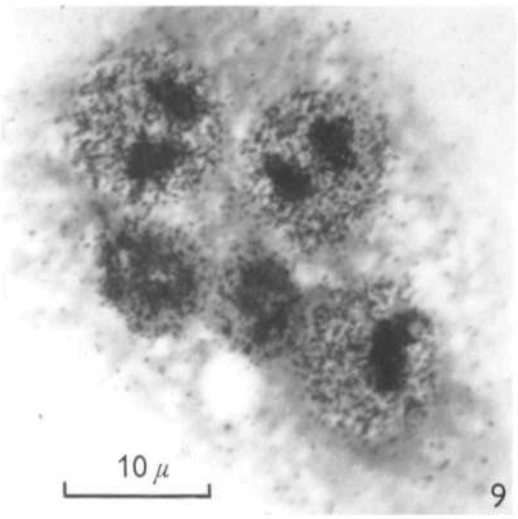
8

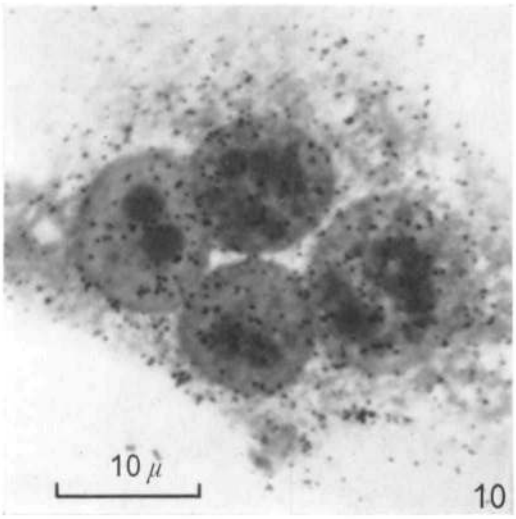
Fig. 9. Autoradiograph of a heterokaryon containing 3 HeLa and 2 Ehrlich nuclei, from a 24-h culture exposed for 2 h to tritiated uridine. All the nuclei are heavily labelled and there is some labelling over the cytoplasm.

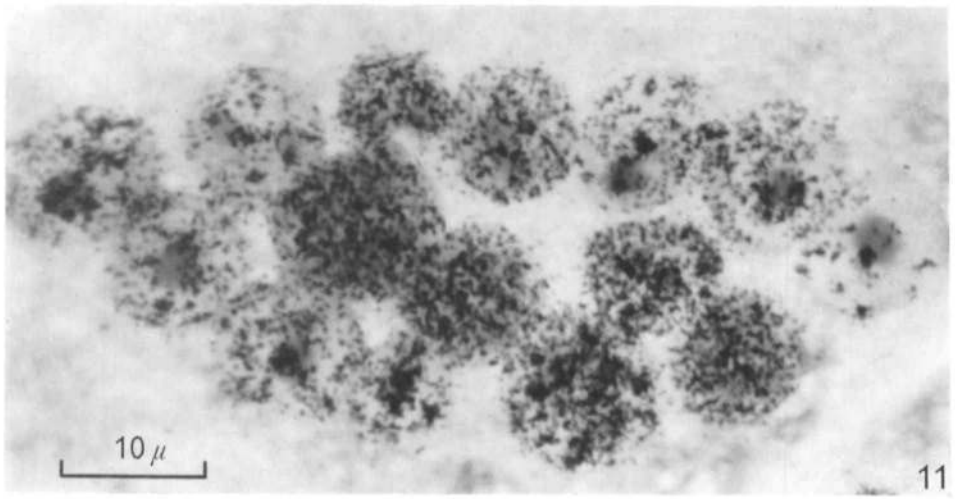
Fig. 10. Autoradiograph of a heterokaryon containing 2 HeLa and 2 Ehrlich nuclei, from a culture exposed for 2 h to tritiated leucine. There is generalized labelling over the whole cell.

Fig. 11. Autoradiograph of a heterokaryon containing 9 HeLa and 6 Ehrlich nuclei, from a 24-h culture exposed for 2 h to tritiated thymidine. All the nuclei are labelled.

Fig. 12. Another heterokaryon from the same preparation as Fig. 11. This cell contains 5 HeLa and 6 Ehrlich nuclei, but only 2 of the Ehrlich nuclei are labelled.


10 μ
9


10 μ
10


10 μ
11

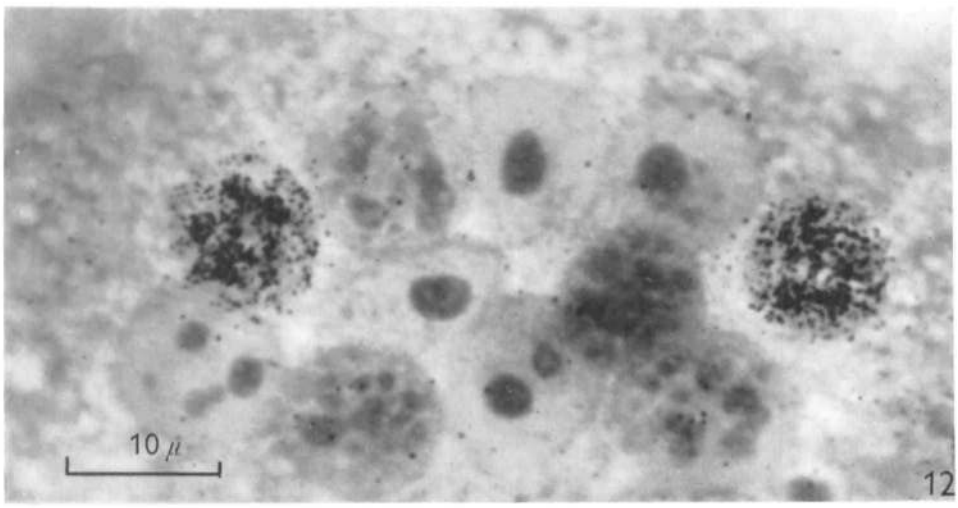
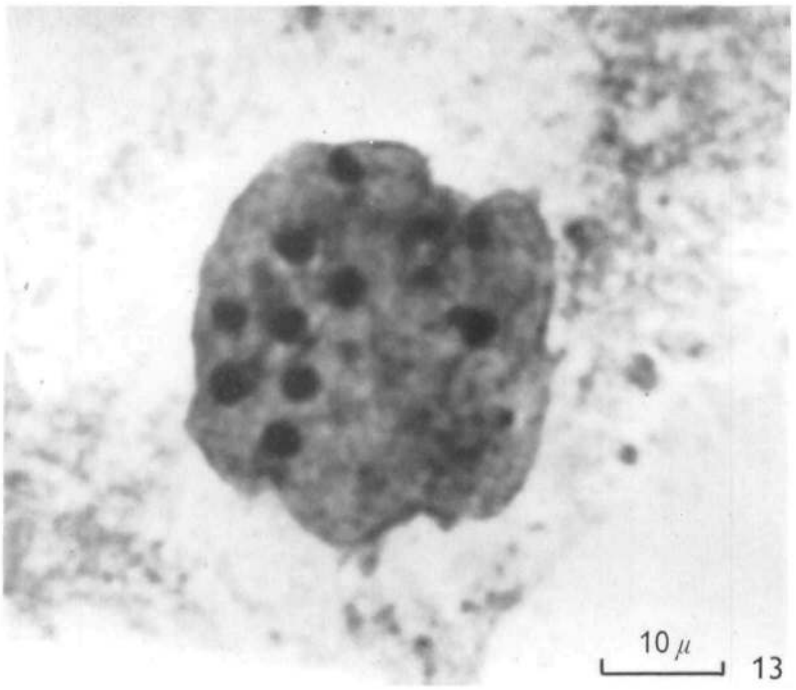

10 μ
12

Fig. 13. A cell in which all the original nuclei have fused together to produce a single large nucleus. This nucleus is irregular in shape and contains many nucleoli.

Fig. 14. A preparation showing, in the same field, two synkaryons in prophase and one dikaryon with both nuclei in synchronous prophase.


10 μ
13

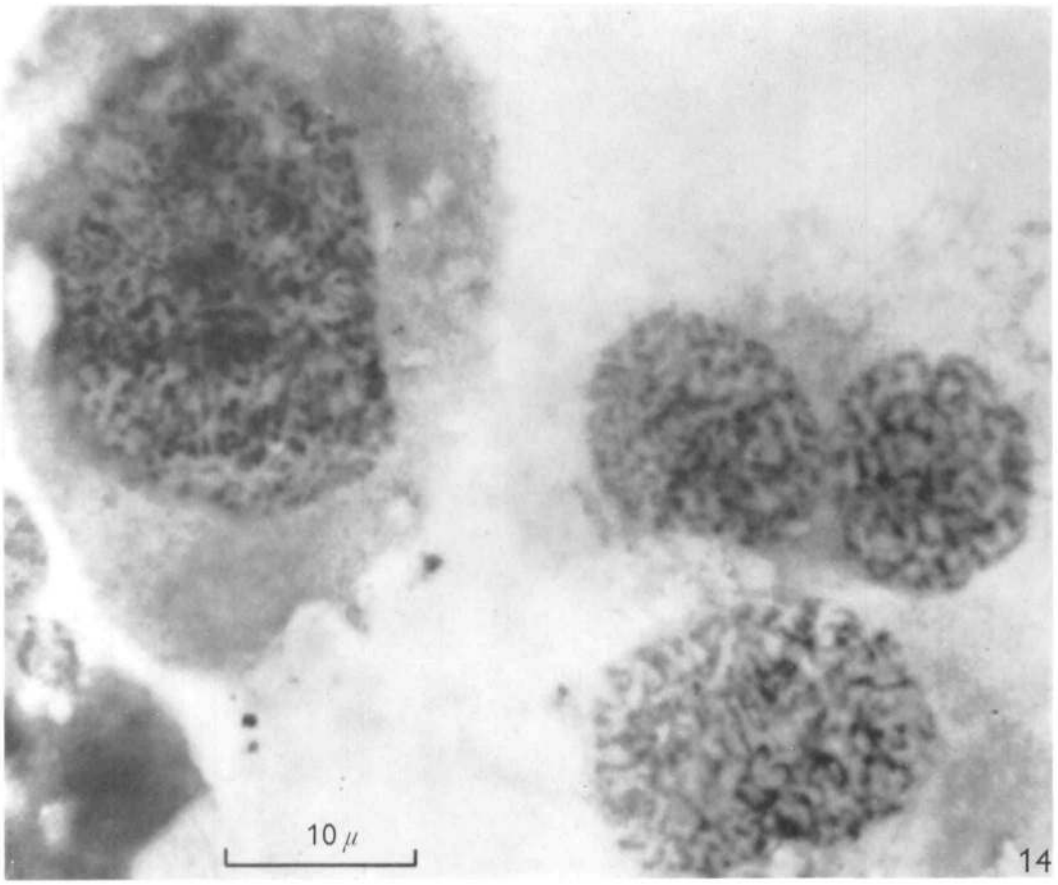
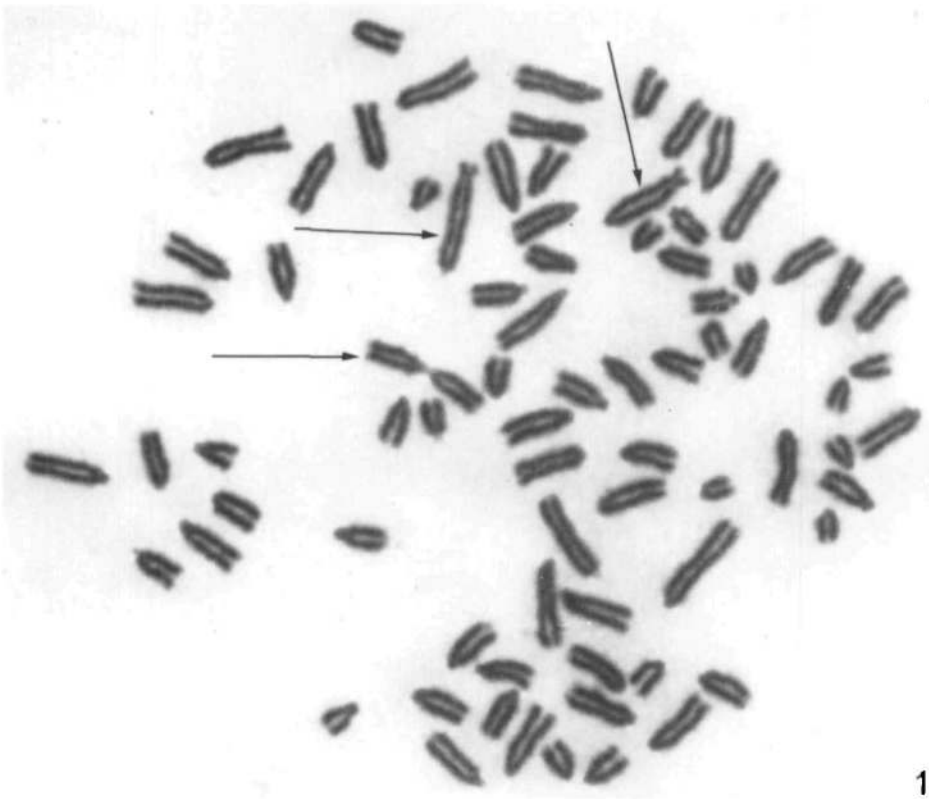

10 μ
14

Fig. 15. Ehrlich ascites cell at metaphase, 77 chromosomes. Arrows indicate the three marker chromosomes described in the text.

Fig. 16. HeLa cell at metaphase, 57 chromosomes. Arrows indicate the three marker chromosomes described in the text.



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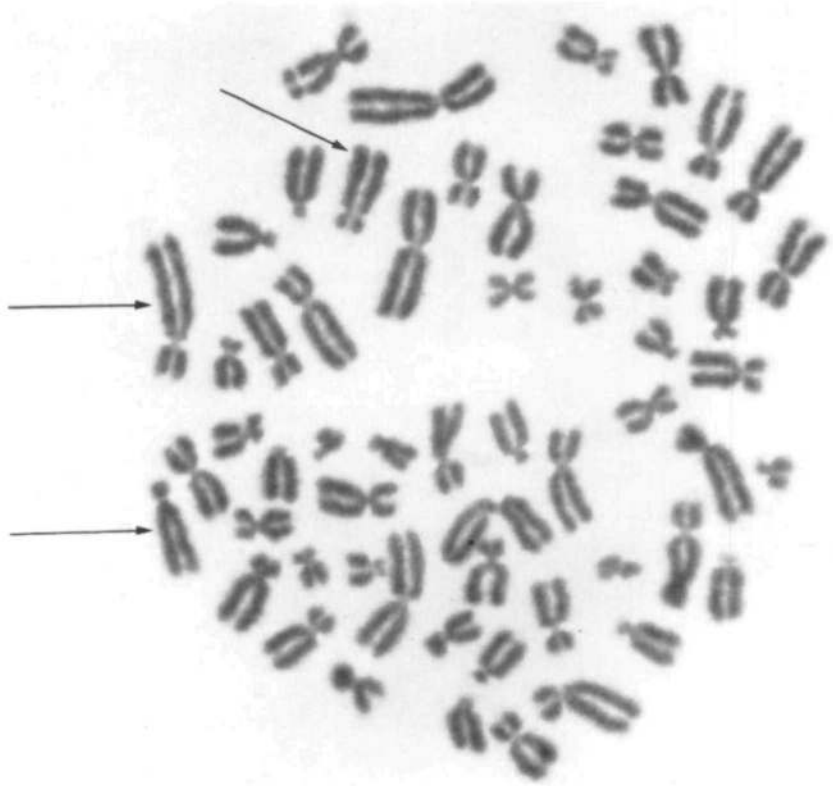
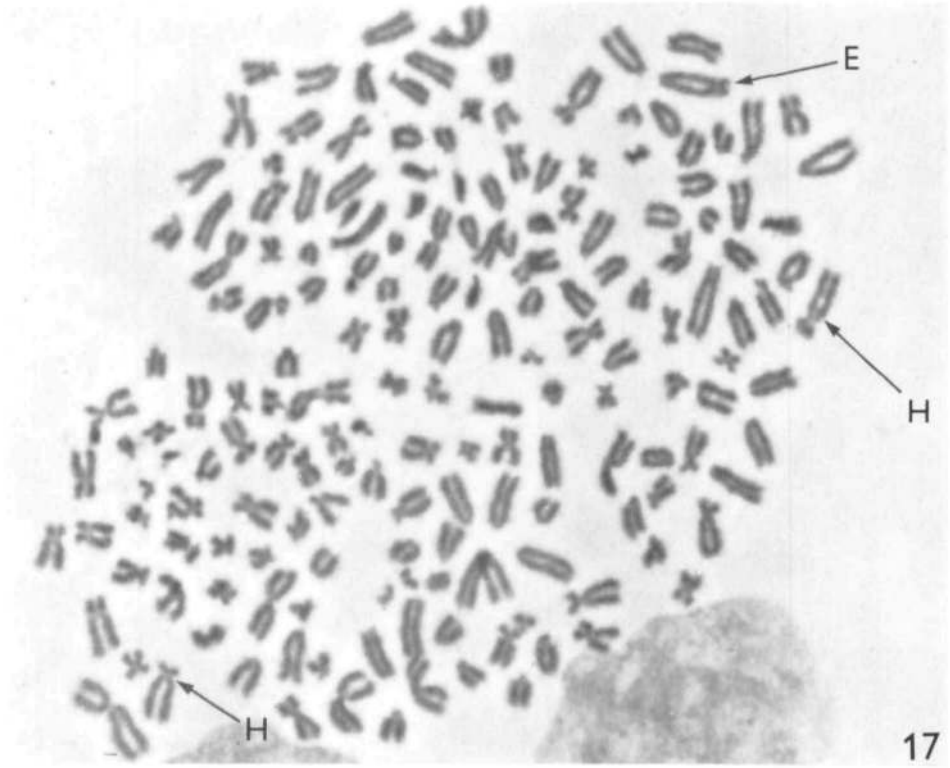


16

Fig. 17. HeLa–Ehrlich hybrid cell at metaphase, 181 chromosomes. Arrows indicate 2 HeLa (H) and 1 Ehrlich (E) marker chromosomes.

Fig. 18. HeLa–Ehrlich hybrid cell at metaphase. Approximately 412 chromosomes (centromeres). Note large number of structural changes.


E
H
H
17

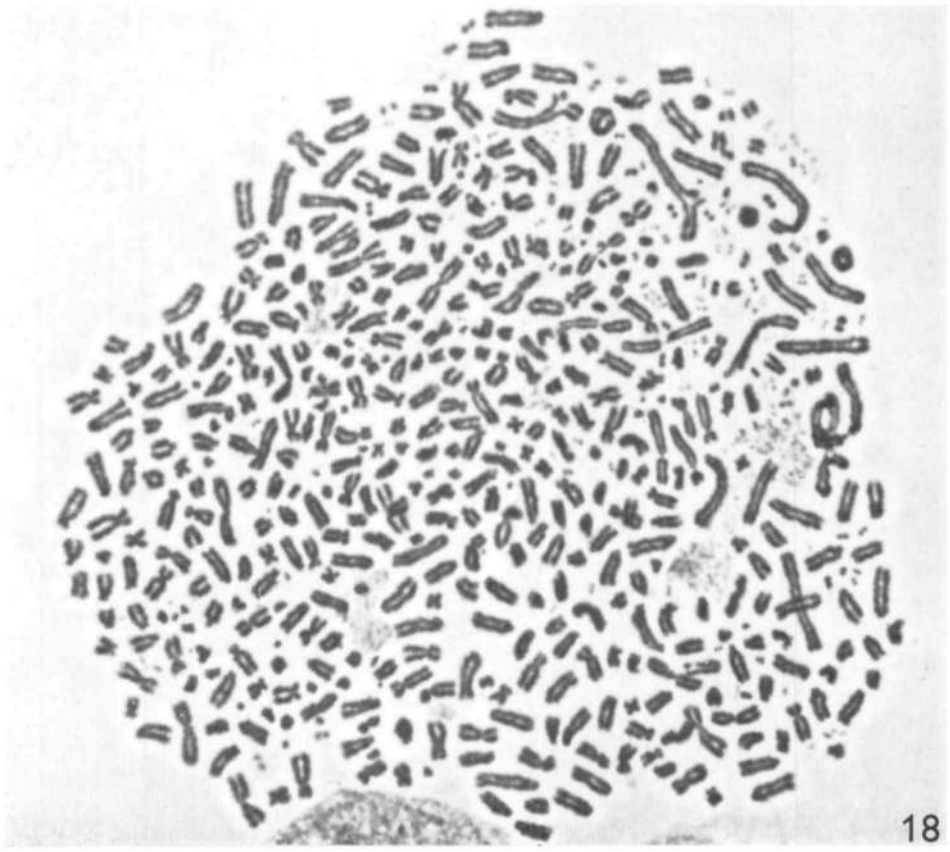

18

Fig. 19 *a–d*. Four frames from a cinemicrographic sequence illustrating dissociation of a heterokaryon. The cell is a dikaryon containing 1 HeLa and 1 Ehrlich nucleus. It divides into two mononucleate cells, one containing a HeLa nucleus and the other an Ehrlich nucleus. (The bright, refractile object in the field is a piece of cell débris.)

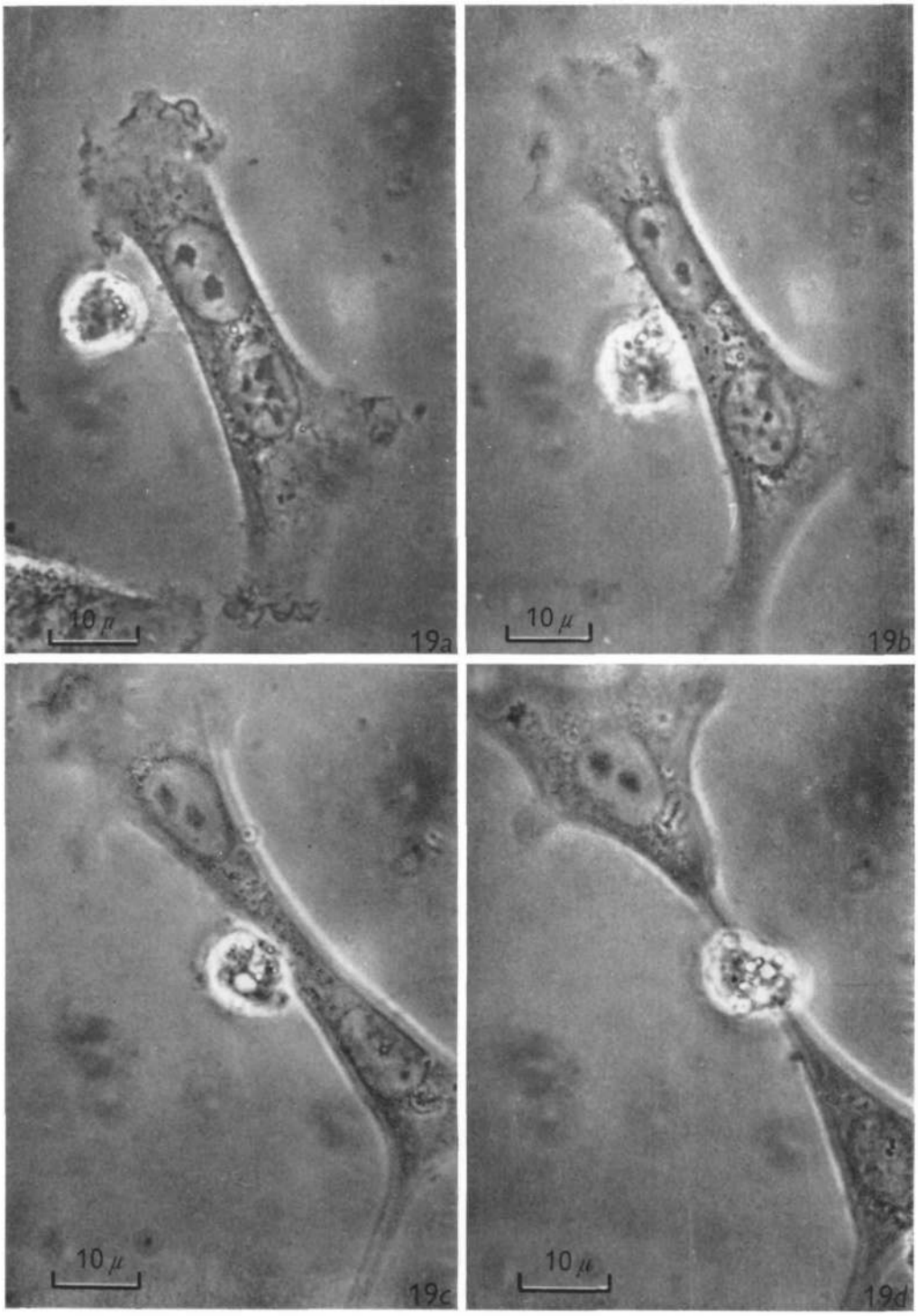
Fig. 20. A heterokaryon containing 1 HeLa nucleus and a number of rabbit macrophage nuclei.

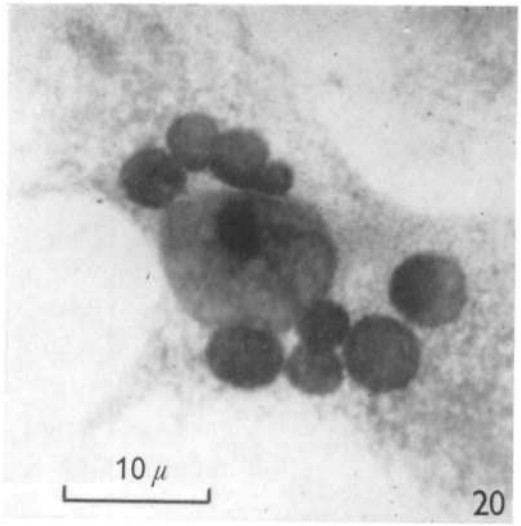
Fig. 21. Autoradiograph of a heterokaryon containing 1 HeLa nucleus and 1 rabbit macrophage nucleus, from a culture exposed for 4 h to tritiated uridine. Both nuclei are heavily labelled, and there is also labelling over the cytoplasm.

Fig. 22. Autoradiograph of a heterokaryon containing 1 HeLa nucleus and 1 rabbit macrophage nucleus, from a 24-h culture exposed for 2 h to tritiated thymidine. Both nuclei are synthesizing DNA.

Fig. 23. A heterokaryon containing 3 HeLa nuclei and 2 rat lymphocyte nuclei.

Fig. 24. A heterokaryon containing 3 HeLa nuclei and 1 rat lymphocyte nucleus which has undergone transformation (marked with an arrow). The transformed lymphocyte nucleus shows three structures resembling small nucleoli, and its chromatin is much more dispersed than that of normal small lymphocyte nuclei.


10 μ
19a
10 μ
19b
10 μ
19c
10 μ
19d


10 μ
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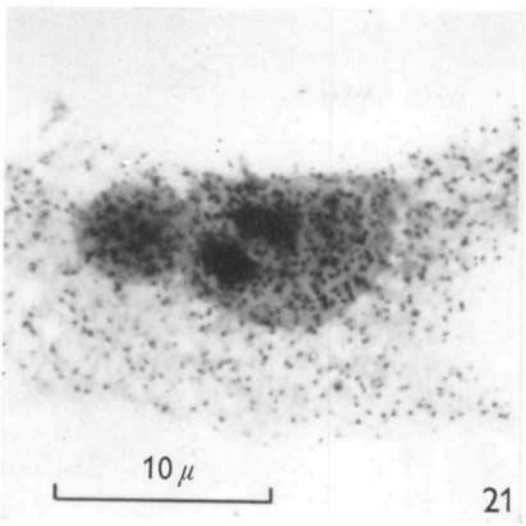
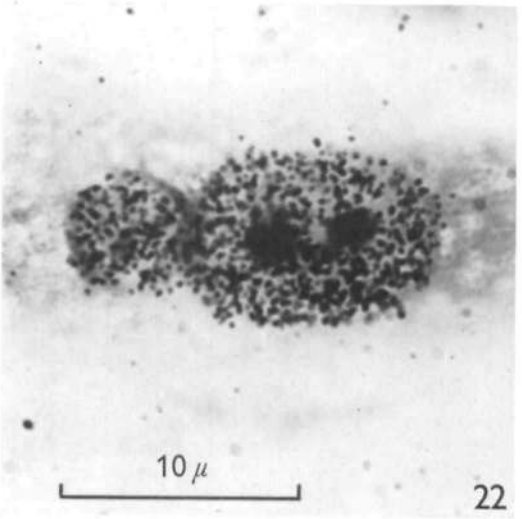

10 μ
21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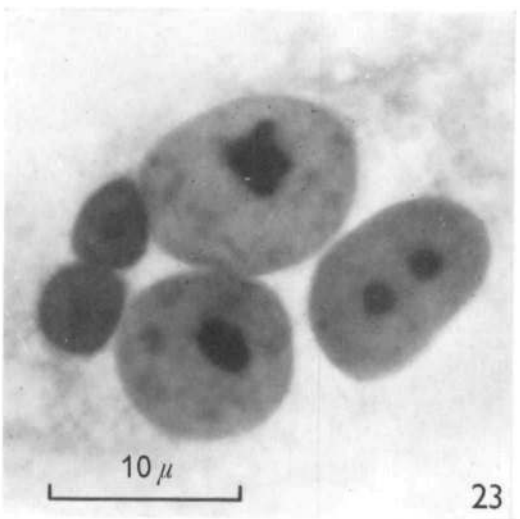
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23

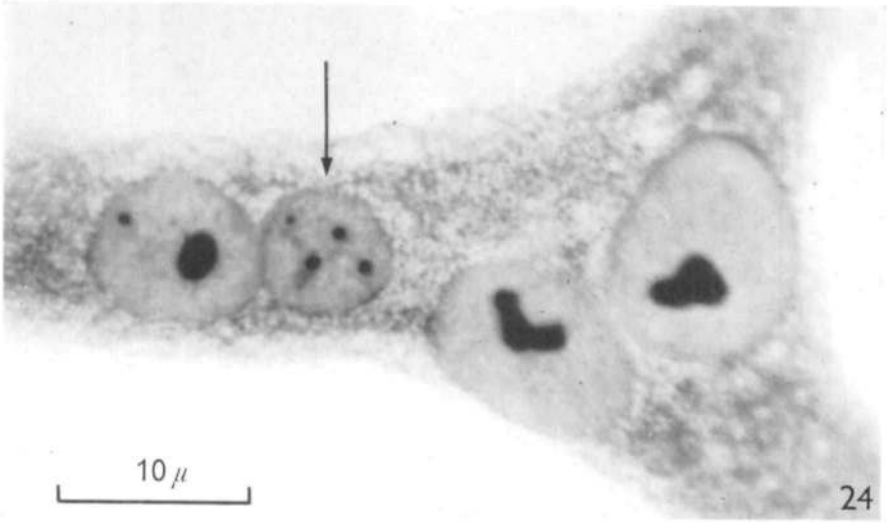
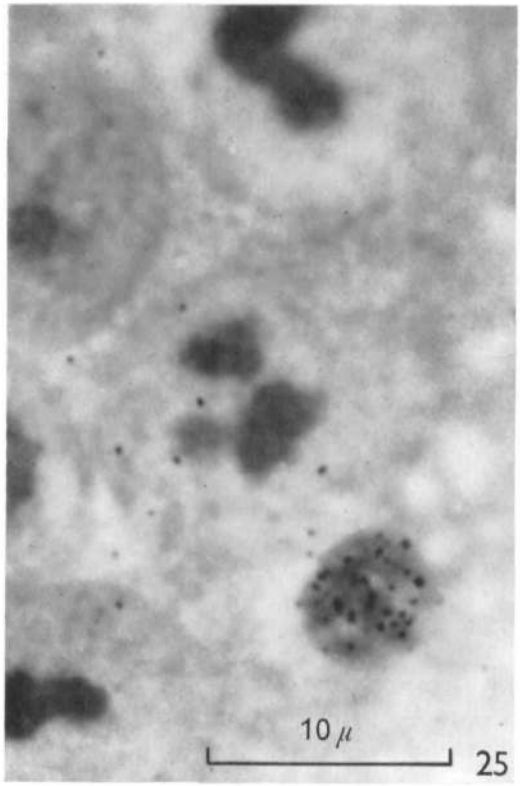

10 μ
24

Fig. 25. Autoradiograph of part of a heterokaryon from a 24-h culture exposed for 2 h to tritiated thymidine. This cell contains a number of HeLa nuclei and 1 rat lymphocyte nucleus. Only the lymphocyte nucleus is synthesizing DNA.

Fig. 26. Autoradiograph of a dikaryon from a 24-h culture exposed for 30 min to tritiated uridine. The cell contains 1 HeLa nucleus and 1 rat lymphocyte nucleus. Both are synthesizing RNA.

Fig. 27. A heterokaryon containing 2 HeLa nuclei and 7 hen erythrocyte nuclei.



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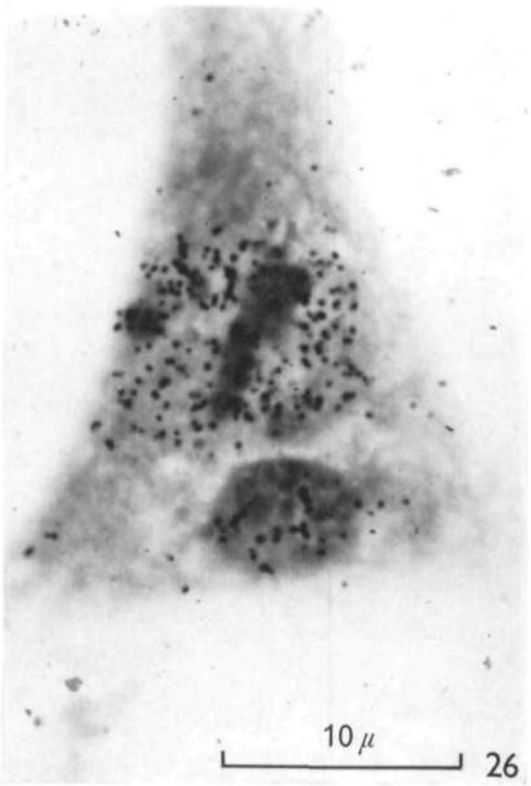

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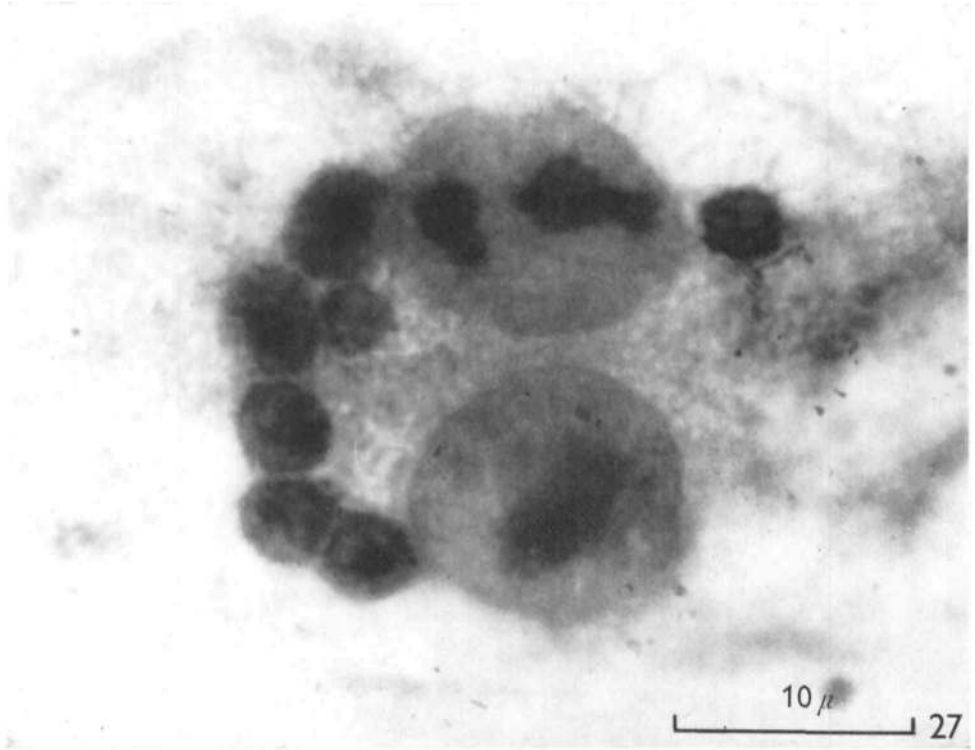
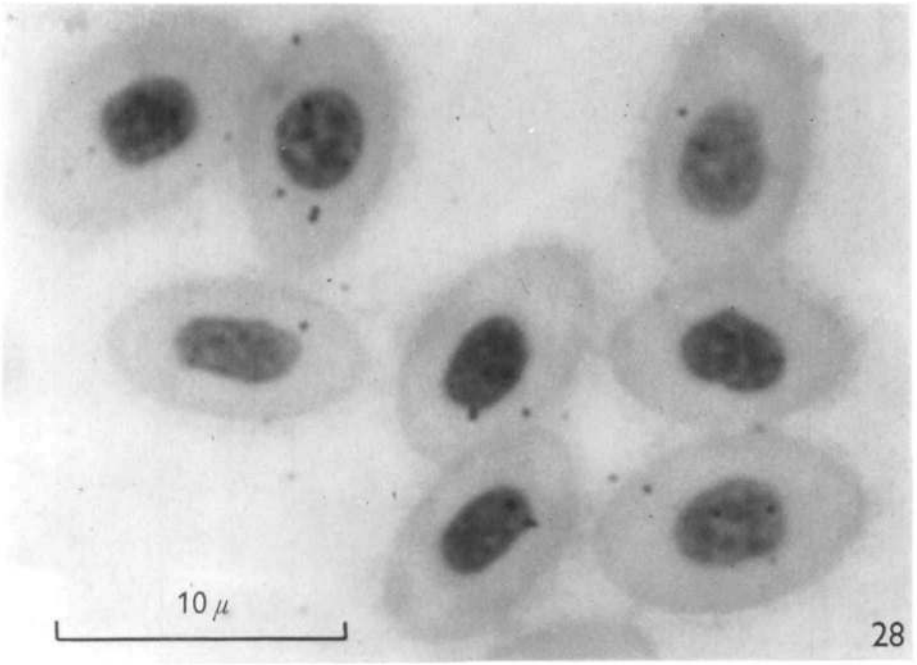


27

Fig. 28. Autoradiograph of a film of hen erythrocytes which had been incubated for 3 h in medium containing tritiated uridine. The autoradiograph was developed after exposure for 7 days. There is no significant labelling of the erythrocyte nuclei.

Fig. 29. Autoradiograph of a heterokaryon from a 24-h culture exposed for 20 min to tritiated uridine. The autoradiograph was developed after exposure for 3 days. The cell contains 4 HeLa nuclei and 4 hen erythrocyte nuclei. All the nuclei are synthesizing RNA.


10 μ
28

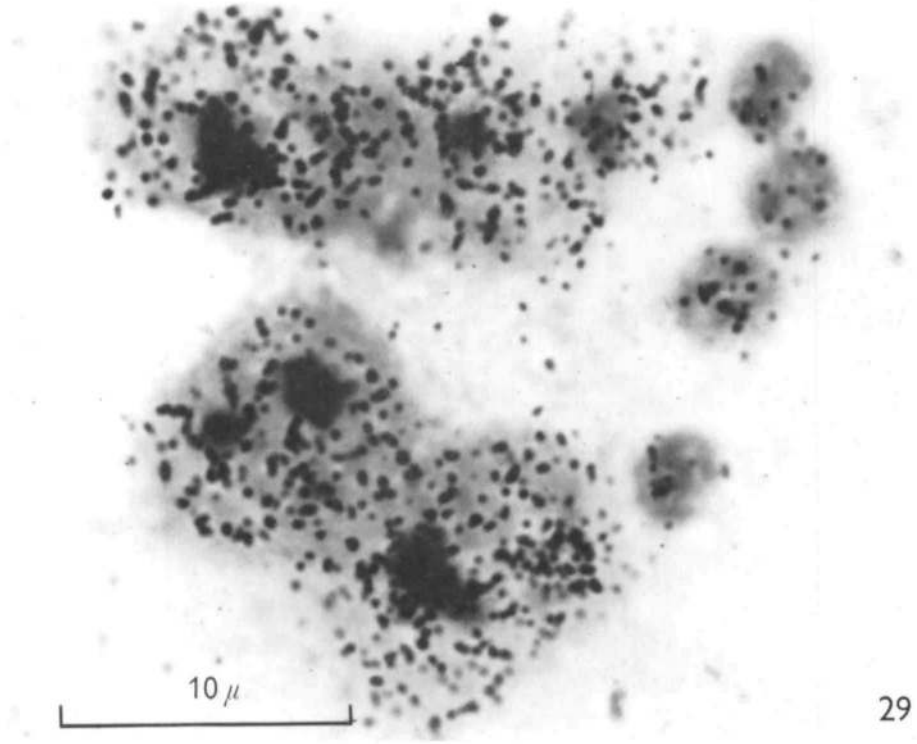

10 μ
29

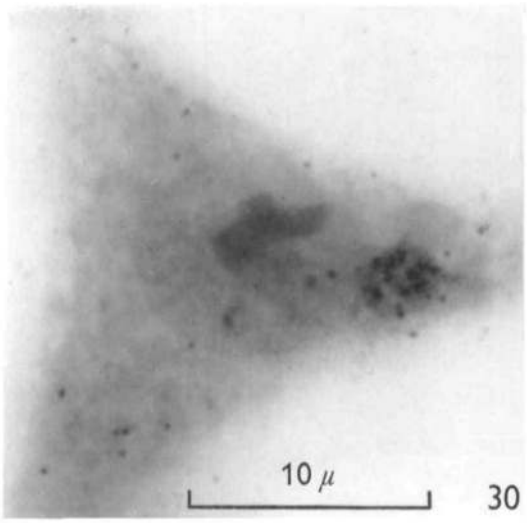
Fig. 30. Autoradiograph of a dikaryon from a 24-h culture exposed for 2 h to tritiated thymidine. The cell contains 1 HeLa nucleus and 1 hen erythrocyte nucleus. The erythrocyte nucleus is synthesizing DNA.

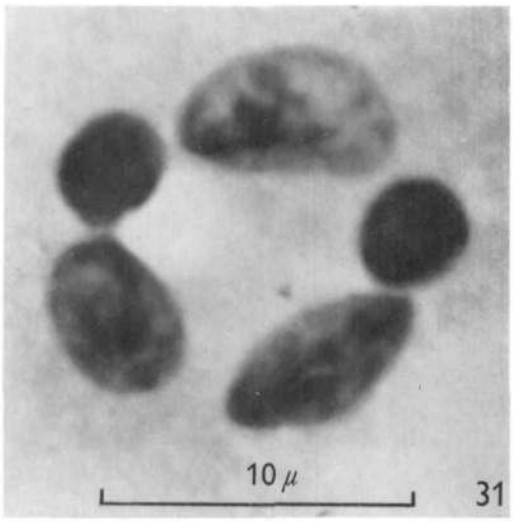
Fig. 31. A heterokaryon containing 3 rabbit macrophage nuclei and 2 rat lymphocyte nuclei, which are smaller and stain more deeply. Note the peripheral distribution of the nuclei in the cell.

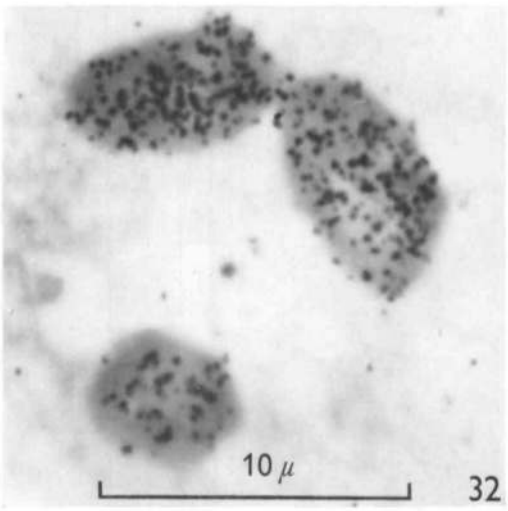
Fig. 32. Autoradiograph of a heterokaryon from a 24-h culture exposed for 30 min to tritiated uridine. The cell contains 2 rabbit macrophage nuclei and 1 rat lymphocyte nucleus. All the nuclei are synthesizing RNA.

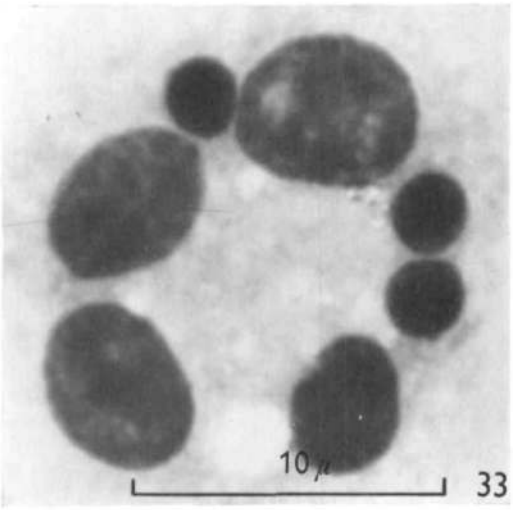
Fig. 33. A heterokaryon containing 4 rabbit macrophage nuclei and 3 hen erythrocyte nuclei, which are much smaller. Note the peripheral distribution of the nuclei in the cell.

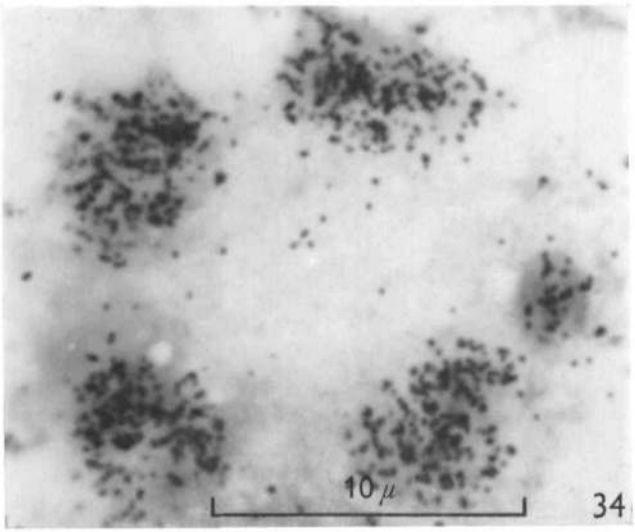
Fig. 34. Autoradiograph of a heterokaryon from a 24-h culture exposed for 30 min to tritiated uridine. The cell contains 4 rabbit macrophage nuclei and 1 hen erythrocyte nucleus. All the nuclei are synthesizing RNA.


10 μ
30


10 μ
31


10 μ
32


10 μ
33


10 μ
34

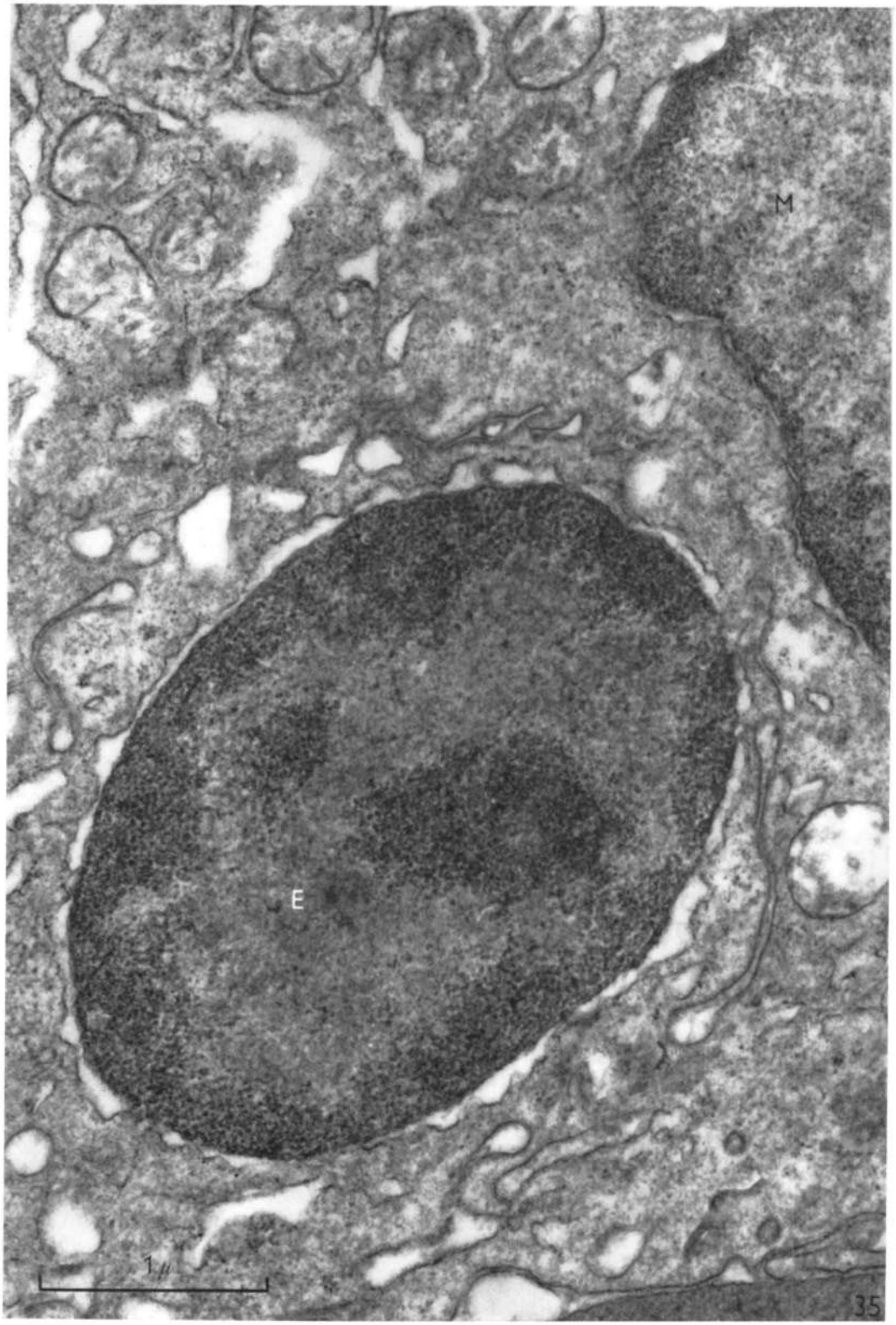


Fig. 35. An electron micrograph of a section through a heterokaryon containing a hen erythrocyte nucleus (E) and a rabbit macrophage nucleus (M). The erythrocyte nucleus shows no evidence of structural damage and has established normal relationships with the cytoplasm of the cell. As in the neighbouring macrophage nucleus, a typical perinuclear cisterna is present, and nucleo-cytoplasmic bridges have been formed.

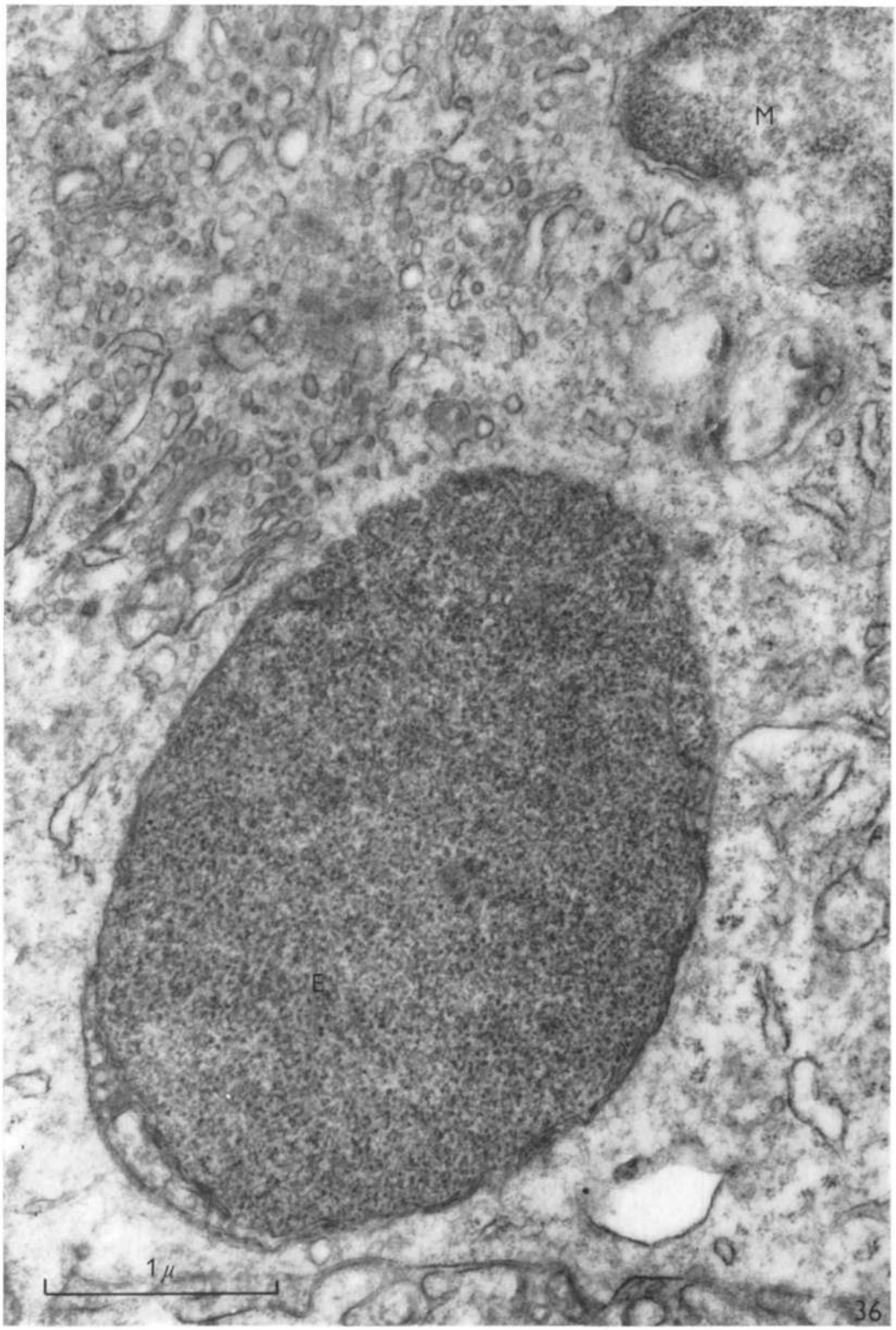


Fig. 36. An electron micrograph of a section through a heterokaryon containing a hen erythrocyte nucleus (E) and a rabbit macrophage nucleus (M). In this case, the erythrocyte nucleus appears to be damaged. It is denser and more homogeneous than the erythrocyte nucleus seen in Fig. 35. There is no perinuclear cisterna and nucleocytoplasmic bridges have not been established.