CYTOCHEMICAL STUDIES OF THE

NUCLEOPROTEINS OF HELA CELLS

INFECTED WITH HERPES VIRUS

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ABSTRACT

The morphological and cytochemical changes in HeLa cells infected with herpes virus have been studied at frequent intervals during infection and related to the growth of virus and the multiplicity of the virus inoculum. Infection with a high multiplicity inoculum produced enlargement and extrusion of small ribonucleoprotein (RNP) bodies in the nucleoli (nucleolini) to form RNP bodies in the nucleoplasm (B bodies) beginning $\frac{1}{2}$ hour after infection. 3 hours after infection, RNP of the pars amorpha appeared to diffuse into the adjacent nucleoplasm, where, $\frac{1}{2}$ hour later, the classical type A inclusion or A body first appeared. The A bodies displaced the B bodies and the nucleoli and eventually filled the nucleus. 6 hours after infection, minute granules containing RNA, DNA, and non-histone protein appeared inside the A bodies (A granules) and increased in number until the late stages of infection, when they disappeared. 18 hours after infection, at the time when the A bodies came to fill the nucleus completely, extrusion of RNP from the nucleus produced cytoplasmic masses which have been termed C bodies. B bodies were formed in the majority of cells before the maturation of infectious virus, but the number of B bodies could not be correlated with the amount of virus in the cell or with the multiplicity of the inoculum. It is suggested that the formation of B bodies may be the result of inhibition of the onset of mitotic division by a mechanism which does not inhibit the formation of RNA in the nucleolini. The nature of the A bodies, the A granules, and the C bodies is discussed and it is concluded that the A granules may represent aggregations of maturing virus in the nucleus. The progression of some C mitotic metaphases to the formation of post-C mitotic multinucleated giant cells is described. These are distinct from syncytia formed by cell fusion.

INTRODUCTION

A number of cytochemical studies of the changes in deoxyribonucleic acid (DNA) in cells infected with herpes virus have been reported (5, 28, 20, 26). These have been supplemented by the biochemical studies of Newton and Stoker (15).

Very little is known, however, about the effects of herpes virus infection upon the ribonucleic acid (RNA) metabolism of the cell beyond the fact that the nucleolar RNA seems to disappear (20) and that the total RNA content per cell is not

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significantly altered (15). The application of the toluidine blue–molybdate stain, which demonstrates DNA and nine different types of ribonucleoprotein in the cell (12), has provided some new information about the changes in the nucleoproteins in cells infected with herpes virus.

MATERIAL AND METHODS

Tissue Culture

The cells used in these experiments were derived from a colony from a line of HeLa cells which had been adapted to grow in calf serum medium by Dr. I. A. Macpherson. A modification (25) of Eagle's medium was used containing 10 per cent calf serum and 10 per cent tryptose phosphate broth. The cells were grown in small culture chambers prepared by sealing cylindrical stainless steel rings onto circular coverslips (22 mm diameter) with paraffin wax as described by Wildy et al. (26).

Virological Methods

HFEM herpes virus was purified by picking a single pock on the chorioallantoic membrane. This clonal line was used after 40 to 43 passages in HeLa cells. The standard inoculum contained 7.5×10^7 pock-forming units (pock u.) per 0.1 ml. Cells in ring cultures were exposed to 0.1 ml of the seed inoculum for 1 hour, after which the cultures were washed thrice with medium and overlaid with 0.1 ml of antiserum prepared by immunizing rabbits with inactivated and activated herpes virus. 1 hour later the antiserum was removed, the cultures were washed three times with medium, and 1 ml of medium was added to each culture. Heat-inactivated virus was substituted in control cultures. In a few experiments where virus titrations were not done, the treatment with antiserum was omitted and the virus inoculum was replaced by the regular medium.

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FIGURE 1

Early and moderately advanced nuclear changes and a normal nucleus 8 hours after infection. Enlarged nucleolini (N) are present at the periphery of two nucleoli in one nucleus in which the A body has not yet appeared. The nucleus of another cell is practically filled by amorphous material (A body). Several B bodies (B) appear to be displaced peripherally by the A body. Some parachromatin (P) still remains between the A bodies and inside the nuclear membrane. The other nucleus appears normal. TBM, stage pre-I. X 1500.

FIGURE 2

Enlarged nucleolini (arrows) in one cell 4 hours after low multiplicity infection. The nucleolini in the other cells are not enlarged and cannot be clearly seen in the photograph. TBM, stage pre-I. X 1500.

FIGURE 3

Three enlarged nucleolini at the edge of a nucleolus (arrow). TBM, stage pre-I. X 1500.

FIGURE 4

A group of enlarged nucleolini at the edge of the nucleolus (arrow) and two clusters of similarly stained RNP granules (B bodies) in the nucleoplasm. TBM, stage pre-I. X 1500.

FIGURE 5

Three B bodies in the nucleoplasm and a dumbbell-shaped extrusion of a nucleolinus (arrow) from the nucleolus. TBM, stage pre-I. X 1500.

FIGURE 6

Diffusion of RNP of the pars amorpha into the A bodies (A). The edges of the nucleolus in the normal cell (arrow) are well defined. Note that diffusion of the pars amorpha occurs in cells which do not contain A bodies. HeLa cells, 4½ hours after high multiplicity infection. TBM, stage III. X 1000.
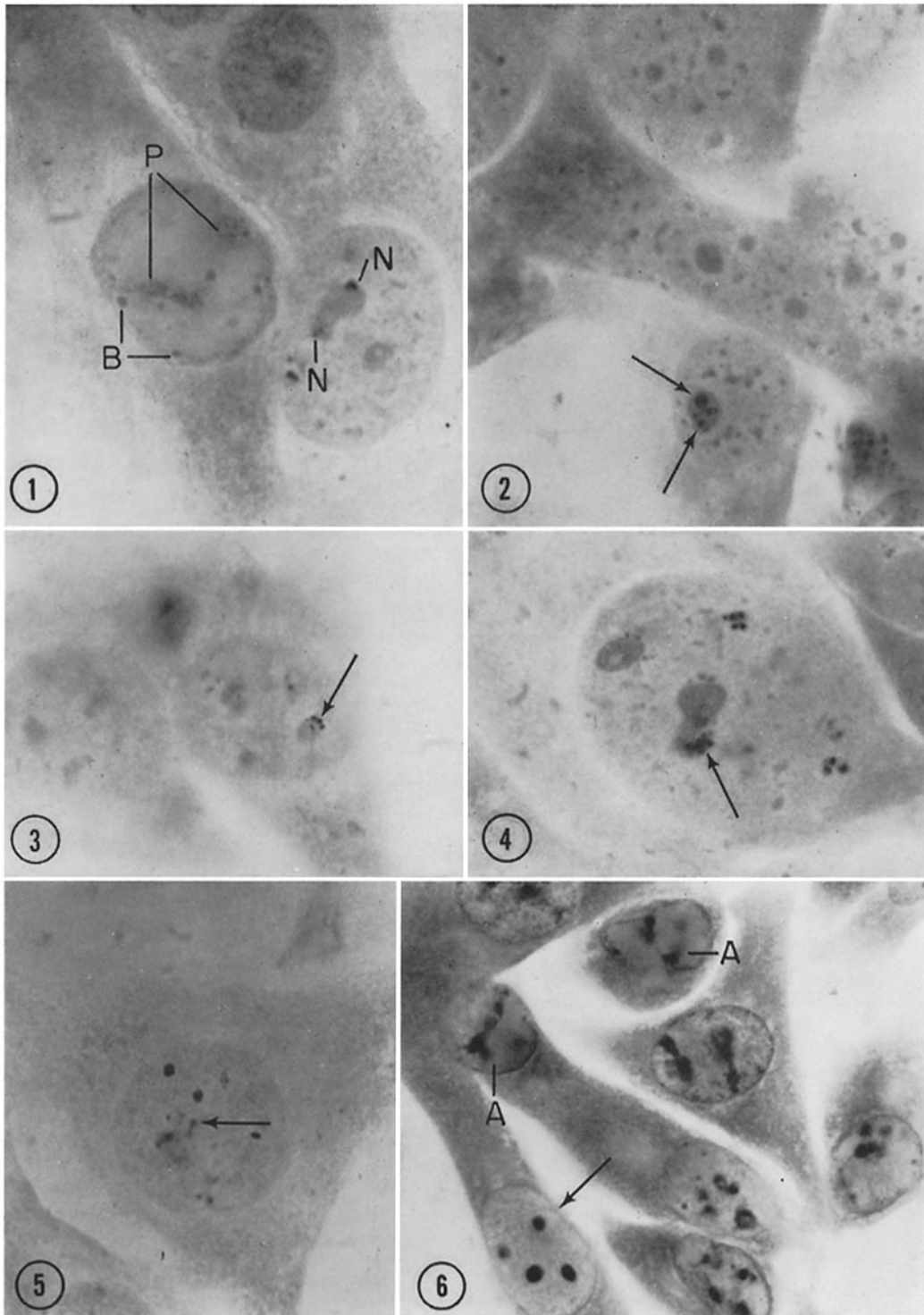
have been described (23). The results were expressed in pock-forming units. Virus was extracted from cells in a Dawe ultrasonic bath.

Cytochemistry

Cultures were rinsed briefly in physiological saline before fixation. Nucleoproteins were stained by the toluidine blue–molybdate (TBM) procedure using 0.008 per cent toluidine blue (National Aniline, 60 per cent dye content, NU 2) for stages pre-I, I, and II of staining, and 0.006 per cent of the same dye for stage III preparations (12). The specificity of the staining for nucleic acids was checked by digestion with deoxyribonuclease (DNase) and ribonuclease (RNase). Preparations were treated for 2 hours at 37°C in 0.01 per cent DNase (Worthington, once crystallized), dissolved in 0.02 M tris(hydroxymethyl)-amino methane buffer containing 0.45 M magnesium chloride and 0.005 M calcium chloride brought to pH 7.3 with N hydrochloric acid. When fixation for long periods in formol sublimate was required (e.g., 60 minutes at 37°C for stage III, TBM), preparations were fixed for 3 minutes, treated with the enzyme, and then fixed for the remaining 57 minutes to produce the necessary inactivation of protein-bound amino groups for staining (12). This modification was required because the enzyme had no effect on cells fixed for long periods in formol sublimate. Control preparations were incubated in the buffer. Digestion with RNase (Worthington Biochemical Corp.) was carried out overnight at 37°C in Sorensen's buffer at pH 6.8. RNase digestion had no effect on the Feulgen reaction. All staining by TBM was inhibited by treatment with DNase followed by RNase and by extraction with 5 per cent trichloroacetic acid at 90°C for 1 hour.

Coverslips were fixed for 3 minutes in formol sublimate and stained by the Feulgen method for DNA (16), by the alkaline fast green method for histones (1), and by the ninhydrin-Schiff procedure for protein-bound groups (4). All Feulgen-positive material was removed by digestion with DNase.

Cytological Analysis

One thousand cells were counted to determine the mitotic index, and the mitoses were grouped as normal or abnormal as indicated in the results.

Two hundred cells in interphase were counted to

FIGURE 7

TBM, stage III, after digestion with deoxyribonuclease. The material which appears to be diffusing from the nucleoli is not digested by DNase, and is, therefore, not nucleolus associated chromatin. Some B bodies (arrows) are also stained. HeLa cells, 4 hours after high multiplicity infection. × 1100.

FIGURE 8

Multiple prominent B bodies (B), many of which appear to be displaced peripherally by the amorphous A bodies. Some parachromatin (P) remains between the A bodies. HeLa cells, 24 hours after low multiplicity infection. TBM, stage pre-I. × 1100.

FIGURE 9

Two enlarged nuclei completely filled by A bodies. The multiple micronuclei in another cell are almost certainly the result of C mitosis, as illustrated in reference 6. HeLa cells, 28 hours after high multiplicity infection. TBM, stage III. × 1100.

FIGURE 10

Cytoplasmic masses of RNP (C bodies) (arrows) which appear to have been extruded from the nucleus. HeLa cells, 28 hours after high multiplicity infection. TBM, stage I. × 1150.

FIGURE 11

Two nuclei filled by A bodies. Many minute granules (A granules) are present in the A bodies. HeLa cells, 25 hours after high multiplicity infection. TBM, stage III. × 1100.

FIGURE 12

Very numerous A granules inside A bodies. (Note that the granules are much more numerous than the photograph would suggest. Only those which are in one focal plane are shown here.) TBM, stage III. × 1500.
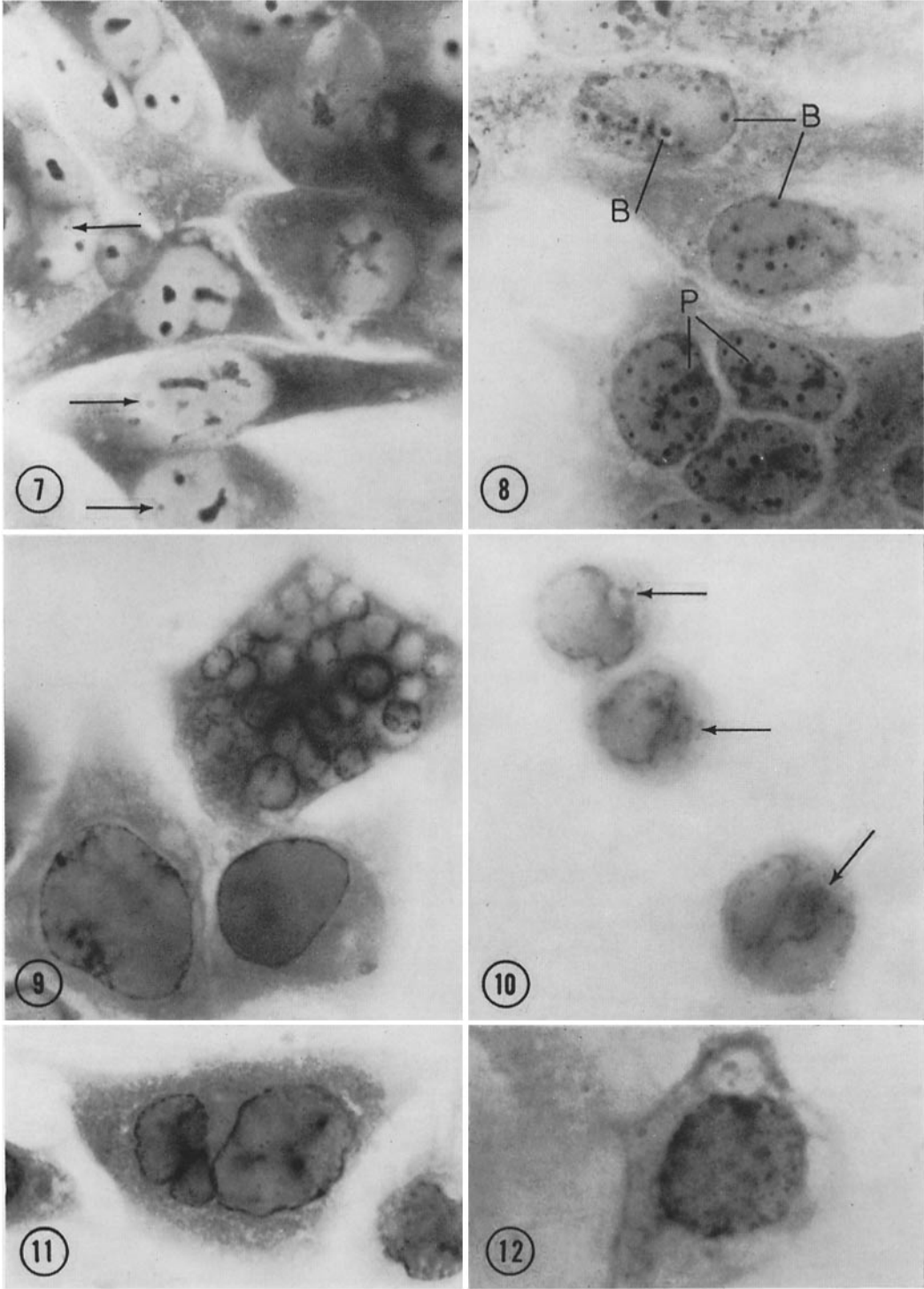
determine the average number of ribonucleoprotein (RNP) bodies (B bodies) and type A inclusions (A bodies) in the nucleoplasm.

Cell counts were made on replicate cultures as previously described (26).

Plan of Experiments

Experiments were designed to study the morphological and cytochemical development of the cellular changes and to relate them to intracellular growth and release of virus from the cell.

chemical analysis was also made on regular cultures infected 4 and 25 hours previously with a high multiplicity inoculum (> 100 pock u. per cell).

3. To investigate the effect of multiplicity of infection on the evolution of the lesions, cultures were infected with threefold dilutions of the seed inoculum, up to 1:2487, which was less than 1 pock u. per cell. Preparations were stained by the TBM method at 4, 8, 10, 12, 14, and 25 hours after infection.
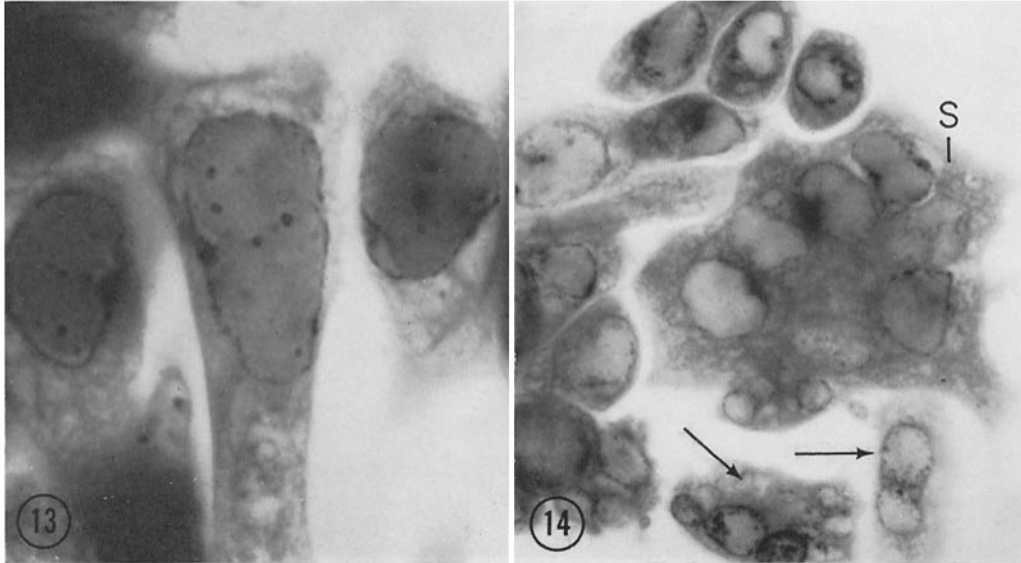
4. Because extensive changes were observed in

FIGURE 13

Large A granules, some of which resemble hollow spheres or rings. TBM, stage III. × 1500.

FIGURE 14

Syncytium (S) in which the nuclei are filled by A bodies. The two cells (arrows) below the syncytium are probably post–C mitotic reconstruction forms (cf Fig. 2.8 in reference 6, produced by colchicine). Note the vacuolation and rounding of the cytoplasm of cells outside the syncytium. TBM, stage III. × 800.

1. Cells were exposed to a high multiplicity of virus (750 pock u. added per cell). Cell counts, virus titration, and cytochemical studies using all stages of the TBM procedure and the Feulgen stain were made at 4, 6, 8, 16, 20, 24, 28, and 41 hours after infection.

2. All the cytochemical procedures with appropriate enzyme controls were carried out on plaques produced in sheets of contiguous cells 40 hours after exposure to a low multiplicity inoculum (< 1 pock u. per cell). In this way, the cytochemistry of all stages of the evolution of the lesions was determined. Since the changes could be roughly divided into early and late and were not synchronous, a complete cyto-

the cells before the formation of infectious virus, studies were made at half-hourly intervals up to 6 hours after addition of 590 pock u. per cell and then at less frequent intervals up to 29 hours after infection. In another experiment, the later stages of infection were investigated at 2-hourly intervals from 18 hours to 26 hours and then at less frequent intervals to 65 hours after infection. In these experiments, the most illustrative staining procedures were used, viz., stages pre-I and III, TBM, and the Feulgen method. Virus titrations were performed, beginning 2½ hours after infection.

RESULTS

Morphological Changes

Although primary infection of the vast majority of cells was produced by high multiplicity inocula, the rate of development of the lesions varied considerably from cell to cell in the same culture. Thus, 4 hours after infection, before secondary infection of cells could occur, very early and moderately advanced changes were seen in the same culture (Fig. 1). Since repeated cytochemical analysis of the same cell was impossible, the pathogenesis of the infection was deduced in part from the proportion of cells showing a given change at intervals after infection and in part from the progressive disruption of nuclear structure. The evolution of the lesions was also observed in the plaques, where the earliest changes were found peripherally and all gradations occurred toward the center, where the lesions were most advanced. None of the changes described in non-dividing virus-infected cells were seen in control cultures, inoculated with heated virus. Occasional abnormalities of mitosis, resembling those produced by colchicine (C mitoses), were noted in the control cultures (Table II).

Cells in Interphase

Two types of RNP are demonstrable in the nucleolus by the TBM stain (stage pre-I) (12). One consists of the pars amorpha and the other comprises a number of minute granules which appear to correspond to the nucleolini of classical cytologists (9). The first detectable abnormality of the cells infected with herpes virus was an enlargement of the nucleolini (Fig. 2). The nucleolini of control uninfected HeLa cells of this strain were extremely minute when stained by the TBM method used in these experiments, and they could be detected in only a few cells. It was thus easy to recognize enlargement of the nucleolini in the infected cells (Fig. 2). The enlarged nucleolini aggregated along the periphery of the pars amorpha and, either singly or in groups, became detached into the nucleoplasm (Figs. 1, 3, 4, and 5). No other change in the nucleoplasm could be detected at this point. Subsequently, larger round or ovoid structures were formed in the nucleoplasm by aggregation of clusters of small granules or by enlargement of single bodies derived from the nucleolini (Figs. 4 and 5). These structures which

are derived from nucleolini will be termed B bodies to distinguish them from the larger masses of amorphous DNA, which may shrink from the nuclear membrane during fixation (3) to produce an artifact known as the type A inclusion. The DNA masses will be referred to as A bodies.

Shortly after the appearance of B bodies, the edges of the pars amorpha became ill defined (Figs. 6 and 7) and areas of rarefaction of the RNP of the nucleoplasm (parachromatin) appeared adjacent to the nucleoli. Subsequently, the DNA of the chromatin also disappeared in
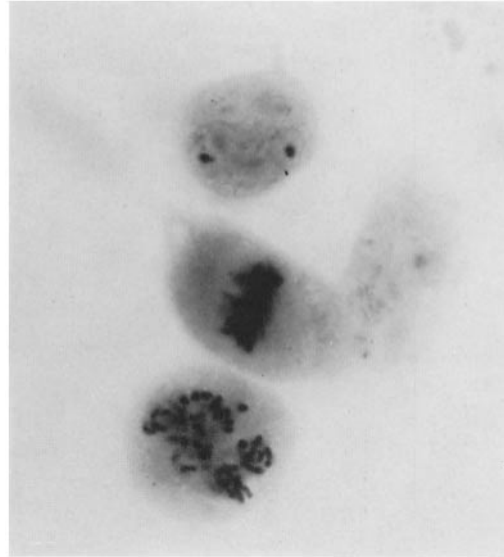

FIGURE 15

Irregular grouping of the chromosomes in one metaphase and clumping of chromosomes in another. Two B bodies are present in the cell above. TBM, stage pre-I. × 1200.

these areas. The RNP of the pars amorpha appeared to diffuse into the areas of rarefied nucleoplasm (Fig. 6). In this way, the smaller nucleoli became completely dispersed. Subsequently, homogeneous, optically dense material filled the areas of rarefaction, and the dense masses increased in size to form the classical type A inclusions or A bodies (Figs. 1, 6, and 9). In doing so, the A bodies displaced and often indented the B bodies and the remaining nucleoli (Figs. 1 and 8). The nucleoli were often compressed into a tricorn shape by two large A bodies and the nuclear membrane. Occasionally the pars amorpha fragmented and rounded fragments appeared among the B bodies. Since the fragments

TABLE I

Cytochemical Changes in HeLa Cells Infected with Herpes Virus (High Multiplicity)

Lesion	Toluidine blue-molybdate (TBM)						Feulgen	Time of appearance	Course	Site
	Pre-I	RNase pre-I	DNase pre-I	III	RNase III	DNase III				
Enlarged nucleolini and B bodies	Purple +++	0	Purple +++	0 to green +	0	0 to green +	0	½ hr.	Continue to form until nucleolus destroyed	Nucleolus, nucleoplasm, and cytoplasm
Diffusion of pars amorpha	Green +	0	Green +	Purple ++++	0	Purple ++++	0	3 hr.	Continues to diffuse until nucleolus destroyed	Diffuses into A body
A body	0 to purple +	0	0	Purple + to ++++	Reduced	Reduced	0 to +++	3½ hr.	Increasing Feulgen-positive	Juxtanucleolar; then fills nucleolus
A granules	0 to purple +	0	0 to purple +	Purple +++	Purple +	Purple +++	Early 0 Middle ++ Late 0	6 hr.	Increase in number then decrease	Inside A bodies
C body	Purple +	0	Purple +	0	0	0	0	18 hr.	Continue to form to 65 hr.	Extruded from nucleoplasm

+ to ++++ indicates intensity of staining. 0, unstained or not recognizably stained.
All staining by TBM and Feulgen prevented by digestion with DNase followed by RNase.
Alkaline fast green, all negative; ninhydrin-Schiff, all positive.

stained like the pars amorpha, they could be readily distinguished from the B bodies (vide infra). B bodies continued to be formed until the pars amorpha fragmented or disappeared. The parachromatin was displaced peripherally by the enlarging A bodies and was not uncommonly compressed between them (Figs. 1 and 8). The A bodies increased in size until they filled the nucleus and produced considerable nuclear enlargement (Fig. 9). The remains of the nucleolus and some associated parachromatin were compressed against the nuclear membrane, usually

B bodies were also observed in the cytoplasm and may have been extruded from the nucleus.

Shortly after the formation of the A bodies, minute granules were observed inside them. These granules (A granules) increased in number and slightly in size (Figs. 11 and 12). For the most part, A granules were spherical and solid, but occasionally they resembled hollow spheres or rings (Fig. 13). As indicated below, they differed in several respects from B bodies.

In the later stages of infection, the nuclei shrank, the nuclear membrane became irregularly
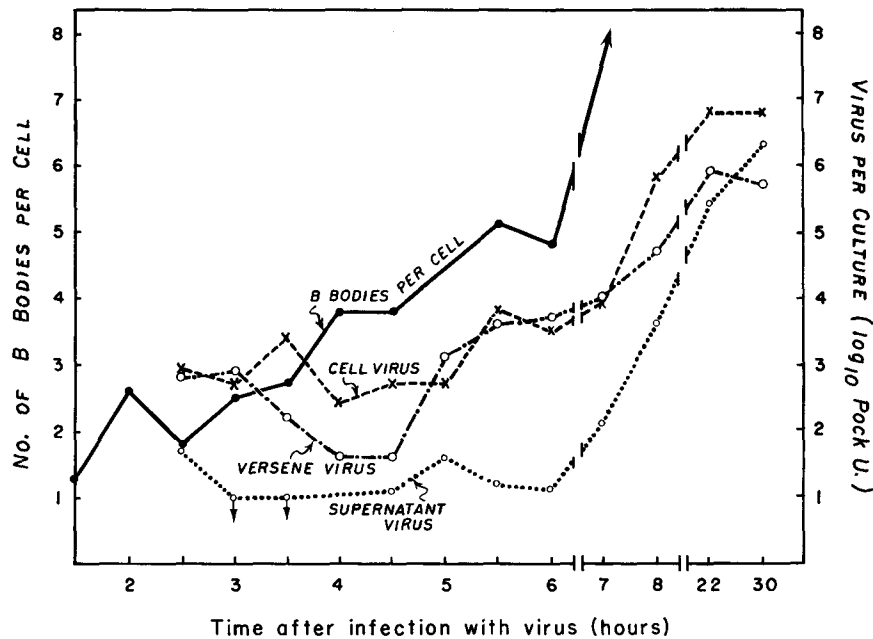

FIGURE 16

Relation of average number of B bodies per cell to virus titer ($\log_{10}$ pock u.).

adjacent to the Golgi zone, where the cytoplasmic RNP was less intensely stained. The juxtanucleolar nuclear membrane became very irregular, and minute projections from the nucleus containing parachromatin were seen. The presence of mushroom-like masses of amorphous material resembling the parachromatin in the Golgi area of the cytoplasm suggested that this material was being extruded from the nucleus. The cytoplasmic masses of RNP apparently derived from the parachromatin (Fig. 10) represent a third type of new formation in the infected cells and will be termed C bodies. Occasional structures resembling

thickened, and the number of A granules and B bodies diminished. The majority of nucleoli became diffusely dispersed and the nucleolini disappeared. Occasional rounded fragments of pars amorpha persisted in an occasional cell. The C bodies appeared to disperse into the cytoplasm after formation, since they were never present in the cytoplasm of more than a small percentage of cells. They continued to be formed, however, throughout the entire period of observation. Not infrequently artifactitious shrinkage of A bodies gave rise to the appearance of the classical type A inclusion.

Some irregular peripheral clumping of chromatin was observed in an occasional cell in the later stages of infection. This was readily distinguished from the B bodies by the presence of Feulgen-positive DNA.

Apart from the presence of C bodies and occasional structures resembling B bodies, the cytoplasmic changes were minimal. Syncytia were inconspicuous except in very dense cultures where they had apparently been formed by fusion of cells; these cells showed advanced nuclear changes with A bodies filling the nucleus (Fig. 14).

changes in metaphase, the usual post–C mitotic reconstruction stages were observed and a certain number of cells underwent karyorrhexis. These changes will be analyzed more fully in relation to time after infection. Cell counts at intervals after infection confirmed the existence of mitotic arrest. In one experiment, the control cultures increased from 1 × 10⁵ cells per ml at the beginning of the experiment to 5.3 × 10⁵ per ml 41 hours later. The virus-infected cultures increased to 1.9 × 10⁵ cells per ml in the same period.
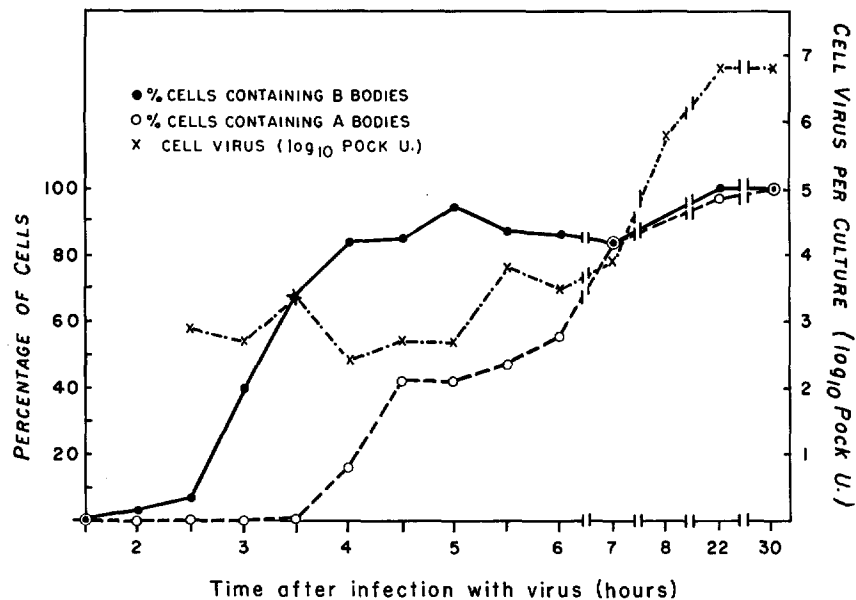

FIGURE 17

Relation of proportion of cells containing A and B bodies to cell virus content.

In sparse cultures syncytia were not formed. Nuclear disorganization of cells which were not included in syncytia was accompanied by variable vacuolation of the cytoplasm and some rounding of the cell (Fig. 14). Large cells with multiple micronuclei were formed as a result of C mitotic arrest (Figs. 9 and 14) (vide infra).

MITOSIS

As previously described (26), infection with herpes virus very quickly produced varying degrees of C mitotic metaphase arrest with failure of spindle formation and irregular scattering of chromosomes (Fig. 15). Slightly later, the onset of prophase was inhibited. Following C mitotic

Cytological and Cytochemical Changes in High Multiplicity Infection

INTERPHASE

The results of three experiments in which cells were infected with a multiplicity of >100 pock u. per cell are summarized in Table I. A very few cells with enlarged nucleolini and occasional B bodies were observed ½ hour and 1 hour after infection. The proportion of cells containing B bodies began to increase significantly 2 hours after infection (Fig. 16) until 5 hours after infection, when 95 per cent of cells contained B bodies. The number of B bodies per cell increased slowly until 6 hours after infection, after which

they began to fragment and became too numerous to count (Fig. 16). In the later stages of infection, the number of B bodies in the nucleus decreased, but they persisted in all cells up to 65 hours after infection.

The B bodies, like the nucleolini, contained RNA which was maximally stained in stage pre-I TBM preparations (Table I). The staining properties of the B bodies were not significantly affected by digestion with DNase (Fig. 7). Although the nucleolini could not be differentially stained in the densely staining nucleolus in ninhydrin-Schiff preparations, the B bodies were positive and therefore contained RNP. They did not stain by the alkaline fast green method for histones, which stained the marginated chromatin. The relation of the percentage of cells containing B bodies and the number of B bodies per cell to the production of virus is illustrated in Figs. 16 and 17. A significant increase in intracellular virus was first noted 5½ hours after infection, by which time practically all cells contained B bodies (Fig. 17). The number of B bodies per cell increased slowly up to 6 hours after infection, after which the results were unreliable. Comparison with the log scale of virus titer indicates that there is little correlation between the number of B bodies and the cell or Versene-associated virus, and thus little correlation with virus production (Fig. 16). Attempts to correlate the decrease in the number of B bodies in the later stages of infection with a drop in the cell virus content were unsuccessful. In the first place, no significant decrease in the cell virus content was observed, and, secondly, the B bodies were too numerous to count with accuracy.

Diffusion of the RNP of the pars amorpha into the nucleoplasm preceded the formation of A bodies (Table I; Fig. 6). This type of RNP appeared to be incorporated into the A bodies as they formed, so that the latter contained RNA, DNA, and non-histone protein which stained by the ninhydrin-Schiff procedure (Table I).

A bodies were first detected 3½ hours after infection. Since the intensity of staining of A bodies in stage III TBM preparations was reduced by pretreatment with RNase or with DNase, they contained both nucleic acids. They did not stain at all after digestion with DNase followed by RNase or after extraction with hot trichloroacetic acid. The RNA of the pars

amorpha, on the other hand, was not removed by digestion with DNase and was completely removed by digestion with RNase. When first detected, the A bodies stained weakly purple in stage III TBM preparations and not at all by the Feulgen method. During the time period studied, they became progressively more deeply colored by TBM and the Feulgen stain. 5½ hours after infection, when an increase of infectious virus was first detected on the cultures, 47 per cent of the cells contained A bodies (Fig. 17). The percentage of cells containing A bodies increased until 97
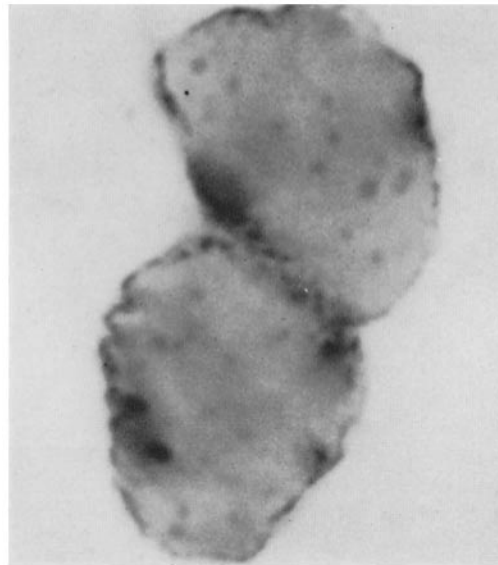

Figure 18

Numerous Feulgen-positive A granules inside the less intensely stained amorphous A bodies. Feulgen stain. × 2300.

per cent of the cells contained A bodies 22½ hours after infection (Fig. 17). Multiple granules were first detected in the A bodies 6 hours after infection. These granules increased in number and slightly in size until about 41 hours after infection, after which increasing numbers of shrunken nuclei containing few or no granules were seen. The granules contained DNA, RNA, and non-histone protein. They were stained most strongly in TBM stage III preparations (Table I). The intensity of staining by TBM was reduced after digestion with RNase but not by DNase. Treatment with DNase followed by RNase, or extraction with hot trichloroacetic acid, prevented all staining by TBM. The granules were first stainable

by the Feulgen method 16 hours after infection (Fig. 18). After 41 hours, they could be detected only rarely in Feulgen-stained preparations. Staining by the Feulgen procedure was completely inhibited by DNase digestion.

C bodies were extruded from the nucleus from 18 hours after infection until the end of the period of observation, but were most conspicuous between 20 and 28 hours after infection (Fig. 10). They consisted of non-histone RNP and were optimally stained in stage pre-I and stage I TBM preparations.

No significant changes were observed in the other types of RNP demonstrable by the TBM procedure with the exception of those resulting from disorganization of nuclear architecture. The parachromatin, perichromosomal, and chromosomal RNP were displaced along with the chromatin and accumulated at the nuclear membrane and around compressed nucleoli. The cytoplasmic RNP was unaffected.

Mitosis

Inhibition of the onset of mitosis and the production of C mitosis have been fully documented by Stoker and Newton (22) and Wildy *et al.* (26). The present experiments demonstrated the progression of many of the C mitotic metaphases (Fig. 15) to abnormal anaphases with rounding up of the chromosomes singly or in groups (Fig. 19) and the formation of multiple micronuclei without cytokinesis (Figs. 21 to 24). Cytokinesis was not always inhibited to the same degree as spindle formation, and sometimes irregular cytoplasmic division with unequal distribution of nuclear material resulted (Fig. 25). For mitotic counts, the mitotic figures were separated into normal and abnormal. Abnormal metaphases were of the classical C mitotic types (6). Rounding up of the scattered or clumped chromosomes without cytokinesis was classified as abnormal anaphase (Fig. 19). When one or more aberrant micronuclei developed a nuclear membrane with a less dense nucleoplasm containing one or more micronucleoli, the cells were scored as abnormal telophase (Figs. 20 to 23). Finally, when all the micronuclei had completed the reconstruction phase and often contained A and B bodies (Figs. 9 and 14), the multinucleated cells were not included in the mitotic count. Minor mitotic

FIGURE 19

Rounding up of chromosomes singly (arrow) and in clumps following failure of spindle formation and cytokinesis (C anaphase) (*cf* Fig. 2.8 in reference 6, produced by colchicine). TBM, stage III. × 1500.

FIGURE 20

Rounding up of chromosomes following C mitotic metaphase arrest in a small syncytium (C anaphase). TBM, stage III. × 1200.

FIGURE 21

Post-C mitotic micronuclei beginning to develop a recognizable nuclear membrane (*cf* Fig. 2.8 in reference 6, produced by colchicine). TBM, stage III. × 1500.

FIGURE 22
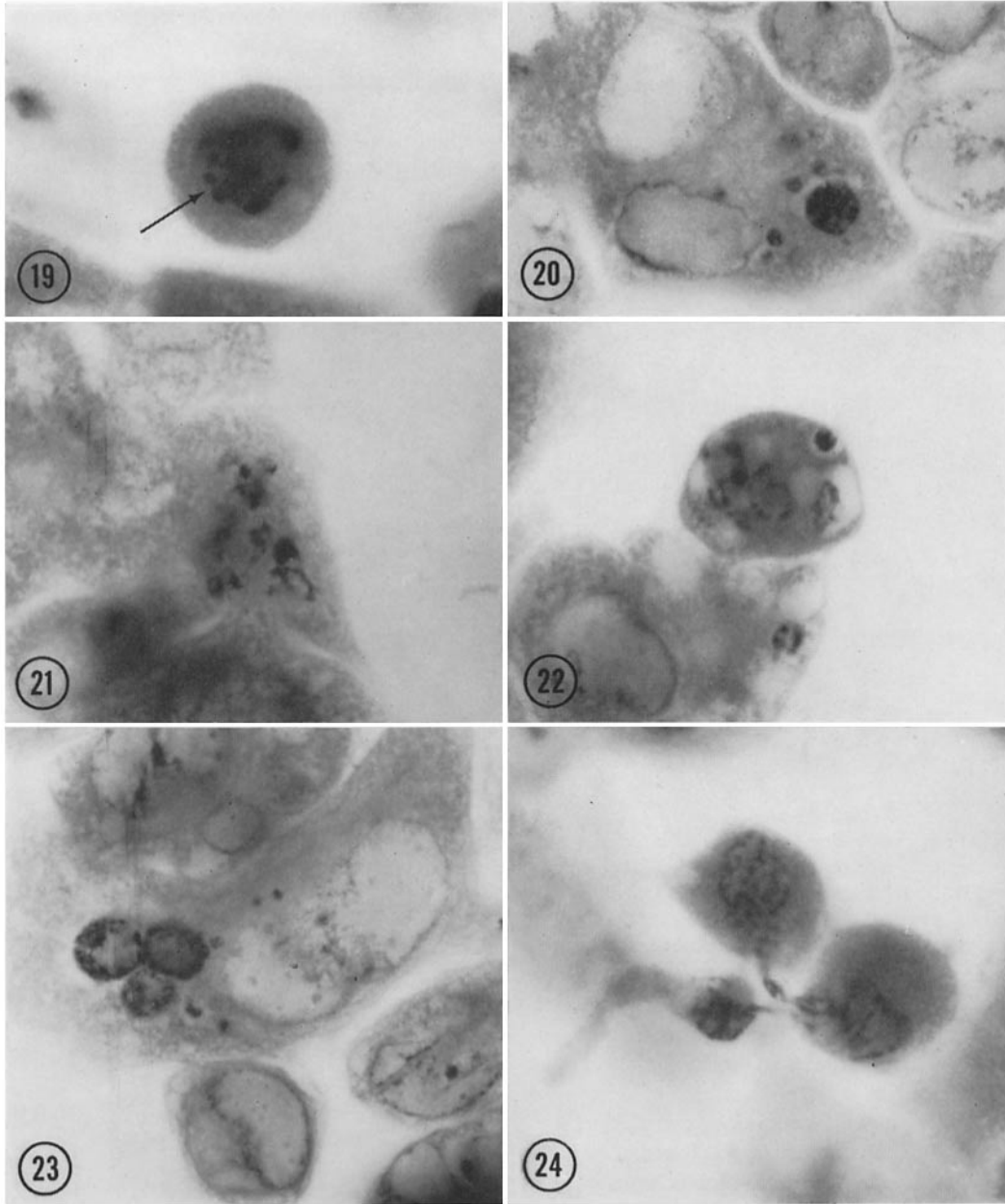
Later stage of reconstruction of C mitotic micronuclei than shown in Fig. 21 (C telophase). TBM, stage III. × 1500.

FIGURE 23

More complete reconstruction of C mitotic telophase. Compare with post-C mitotic interphases in Figs. 9 and 14. TBM, stage III. × 1200.

FIGURE 24

Complete cytokinesis with incomplete and unequal division of the nucleus (C telophase). TBM, stage III. × 1500.

abnormalities, such as tripolar mitoses and occasional lagging chromosomes, were not included in the abnormal group. The results of such a count on cells infected with a multiplicity of 750 pock u. per cell are shown in Table II. The number of cells undergoing normal mitosis was diminished 4 hours after infection and the proportion of abnormal mitoses was significantly increased. The diminution of the number of cells in prophase indicates that inhibition of the onset of mitosis was almost complete 6 hours after infection. Increasing numbers of abnormal

centage of abnormal mitosis from 4 to 67 per cent was noted 1½ to 2½ hours after infection, and the same progression of C mitotic metaphases to C mitotic anaphases and telophases was observed. Comparison of Fig. 26 with Fig. 17 indicates that the increase in mitotic abnormalities at 2½ hours preceded the formation of infectious virus, but that changes in the nucleolini and the formation of B bodies had already taken place in a number of cells. A bodies were not formed until 1 hour later.
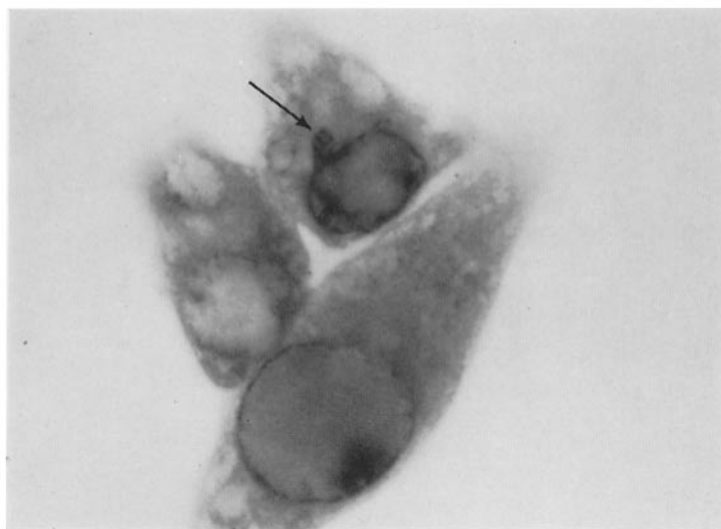

Figure 25

Nuclear protrusion (arrow) which may be due to failure of incorporation of an aberrant chromosome or which might represent some type of nuclear budding or so called "amitotic division." TBM, stage III. × 1500.

metaphases were followed by increasing numbers of abnormal anaphases and telophases. The number of abnormal metaphases decreased 16 hours after infection, partly because the pre-prophase block prevented the formation of new metaphases, and partly because the cells were progressing to abnormal anaphase and telophase. Only a small percentage of abnormal mitotic figures was observed in control cultures (Table II).

In a second experiment, cells were examined at half-hourly intervals for the first 6 hours after infection and at less frequent intervals thereafter (Fig. 26). Again preprophase block was complete 6 hours after infection. A sharp rise in the per-

Effect of Multiplicity of Virus Inoculum on Development of Lesions

In the cultures that were inoculated with threefold dilutions of the seed inoculum from 1:3 to 1:2487, fewer cells were primarily infected by the high dilution. Dilution of the inoculum resulted in a retardation of the onset of cellular changes and in their subsequent development. The number of B bodies and A bodies per cell was unaffected by the multiplicity of the inoculum. In cultures inoculated with the more dilute inocula, the B bodies tended to be somewhat larger than those in cells infected with more viruses, probably because their disintegration was delayed.

TABLE II

Analysis of Normal and Abnormal Mitosis in Herpes-Infected and Uninfected HeLa Cells

Time after inoculation (hrs.)	Mitoses per cent									Total mitoses per cent	Percentage of abnormal mitoses
	Normal					Abnormal					
	P	M	A	T	Total	M	A	T	Total		
Infected:											
4	0.3	0.1	0	0.1	0.5	0.2	0	0	0.2	0.7	29
6	0	0.2	0	0.1	0.3	3.3	0	0	3.3	3.6	92
8	0.1	0.2	0	0.1	0.4	2.8	1.1	0.4	4.3	4.7	91
12	0	0.1	0	0.1	0.2	1.3	1.0	0.5	2.8	3.0	93
16	0	0	0	0	0	0.7	0.4	0.5	1.6	1.6	100
20	0	0	0	0	0	0	0.7	0.7	1.4	1.4	100
24	0	0	0	0	0	0	1.4	0.8	2.2	2.2	100
28	0	0	0	0	0	0	1.1	1.1	2.2	2.2	100
40½	0	0	0	0	0	0	0.4	0.5	0.9	0.9	100
Uninfected: 4–40½											
Mean	0.92	2.86	0.7	1.28	5.77	0.07	0.01	0.03	0.12	5.90	2.1
Range	0.3–1.5	0.4–5.3	0.2–0.9	0.2–2.0	1.9–9.4	0–0.2	0–0.1	0–0.2	0–0.4	2.0–9.5	0–5

P, prophase; M, metaphase; A, anaphase; T, telophase.

DISCUSSION

Enlargement of nucleolini and extrusion of this type of RNP from the nucleolus into the nucleoplasm to form B bodies was observed in the majority of cells before the formation of infectious virus. Reexamination of preparations of P388D$_1$ mouse lymphoma cells infected with polyoma virus (10), HeLa cells infected with parainfluenza type 3 virus (13), and Ehrlich ascites cells in which mitosis had been suppressed by large doses of colchicine (8) indicated that a similar phenomenon

the formation of RNP of the nucleolini ceased, and the nucleolini and later the B bodies disappeared. The inhibition of cell division and the enlargement and, later, inhibition of the formation of the RNP of the nucleolini by FUDR was the result of interference with DNA synthesis, since these effects were reversed by thymidine (11). The changes in the nucleolini of cells infected with herpes virus resemble those of cells treated with FUDR except that the formation of B bodies from nucleolini continued until the nucleolus was destroyed. In 1864, Balbiani observed the extrusion
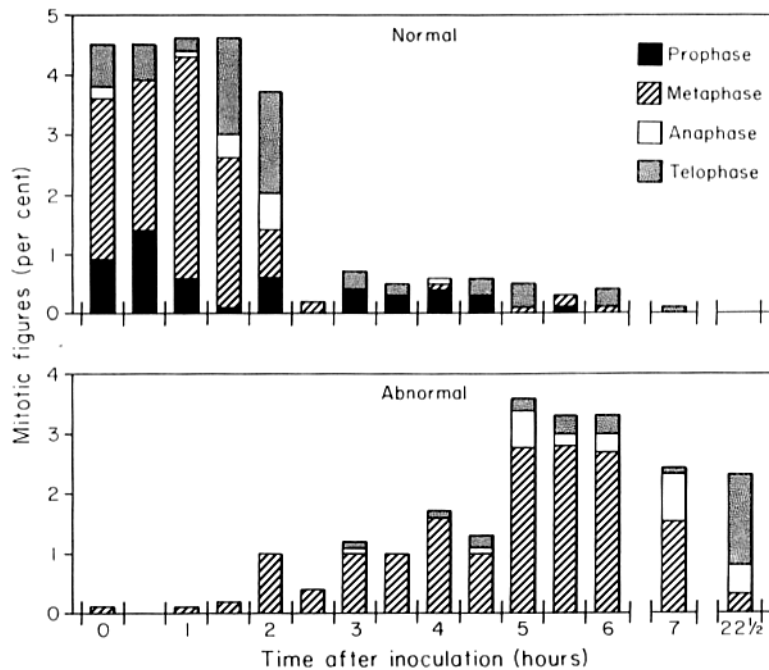

FIGURE 26

Analyses of mitoses in HeLa cells infected with a high multiplicity inoculum of herpes virus.

occurred in these systems. Since the changes in the nucleolini were produced by DNA and RNA viruses and by colchicine, it seems highly unlikely that the B bodies were directly related to synthesis of virus. Recent studies of the effect of 5-fluoro-deoxyuridine (FUDR), an inhibitor of thymidylate synthetase, on HeLa cells have provided some suggestive information regarding the nature of the changes in the nucleolini. FUDR inhibited DNA synthesis and cell division (21). This was accompanied, first, by enlargement of the nucleolini and the production of RNP aggregations in the nucleoplasm, resembling B bodies. Subsequently,

of nucleolini into the nucleoplasm (2). In uninfected cells, the nucleolini were small and minute granules of similarly staining RNP were present in the nucleoplasm. If the synthesis of the RNP of the nucleolini continued and the passage of this material from the nucleolus through the nucleoplasm to the cytoplasm was slowed, accumulation of this material in the nucleoli, the nucleoplasm, and occasionally the cytoplasm could account for the phenomena observed in cells infected with herpes virus. Cell division was inhibited in each of the systems in which the changes in nucleolini were observed. It is of interest that cell division

was also inhibited in HeLa cells infected with vaccinia virus, but the nucleolini were unaffected (11). There may, therefore, be some factor common to the systems in which cell division and changes in the nucleolini are observed. In the case of FUDR, this is certainly an inhibition of DNA synthesis. Wildy *et al.* (26) have demonstrated an early decrease in DNA content of cells infected with herpes virus. This is followed by an increase in the amount of DNA per cell. It is not clear, however, whether this DNA is native to the cell, viral, or a virus-induced specific product. It seems probable, however, from the morphological changes produced, that it is not chromosomal and that a disturbance of the normal synthesis of DNA is present. Alteration of the synthesis of normal DNA could certainly account for the inhibition of mitotic division, which is complete about 6 hours after infection.

Diffusion of RNP of the pars amorpha type into the A bodies was suggested by the manner in which the nucleolar margin became irregular and ill defined adjacent to the areas of rarefaction of the nucleoplasm where the A bodies were forming, and by the appearance of increasing amounts of RNP with identical staining properties in the A bodies. A similar phenomenon has been observed in cells infected with polyoma virus (10).

Electron microscopic studies of cells infected with herpes or herpes B virus (18, 24, 14, 7) would suggest that the virus does not form diffusely throughout the nucleus, but in localized foci, and sometimes in crystalline arrays. It seems unlikely that the A bodies consist of masses of virus. Newton and Stoker (15) have shown that the amount of DNA synthesized by cells infected with herpes virus is much in excess of the amount in the virus particles. The A bodies may, therefore, represent the excessive synthesis of DNA in the infected cells. The A granules are, however, of such dimensions that they may be aggregations of viral particles. They were first detected about the time when the cell virus content began to increase significantly, and became more numerous until the viral content of the cell was maximal. The fact that the A granules were Feulgen-positive and contained non-histone protein is also consistent with their viral nature. Subsequent studies with the electron microscope of hamster cells infected with herpes virus *in vitro* suggest that the viral particles are formed around the periphery of electron-opaque bodies, which appear to be of about the same size as the A granules (27).

The significance of the extrusion of nuclear RNP to form the C bodies in a relatively late stage of infection is not clear. Irregularities of the nuclear membrane similar to those which accompany this change have been described in cells infected with herpes (14, 7). Light microscopic studies cannot, however, determine whether release of virus particles takes place at the time of formation of C bodies.

The occurrence of C mitosis in cells infected with herpes virus may be attributed to a failure of the function of the centrioles, as in colchicine poisoning (6). On the other hand, the amorphous RNP of the nucleoplasm increases during prophase and diffuses into the spindle at the onset of metaphase (12). It has been suggested that this material plays a role in the synthesis of the proteins of the spindle (12). This amorphous RNP disappears in the areas of the nucleoplasm where the A bodies form, and a deficiency of this material in the spindle zone may contribute to the failure of formation of the spindle fibers. The very early increase in the percentage of abnormal mitosis, 2½ hours after infection, before the formation of A bodies, would suggest, however, that this is not the primary cause of C mitosis.

Two, and possibly three, types of multinucleated cells can be distinguished in cultures infected with herpes virus. First, giant cells are produced by fusion of infected with uninfected cells (19); these have been amply attested by time lapse cinematography (3). Secondly, cells with multiple micronuclei are produced as a result of C mitosis. Thirdly, multinucleated cells may be produced by so called "amitotic division" or nuclear budding as described by Reissig and Kaplan (17) with pseudorabies virus. In the present experiments, the observation of a rare nuclear protrusion (Fig. 25) suggested that such a process might occur. It is, however, equally possible that such apparent nuclear buds may be the result of failure to incorporate an aberrant chromosome after C mitotic failure of spindle formation. Cells showing irregular nuclear projections following C mitosis are adequately illustrated by Eigsti and Dustin (6).

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