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The Replication and Coating of Vaccinia DNA

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The replication and subsequent fate of vaccinia DNA in HeLa cells has been studied by examining the cytoplasmic fraction. Vaccinia DNA replication begins one to one and a half hours after infection, reaches a maximum rate at two to two and a half hours and then decreases sharply. More than 90% of viral DNA is synthesized by four and a half hours. The maximum rate of viral DNA replication is several times that of DNA replication in uninfected cells. The replication of viral DNA requires synthesis of protein, presumably a polymerase, even after viral DNA has been uncoated.

Replicating viral, as well as parental, DNA is associated with very large structures or aggregates, which may be identical with the viral inclusions (factories) identified by microscopic, electronmicroscopic and autoradiographic techniques. Aggregates contain no detectable amounts of viral messenger RNA. They dissociate or break at concentrations of Mg^{2+} of less than 1·5 mM. During the early stages of the infection cycle practically all viral DNA is associated with the aggregates; after three to four hours progressively increasing amounts of viral DNA become dissociated from them. Shortly afterwards, DNA becomes coated with protein in what appears to be a stepwise fashion. This process, which appears to proceed within the aggregates, has been analysed by the use of puromycin. The first stage(s) result in viral DNA becoming larger or heavier without losing its susceptibility to DNase. The next stage(s) result in the formation of particles resistant to DNase, but still smaller or lighter than virions. The proportion of viral DNA in these immature forms, which are very heterogeneous in size and density, remains constant throughout the maturation period. The final stage of the maturation process becomes detectable at five to six hours; very little of the protein(s) necessary for this step is synthesized before five hours. Only about one-third of the total DNA replicated during the early part of the infection cycle is actually incorporated into virions.

Some implications of these findings are discussed.

1. Introduction

Mammalian cells in suspension culture infected with vaccinia virus constitute a favorable system for the study of the replication of a DNA-containing viral genome and its incorporation into mature virus. Although vaccinia DNA does not differ significantly in base composition from host DNA, and does not contain an unusual base, advantage can be taken of the fact that vaccinia DNA replicates in discrete "factories" in the cytoplasm (Cairns, 1960), which can be cleanly separated from nuclear host cell DNA. Further, centrifugation in density gradients of the cytoplasmic fraction from infected cells labeled with radioactive thymidine affords a powerful

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tool for analysing the intermediate stages as viral DNA is coated and incorporated into mature virus.

Previous work on the replication of vaccinia DNA can be divided into three classes. (1) Measurement of thymidine incorporation by biochemical techniques (Magee & Sagik, 1959; Magee, Sheek & Burrous, 1960; Hanafusa, 1960; Kit & Dubbs, 1963). These studies were largely restricted to measuring incorporation into unfractionated cells, and the results were difficult to interpret since the incorporation measured reflected the replication of both host cell and viral DNA, either of which can predominate, depending on conditions. Further, different authors used different virus as well as different cell strains, thus introducing further variables. (2) Measurement of thymidine incorporation by autoradiographic techniques (Cairns, 1960; Kit, Dubbs & Hsu, 1963; Kato, Kurisu & Kamahora, 1962). These studies established that vaccinia virus replicates exclusively in the cytoplasm, and that one of the early effects of infection is inhibition of host DNA replication. However, they yielded no quantitative data. (3) The time-course of viral DNA synthesis has to date been most clearly defined by the use of dFU‡ (Salzman, 1960), which showed that DNA replication started about two hours after infection and preceded maturation by three to four hours. By six hours after infection enough viral DNA had accumulated to yield a normal complement of infectious virus. The question of whether viral DNA replication continued past this time was left open; Kit *et al.* (1963) concluded that it did.

The work described here was carried out with the vaccinia strain WR growing in HeLa S3 cells in suspension culture. In this system, the adsorbed virus is uncoated by a two-stage mechanism (Joklik, 1964*a*,*b*). The first stage is mediated through a mechanism existing in uninfected cells and results in the degradation of the infecting virions to viral cores. The second stage involves the *de novo* synthesis of protein coded by the host genome, with a viral protein acting as the inducer, and results in the breakdown of the viral cores to naked viral DNA. At high multiplicities of infection (200 virus particles adsorbed per cell), the first naked viral DNA molecules are liberated between 30 minutes and one hour after infection. As soon as viral DNA is uncoated, it codes for M-RNA (Becker & Joklik, 1964; Salzman, Shatkin & Sebring, 1964), this synthesis reaching a maximum between three and four hours after infection. Viral M-RNA increases in size as infection proceeds: at one hour its sedimentation coefficient is chiefly 8 to 12 s, at five hours after infection mainly 16 to 22 s.

The present paper deals with the later stages of viral replication, and will describe: (1) the time-course of synthesis of vaccinia DNA, taking advantage of the fact that it replicates in the cytoplasm; (2) the properties of the "factories" within which viral DNA replication occurs; and (3) the "coating" of viral DNA as it is incorporated into mature progeny.

2. Materials and Methods

(a) *Cells, medium and virus*

HeLa S3 cells were grown in suspension culture in Eagle's medium (Eagle, 1959) supplemented with 5% fetal calf serum. The WR strain of vaccinia virus was used. One highly purified virus preparation (Joklik, 1962*a*) was used throughout; it contained $2{\cdot}8 \times 10^{11}$ elementary bodies/ml. and $8{\cdot}5 \times 10^{9}$ p.f.u./ml. on chick embryo fibroblasts. WR labeled with [^{14}C]dT was prepared and purified similarly.

‡ Abbreviations are used: dFU, fluorodeoxyuridine; dT, thymidine; UR, uridine; TCA, trichloroacetic acid; p.f.u., plaque forming unit; M-RNA, messenger RNA; T-RNA, transfer RNA; SDS, sodium dodecyl sulfate; O.D., optical density; O.D. unit, optical density unit (1 O.D. unit is the amount of material having an optical density of 1 when the light path is 1 cm).

(b) *Adsorption*

Procedure A (Becker & Joklik, 1964) was followed. In brief, cells were infected at a concentration of 10^7 cells/ml. in adsorption medium with an added multiplicity of 400 elementary bodies per cell, for 15 min at 37°C. This resulted in 50% adsorption. The cells were then diluted to 10^6 cells/ml. with growth medium and incubation continued. All operations were carried out at 37°C.

(c) *Pulse-labeling*

At various times after infection, [^{14}C]dT or [^{14}C]UR (both 30 mc/m-mole, New England Nuclear Corp.) was added to cells, generally at the rate of 1 to 5 μc per 4×10^7 cells, depending on the experiment. If the extent of incorporation was to be measured at the end of the labeling period, cells were added to crushed frozen saline. If cells were to be incubated further, they were centrifuged, resuspended in fresh prewarmed medium lacking the labeled compound, and incubation continued.

(d) *Breaking of cells*

Cells were collected by centrifugation, washed once with 100 ml. Earle's saline, and allowed to swell for 10 min at 0°C in hypotonic medium, either RSB (10^{-2} M-KCl, 10^{-2} M-tris pH 7·4, 1·5 mM-Mg^{2+}) (Warner, Knopf & Rich, 1963), or 1 mM-phosphate pH 7·4, at a cell concentration of 2×10^7 per ml. The cells were then broken in a Dounce homogenizer (generally 2 ml. at a time) precalibrated for the number of strokes necessary to break over 98% of the cells. The number of strokes was selected so that when uninfected cells labeled with [^{14}C]dT were broken and the homogenate was then centrifuged at 200 *g* for 2 min, less than 2% of the radioactive material was not deposited.

(e) *Sucrose density-gradient centrifugation*

Sucrose gradients were 25 to 40% w/w in RSB or 1 mM-phosphate. Gradients were collected through a needle inserted from above. The effluent was pumped through the flow cell of a Gilford spectrophotometer and the optical density at 260 mμ recorded automatically. Fractions of about 1 ml. were collected and precipitated with 10% TCA after addition of carrier RNA and protein. They were then filtered onto 30 mm Schleicher & Schuell B6 membrane filters by means of a multifilter unit capable of handling 45 samples simultaneously. The membranes were glued to aluminium planchets and counted in a Nuclear Chicago low background gas-flow counter using a thin end window detector. For many gradients both DNase-resistant and DNase-susceptible radioactive material was determined. The fractions collected from such gradients were split and one-half plated directly, while the other half was first incubated with 50 μg/fraction of DNase at 37°C for 30 min.

3. Results

(a) *Viral growth cycle*

The following data on the replication of viral DNA must be related to the viral growth cycle. Vaccinia WR multiplies rapidly and to high titer in our strain of HeLa S3 cells. At 24 hours after infection the average virus content is 5000 to 10,000 virus particles/cell. At five hours the viral yield is 5%, at seven hours 25%, and at 11 hours 40% of that obtained at 24 hours.

(b) *DNA replication in infected cells*

Since the DNA of vaccinia virus replicates in the cytoplasm, experiments designed to examine the kinetics of its formation necessitate the use of cytoplasm uncontaminated with nuclear DNA. Such cytoplasm is provided by homogenizing cells in a precalibrated Dounce homogenizer and centrifuging the homogenate at 200 *g* for two minutes (see Materials and Methods). Figure 1 describes the rate of incorporation

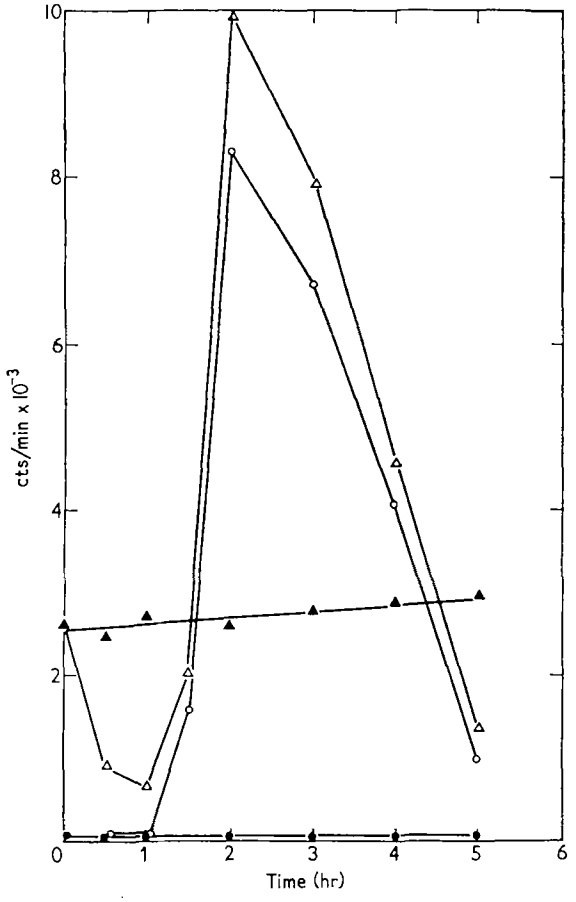

FIG. 1. The incorporation of [^{14}C]dT into uninfected and infected HeLa cells. Cells were infected as described in Materials and Methods. 5 min before the times indicated, samples of $1{\cdot}5 \times 10^7$ cells were exposed to 0·2 μc [^{14}C]dT for 10 min. They were then chilled rapidly, washed with Earle's salt solution, allowed to swell in 2 ml. RSB for 10 min at 0°C and disrupted with a Dounce homogenizer. Samples were plated for radioactivity measurements before and after centrifuging at 200 *g* for 2 min. —▲—▲—, control, total; —●—●—, control, cytoplasm; —△—△—, infected, total; —○—○—, infected, cytoplasm.

of [^{14}C]dT into HeLa cells at various stages after infection with vaccinia virus. The rate of incorporation into uninfected cells remains very nearly constant during the duration of the experiment; only 1 per cent of the total label incorporated is recovered in the cytoplasmic fraction. In infected cells there occurs, beginning at one to one and a half hours after infection, a very large burst of [^{14}C]dT incorporation. Most, if not all, of the incorporated label appears in the cytoplasmic fraction. The maximum rate of incorporation occurs at two to two and a half hours post-infection and often is three times the rate observed for uninfected cells. The rate of [^{14}C]dT incorporation into infected cells diminishes rapidly after three to four hours; over 90% of the cytoplasmic [^{14}C]dT incorporation occurs before four and a half hours after infection. It is not possible to say from this experiment whether incorporation of [^{14}C]dT into nuclei is also stimulated, since, as will be shown below, replicating DNA in the cytoplasm is associated with very large structures, some of which are deposited during the centrifugation, so that the values for the cytoplasm are too low.

However, two observations suggest that replication of host cell DNA is in fact drastically inhibited very soon after infection: (1) during the first one and a half hours after infection, infected cells incorporate as much as 75% less [^{14}C]dT than non-infected cells; (2) infection with virus inactivated with u.v.-irradiation, the DNA of which does not replicate, results in similar inhibition during the first one and a half hours, and permits a study of the further course of inhibition (Joklik & Becker, in preparation). Within three hours after infection with such virus, [^{14}C]dT incorporation into unfractionated cells is only about 3% of that in control cells. We conclude that infection of HeLa cells with vaccinia WR leads to a rapid and complete cessation of the replication of the host cell genome and that the following incorporation of [^{14}C]dT into the cytoplasm of infected cells represents replication of viral DNA.

(c) *Identity of the newly formed cytoplasmic DNA*

It might be argued that one of the effects of viral infection is injury to the nuclear membrane structure, so that the labeled DNA recoverable in the cytoplasmic fraction in fact represents DNA which had leaked out of the nucleus. That this is not the case is shown by the fact that cells prelabeled in DNA and subsequently infected with virus yield no more radioactivity in the cytoplasmic fraction than do prelabeled uninfected cells.

Chase experiments, reported below, demonstrate that the newly formed cytoplasmic DNA is viral DNA. Direct evidence on this point was obtained by comparing cesium chloride equilibrium density-gradient profiles of newly replicated cytoplasmic DNA, host cell nuclear DNA and authentic viral DNA isolated from highly purified virus. The latter was labeled with [^{3}H]dT, the former two with [^{14}C]dT, and each of these was centrifuged together with the authentic viral DNA. The cytoplasmic DNA coincided exactly with authentic viral DNA and could not be differentiated from it. Host cell DNA, on the other hand, was approximately seven drops heavier than viral DNA (Fig. 2), close to the expected result. Vaccinia DNA contains 36% GC (Joklik, 1962*b*) and its density in CsCl is 1·696 g cm^{-3} (Szybalski, Erikson, Gentry, Gafford & Randall, 1963), while human DNA contains 42% GC (Scherrer, Latham & Darnell, 1963) and its density in CsCl is 1·702 g cm^{-3} (Davern, 1960), a difference of 0·006 g cm^{-3}. (This agrees with the relation of Sueoka, Marmur & Doty (1959) that in CsCl there is a density increase of 0·001 g cm^{-3} per 1% increase in GC content.) The gradient in the center of our tubes, where the DNA banded, was about 0·008 g cm^{-3}/10 drops (determined by measuring the refractive index of every tenth drop).

The initiation of cytoplasmic DNA replication requires *de novo* protein synthesis. Cells were infected and at various times from zero to two hours thereafter puromycin was added to a final concentration of 50 μg/ml. At various times the cells were then exposed for 10-minute periods to [^{14}C]dT, and its incorporation into the cytoplasmic fractions measured. (1) If puromycin is added at the same time as virus, there is no replication of DNA in the cytoplasm (Table 1). This can be accounted for by the fact that puromycin inhibits the second stage of uncoating (Joklik, 1964*b*); under these conditions no parental DNA is uncoated and no template DNA is therefore available. (2) When puromycin is added one hour after infection, there is again no replication of DNA in the cytoplasm. This, however, cannot be attributed to failure of parental viral DNA to be uncoated, since the cells by then contain enough uncoating protein ultimately to uncover most of, if not all, the infecting DNA (Fig. 3) (Joklik, 1964*b*). Parental DNA templates are therefore available, yet there is no

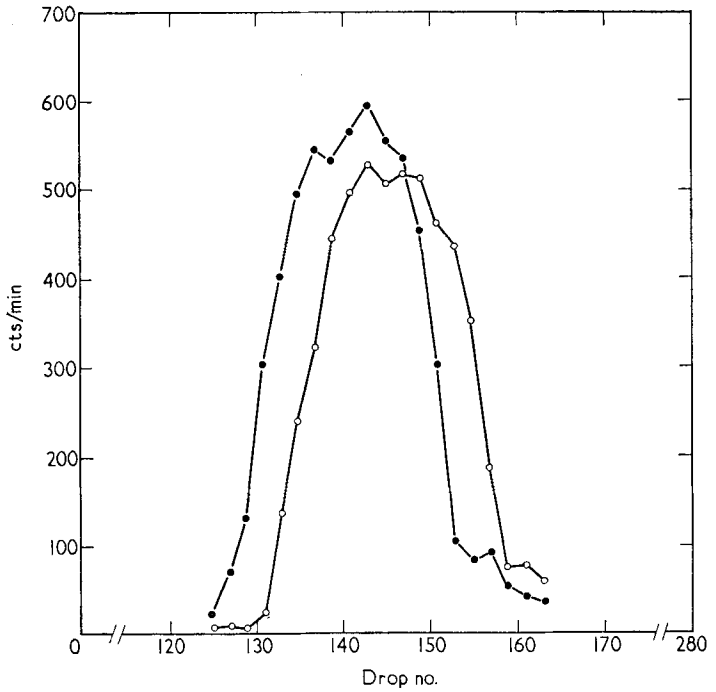

FIG. 2. Radioactivity profiles of HeLa cell DNA labeled with [^{14}C]dT (—●—●—) and vaccinia WR virus DNA labeled with [^{3}H]dT (—○—○—) in CsCl. Gradients were centrifuged for 65 hr at 35,000 rev./min. Single drops were collected. This profile demonstrates the separation obtainable with HeLa cell and WR DNA. DNA extracted from the cytoplasmic fraction of cells infected for 2 hr and then labeled with [^{14}C]dT for another half hour was indistinguishable from WR DNA in a similar gradient. DNA was extracted from WR labeled with [^{3}H]dT by the method previously described (Joklik, 1962c); and from the HeLa cell nuclear fraction (uninfected cells) and cytoplasmic fraction (infected cells) by a method using SDS, phenol extraction and ethanol precipitation.

TABLE 1

Effect of puromycin on the incorporation of [^{14}C]dT into the cytoplasmic fraction

Time of addition of label (hr post-infection)	Cts/min incorporated into				
	uninfected cells	infected cells			
		puromycin not added	puromycin added at 0 hr	puromycin added at 1 hr	puromycin added at 2 hr
0	320	(320)	(320)	(320)	(320)
1	200	200	200	(200)	(200)
2	250	12,640	160	300	(12,640)
3	—	6,640	100	150	2,000
4	300	2,240	150	150	300

Suspensions of infected cells were exposed to puromycin (50 μg/ml.) at 0, 1 and 2 hr after infection. At intervals, samples of 10^7 cells were withdrawn and pulse-labeled for 10 min with 0·1 μc [^{14}C]dT. Uninfected and infected cells were similarly pulse-labeled without the addition of puromycin. The figures represent the amount of radioactive material incorporated into the cytoplasmic fraction during the 10-min pulse. Brackets denote values only determined for the appropriate parent suspension.

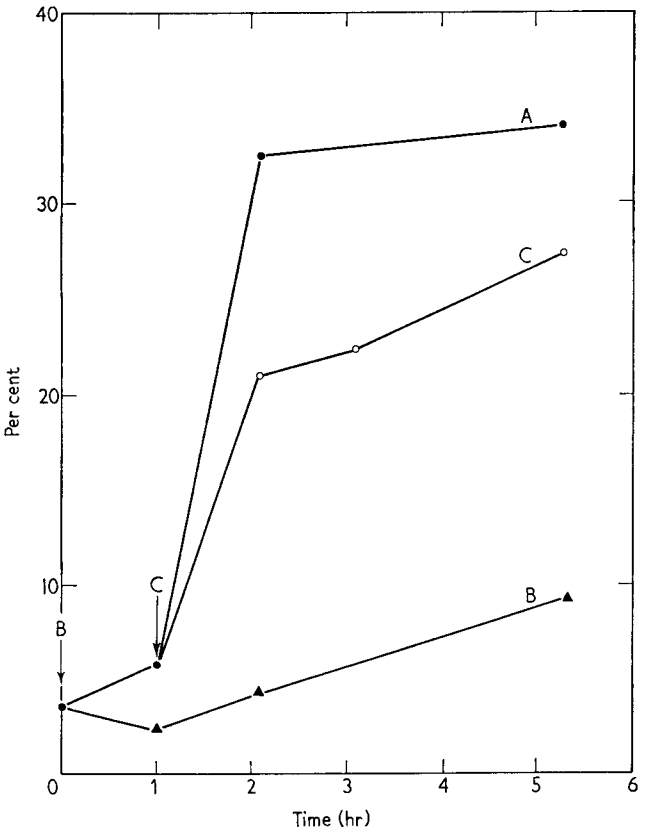

FIG. 3. Uncoating of vaccinia WR labeled with [^{14}C]dT. Multiplicity: 350 virus particles/cell. Curve A: No addition. Curve B: Puromycin added at time 0 (immediately after adsorption). Curve C: Puromycin added 1 hr later. Final concentration of puromycin: 50 μg/ml. The techniques used were as described before (Joklik, 1964*a*). The abscissa represents percentage of viral DNA uncoated.

replication of viral DNA. This is explained by postulating that viral DNA replication requires the formation of a *new* DNA polymerase which is synthesized after one hour after infection. (3) If puromycin is added two hours after infection, when cytoplasmic DNA replication has already begun, it causes a decrease in the rate of that replication as compared with control cells. This suggests either that the new DNA polymerase responsible for the cytoplasmic (viral) DNA replication is unstable, or that replication of viral DNA requires the continuous synthesis of some other protein.

Actinomycin D is known to inhibit uncoating of parental virus (Joklik, 1964*b*) and would also be expected to prevent the formation of the postulated new DNA polymerase. It should thus inhibit the virus-induced cytoplasmic DNA replication, since neither template nor enzyme would be available. This was indeed found to be so: actinomycin D, at 2 μg/ml., completely prevented the incorporation of [^{14}C]dT into the cytoplasm of infected cells (Table 2).

We conclude that the DNA formed between one and a half and five hours after infection and recovered in the cytoplasmic cell fraction according to the techniques described is viral DNA and it will be referred to as such in what follows.

TABLE 2

Effect of actinomycin D on incorporation of [^{14}C]dT into the cytoplasm of infected cells

Actinomycin D	−	+	−	+
Infected	−	−	+	+
Period of pulse, post-infection (min)				
15– 25	40	40	42	38
55– 65	30	20	34	24
115–125	20	18	9086	28
205–215	48	18	5080	30
295–305	40	18	1228	34

The numbers in the body of the table represent cts/min [^{14}C]dT incorporated when $1{\cdot}5 \times 10^7$ cells were pulsed for 10 min with 0·2 μc [^{14}C]dT. Multiplicity of infection: 200 particles/cell. Concentration of actinomycin D: 2 μg/ml., added at the same time as the virus. Conditions of infection, breaking of cells and preparation of the cytoplasmic fraction as outlined in Materials and Methods.

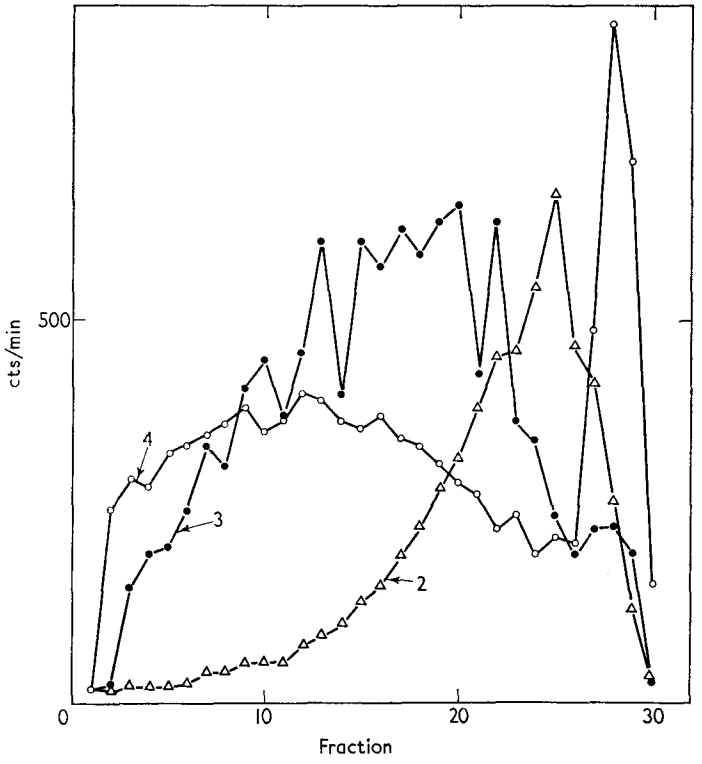

FIG. 4. Size distribution of newly replicated viral DNA. Samples of 5×10^7 cells were pulse-labeled for 10 min with 0·5 μc [^{14}C]dT at 2, 3 and 4 hr after infection. The cytoplasmic fractions (prepared in RSB) were centrifuged into 25 to 40% sucrose gradients in RSB for 8 min at 6000 rev./min. Pellet: 2 hr, 240 cts/min; 3 hr, 510 cts/min; 4 hr, 1070 cts/min.

(d) *Aggregates containing newly replicated viral DNA*

(i) *Size of the aggregates*

The newly replicated viral DNA exists in the form of large aggregates (Fig. 4). The conditions of centrifugation employed here allow only very limited penetration of whole virus into gradients and even less of intact single viral DNA molecules (mol. wt 160,000,000, Joklik, 1962*b*). The size of the aggregates increases considerably between two and four hours after infection. Their size at one and a half hours is significantly less than at two hours.

(ii) *Aggregates as the site of DNA replication*

[^{14}C]dT is incorporated into infected cells with a lag of not more than 30 seconds. Samples of cells infected for three hours were labeled with [^{14}C]dT for one and two minutes, respectively, and their cytoplasmic fractions centrifuged into 25 to 40% sucrose gradients in RSB for two hours at 23,000 rev./min. In both cases 98% of the label was in the pellet. Another sample of cells was pulsed for three minutes and the cytoplasmic fraction centrifuged into a similar sucrose gradient for nine minutes at 9000 rev./min (Fig. 5). Less than 5% of the label was at the top of the gradient where free DNA would be expected.

(iii) *Association of parental viral DNA with the aggregates*

If aggregates are the site of DNA replication, parental viral DNA would be expected to be associated with them. This was found to be the case. Cells were infected with highly purified WR virus, labeled in the DNA moiety with [^{14}C]dT

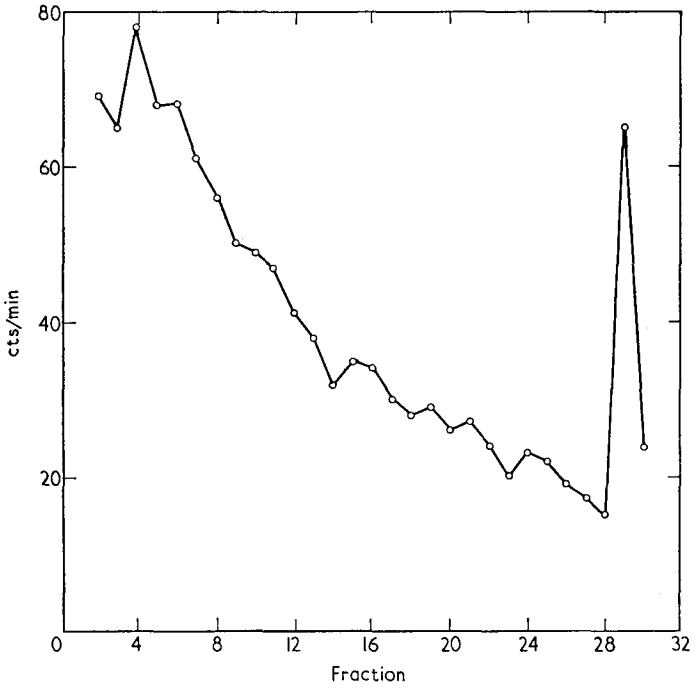

FIG. 5. Size distribution of viral DNA replicated within 3 min. Cells infected for 3 hr were pulsed with [^{14}C]dT for 3 min. The cytoplasmic fraction was centrifuged into a 25 to 40% sucrose gradient in RSB for 9 min at 9000 rev./min. Pellet, 610 cts/min.

(Joklik, 1962*a*); at intervals thereafter the cells were broken, the cytoplasmic fractions separated and centrifuged into 25 to 40% sucrose gradients for eight minutes at 6000 rev./min (Fig. 6). At one hour after infection, all but 10% of the radioactive label was at the top of the gradient and about one-half was resistant to DNase; at this time the first stage of uncoating is almost complete and the second stage is proceeding (Joklik, 1964*a*). One hour later, 61% of the label centrifuged well into the gradient and was associated with aggregates. All of this material was sensitive to DNase, that is, it was free DNA. Of the 39% of the label still at the top of the gradient, two-thirds was now sensitive to DNase, representing uncoated viral DNA

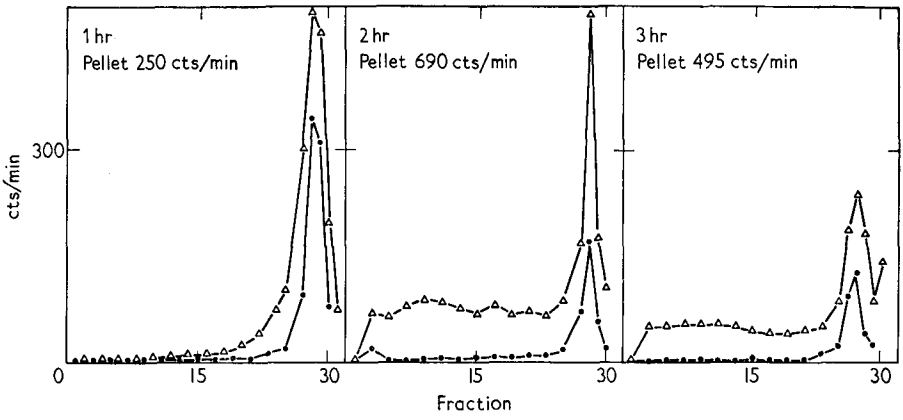

FIG. 6. Association of parental DNA with aggregates. For details see text.

not yet associated with aggregates. One hour later, this top fraction had decreased to 25% of the total, and three-quarters of it was sensitive to DNase. Again all label in the aggregate fraction was sensitive to DNase. Between 10 and 20% of the label was regularly recovered at the bottom of the gradient in the form of a pellet, and was attributable to virus adsorbed on large cell membrane debris. The conclusion is that soon after uncoating, parental viral DNA becomes associated with very large aggregates having sedimentation characteristics similar to those with which replicating viral DNA is associated.

(iv) *Absence of polyribosomes in the aggregates*

We have previously reported (Becker & Joklik, 1964) that M-RNA is copied from viral DNA during the early part of the infection cycle. It was of interest to establish whether not only the replicating DNA but also protein-synthesizing structures, that is, polyribosomes, were associated with the aggregates: in other words, whether the aggregates represented integrated units for the synthesis of viral nucleic acid and protein, as are the "virus synthesizing bodies" in the case of poliovirus (Becker, Penman & Darnell, 1963; Penman, Becker & Darnell, 1964). Figure 7 shows that this is not so. The sedimentation profiles for newly synthesized DNA and RNA are quite different. No newly synthesized RNA is detectable in the aggregates at two and a half hours after infection, the time at which both viral DNA and viral M-RNA synthesis proceed at the maximum rate. The aggregates therefore contain no polyribosomes formed by viral M-RNA.

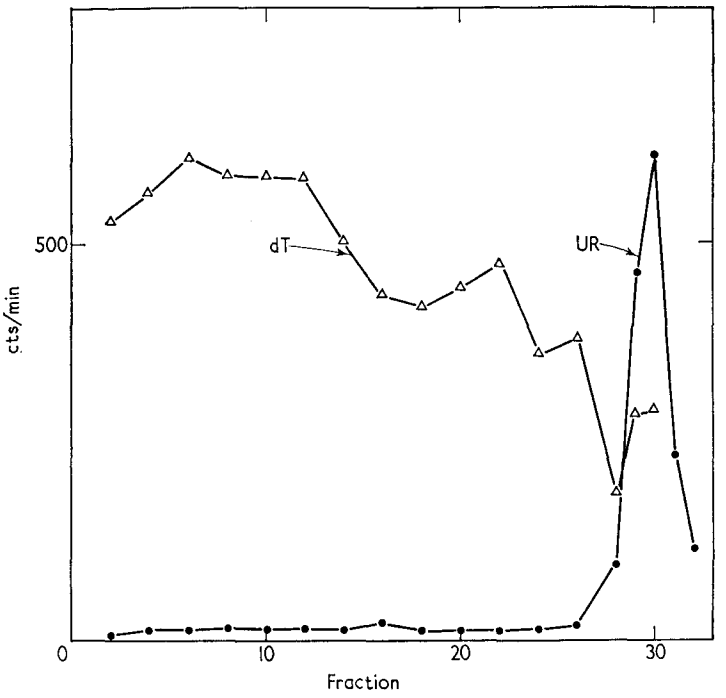

FIG. 7. Failure of newly formed viral messenger RNA to become associated with aggregates. Two lots of 3×10^7 cells each were infected by the standard procedure and labeled at 3 hr with 0·2 μc [^{14}C]dT or 0·2 μc [^{14}C]UR respectively for 10 min. The cells were broken in RSB and centrifuged into 25 to 40% sucrose gradients in RSB for 6 min at 6000 rev./min. Pellet: UR, 585 cts/min; dT, 5030 cts/min.

(v) *Factors affecting the stability of the aggregates*

In contrast to the "virus synthesizing bodies" in cells infected with poliovirus, the aggregates containing vaccinia virus DNA are not disrupted by sodium deoxycholate. They are, however, very sensitive to sonication: 30 or 60 seconds treatment with a Mullard MSE ultrasonic disintegrator caused all labeled DNA to band at the top of the gradient, while treatment for 15 seconds was almost but not quite as efficient (Fig. 8). Further, the aggregates break down in the absence of magnesium. In all experiments described so far, cells have been broken in RSB (see Materials and Methods) and centrifuged into gradients of 25 to 40% sucrose in RSB. If the cells are instead homogenized in 1 mM-phosphate, pH 7, and centrifuged into 25 to 40% sucrose gradients made up in 1 mM-phosphate, the aggregates, although not completely dispersed, are very much smaller (Fig. 8).

The effect of magnesium on the stability of the aggregates is further illustrated in Table 3. Cells were labeled with [^{14}C]dT from two and a quarter to three and a quarter hours after infection. They were then broken in various media, the cytoplasmic fractions (the supernatants after centrifuging for two minutes at 200 *g*) centrifuged for 20 minutes at 2000 *g*, and the percentage of radioactivity in the pellets determined. If breaking of cells is carried out in RSB, which contained 1·5 mM-magnesium, 70% of the label is in structures of such a size that they are deposited by centrifugation for 10 minutes at 2000 *g*. If the magnesium concentration

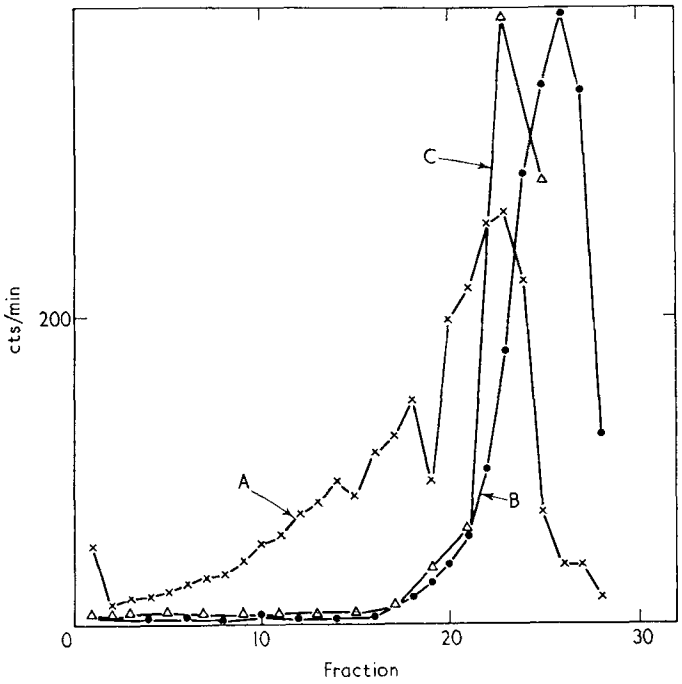

FIG. 8. The effect of sonication and absence of magnesium on the stability of aggregates. Cells: 2×10^7 cells/sample, pulse-labeled with 0·1 μc [^{14}C]dT for 10 min $2\frac{1}{2}$ hr after infection. Cytoplasmic fractions were prepared by breaking in either RSB or 1 mM-phosphate and centrifuged into 25 to 40% sucrose in either RSB or 1 mM-phosphate. Centrifugation: 8 min at 6000 rev./min. For further details see text. Curve A, RSB—RSB/sucrose; pellet, 130 cts/min. Curve B, RSB—RSB/sucrose, sonicated 30 sec; pellet, 85 cts/min. Curve C, phosphate—phosphate/sucrose; pellet, 50 cts/min.

TABLE 3

Effect of magnesium concentration on the stability of the aggregates

Cells disrupted in	Percentage of cts/min in cytoplasm deposited by centrifuging for 20 min at 2000 *g*
10^{-2} M-tris, 10^{-2} M-KCl, $1{\cdot}5 \times 10^{-3}$ M-Mg^{2+} (RSB)	70
10^{-2} M-tris, 10^{-2} M-KCl, $0{\cdot}3 \times 10^{-3}$ M-Mg^{2+}	40
10^{-2} M-tris, 10^{-2} M-KCl	15
10^{-3} M-phosphate	13
10^{-3} M-phosphate, then Mg^{2+} to $1{\cdot}5 \times 10^{-3}$ M	60

Cytoplasm: supernatant fraction after centrifuging for 2 min at 200 *g*.
Cells were labeled with [^{14}C]dT from $2\frac{3}{4}$ to $3\frac{1}{4}$ hr after infection, and then broken.

is diminished to 0·3 mM, this value decreases to 40%; and if magnesium is omitted altogether it decreases to 15%, the same value as in 1 mM-phosphate. The operative variable is thus magnesium concentration, not ionic strength. If the cells are broken in 1 mM-phosphate and magnesium is then added to a final concentration of 1·5 mM, and the preparation is examined after 10 minutes at 0°C, the amount of material in the pellet is again high, 60%. The dissociation is thus reversible.

(vi) *The fate of the aggregates in the infected cell*

It has been shown above (section 3(d)(i)) that DNA being synthesized two, three and four hours after infection is associated with progressively larger structures. It was of interest to determine in what manner the size of the structure with which a particular set of viral DNA molecules was associated changed as infection progressed. Accordingly, cells were labeled with [^{14}C]dT from one and three-quarters to two hours after infection and then transferred to medium lacking [^{14}C]dT. Under these conditions, labeled thymidine compounds in the acid-soluble pool are soon exhausted; the amount of radioactivity incorporated into DNA increases by less than 15% after removal of the labeled precursor. At intervals after the transfer, samples of cells were removed, broken in RSB and cytoplasmic fractions prepared by centrifugation for two minutes at 200 *g*. The radioactive material in the whole cytoplasmic fraction and in the supernatant fractions after centrifugation for 5 minutes at 475 *g*, 10 minutes at 850 *g* and 20 minutes at 2000 *g* was then measured to give estimates of the size of the aggregates with which the DNA molecules that had been synthesized between one and three-quarters and two hours after infection were subsequently associated (Fig. 9). The aggregates proved to be largest at three and four

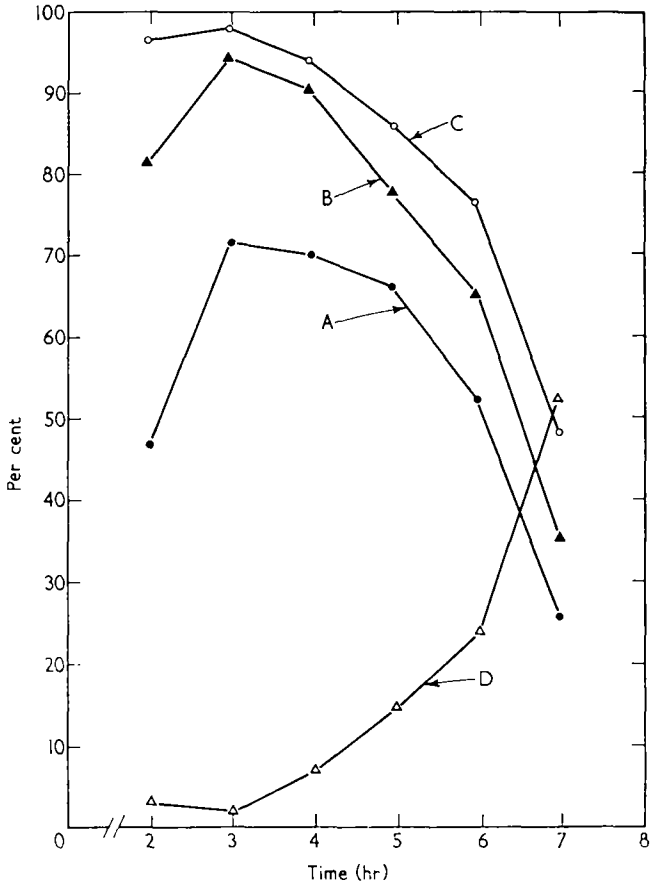

FIG. 9. Size distribution of aggregates during infection cycle. A: percentage of radioactive label deposited by centrifuging for 5 min at 475 *g*; B: 10 min at 850 *g*; C: 20 min at 2000 *g*; D: percentage of label in supernatant fraction after centrifuging for 20 min at 2000 *g*.

hours after infection (compare Fig. 4), and then begin to break up. At seven hours after infection, over 50% of the DNA can no longer be pelleted by centrifuging at 2000 *g* for 20 minutes, in contrast to the 2% not pelleted at three hours.

We conclude that the aggregates described above are probably the "factories" observed by autoradiographic and electronmicroscopic techniques. This conclusion will be further justified below.

(e) *Incorporation of viral DNA into virus particles*

(i) *Coating*

Coating is studied by measuring the amount of viral DNA replicated early during the infection cycle (and then completely susceptible to DNase) which becomes resistant to DNase. Figure 10 shows the results of such an experiment. Almost all

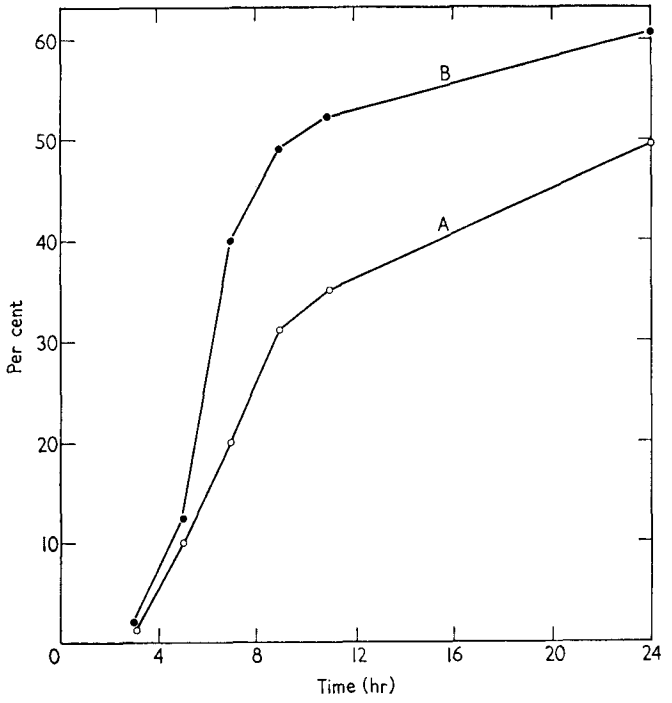

FIG. 10. Coating of viral DNA replicated during 1½ to 2½ hr after infection. 6×10^7 cells were labeled with 2 μc of [^{14}C]dT from 1½ to 2½ hr after infection. The cells were then washed, resuspended, and incubation continued. At various times samples were taken, the cells broken in RSB, the cytoplasmic fractions prepared and the susceptibility of the labeled viral DNA to DNase estimated both in the whole cytoplasmic fraction and in the deposit after centrifuging for 10 min at 2000 *g*. DNase: 50 μg/ml.; magnesium concentration during enzyme digestion: 10 mM; incubation time: 37°C for 30 min. Abscissa: per cent of DNA resistant to DNase. Curve A: whole cytoplasmic fraction; curve B: deposit.

viral DNA replicated between one and a half and two and a half hours after infection remains digestible by DNase up to the fifth hour. This DNA then becomes progressively more resistant to DNase, until at 24 hours after infection about 50% of it is not digestible by DNase. DNA in aggregates becomes resistant more rapidly than the cytoplasmic DNA. Most of the coating process in aggregates occurred between five

and ten hours; for the cytoplasm as a whole, it continued beyond ten hours. It is likely that the development of resistance to DNase represents coating of viral DNA by structural viral protein, and that this process occurs predominantly in large aggregates.

(ii) *Incorporation of viral DNA into immature and mature virus progeny*

The object of the next series of experiments was to characterize the particles, into which viral DNA replicated during the early portion of the infection cycle, are eventually incorporated. The plan of the experiments was as follows: viral DNA replicating between two and a quarter and two and three-quarter hours after infection was labeled with [^{14}C]dT. The label was then removed and incubation continued. At times thereafter samples of the infected culture were taken, the cells broken in 1 mM-phosphate, and the whole homogenates centrifuged into 25 to 40% sucrose gradients in 1 mM-phosphate for 60 minutes at 15,000 rev./min. Under these conditions, virions banded near the bottom of the gradient. Gradients were collected and plated before and after treatment with DNase.

Centrifuging whole homogenates in 1 mM-phosphate yielded gradients with the optimum distribution of label. If cells were broken in RSB, about 50% of the DNA remained at the top of the gradients, while the rest was pelleted (this agrees with Fig. 9). If the cells were broken in 1 mM-phosphate and the cytoplasmic fraction then isolated and sonicated in order to disperse all aggregates, less label banded in the immature particle fraction (see below). If the cells were broken in 1 mM-phosphate and the cytoplasmic fraction rather than the whole homogenate was centrifuged, results were very similar to the ones described below, indicating that the extra manipulation could be eliminated. Controls showed that under the conditions of centrifugation employed the small amount of labeled nuclear DNA present in these cells is deposited in the pellet.

Figure 11 shows the patterns obtained with cells removed at 5½, 7½, 11 and 24 hours after infection. The following bands can be distinguished. (1) A virion band in the lower part of the gradient. Label in this band is completely resistant to DNase. (2) Free DNA remaining at or near the top of the gradient. This material is digested by DNase to the extent of 95 to 99%. (3) Material in the intervening region. This material is partially sensitive to DNase digestion and will be referred to as the "immature particle" or "sub-virion" region; it represents material containing viral DNA (since it is labeled) in completely or partially coated form, heavier or larger than free molecules but smaller or lighter than virions. It is this material which is partially sensitive to sonication (see above).

Table 4 shows the relative amounts of label in virions, sub-virions and naked DNA, calculated by analysing the radioactivity profiles. The amount of labeled DNA in the virion fraction increases in proportion to the optical density at 260 mμ (a measure of the total number of particles). The amount of label in the sub-virion fraction remains fairly constant from 5 to 24 hours. At no time is there a significant accumulation of immature virus particles.

Table 5 provides evidence that the material in the virus peak is indeed composed of virions. Cells infected for 7½, 12 and 24 hours were homogenized in 1 mM-phosphate and the whole homogenate centrifuged into 25 to 40% sucrose gradients in 1 mM-phosphate. Fractions containing the virion bands were pooled and the optical density and number of p.f.u. determined. From the optical density the number of elementary

bodies was calculated according to the relation 1 O.D. unit at 260 mμ = $1{\cdot}2 \times 10^{10}$ elementary bodies (Joklik, 1962*b*). The specific infectivity in terms of infectivity per elementary body was constant, demonstrating that the virus peak does indeed consist of virions.

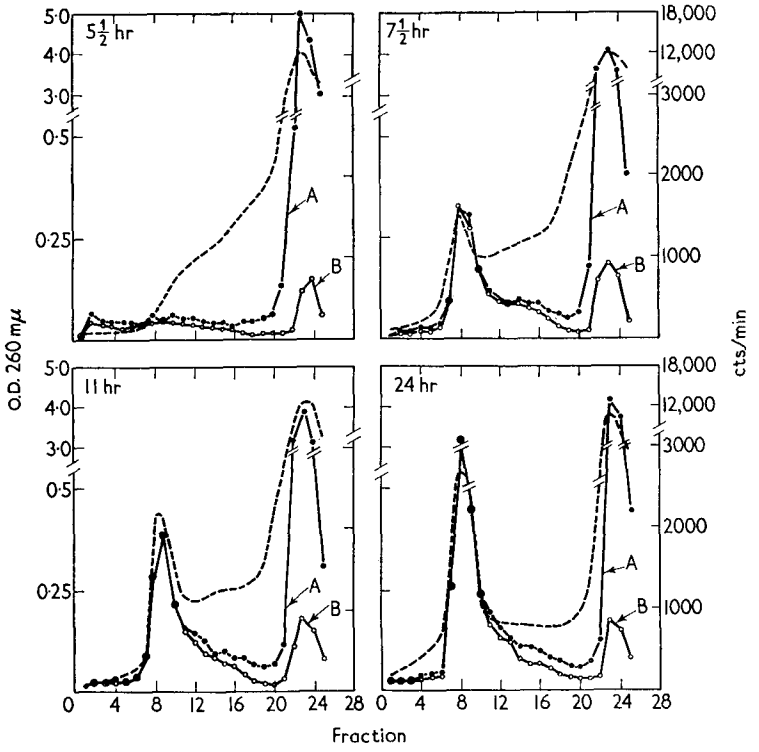

FIG. 11. Distribution of viral DNA replicated during the interval 2½ to 2¾ hr after infection in sucrose density gradients. For details see text. Gradients were divided into two portions and plated before (A) and after (B) treatment with DNase (50 μg/1 ml. fraction). Broken curves: O.D. at 260 mμ.

TABLE 4

Incorporation of viral DNA into immature and mature virus progeny

Fraction	Time after infection (hr) 5½	7½	9	11	24
Virions	1	8·8	10·75	13·35	28·5
Subvirions: res. to DNase	3·6	6·15	6·7	6·55	7·35
Subvirions: susc. to DNase	1·8	1·6	1·7	1·75	1·8
Free DNA	94·5	83·45	80·85	78·35	62·35

The numbers in the body of the table represent percent of total cts/min recovered in the gradients present in the indicated fraction.

TABLE 5

Analysis of the virion bands isolated from gradients

Cells harvested at (hr)	O.D. units at 260 mμ	Virus particles	p.f.u.	Virus particles per p.f.u.
$7\frac{1}{2}$	3·3	4×10^{10}	$1{\cdot}5\times10^{9}$	27
$11\frac{1}{2}$	6·7	8×10^{10}	$3{\cdot}8\times10^{9}$	22
$23\frac{1}{2}$	14·2	$1{\cdot}7\times10^{11}$	6×10^{9}	28

3×10^{7} cells were analysed at each of the three times indicated.

(iii) *Withdrawal of viral DNA from the replicating pool*

The foregoing experiments indicate that the bulk of vaccinia DNA is replicated in the cytoplasm from one and a half to five hours after infection, while coating of viral DNA begins at about five hours. This suggests that there is no significant withdrawal from the pool of replicating DNA until replication has almost ceased. This was confirmed in two ways.

(1) Replicating viral DNA was pulse-labeled with [^{14}C]dT from $1\frac{3}{4}$ to 2, $3\frac{1}{2}$ to $3\frac{3}{4}$ and $4\frac{3}{4}$ to 5 hours after infection. After each pulse the label was removed by centrifugation and incubation resumed. At seven hours the cells were homogenized in 1 mM-phosphate and the whole homogenate centrifuged into 25 to 40% sucrose gradients in 1 mM-phosphate. Total counts and counts resistant to DNase digestion were then determined. The optical density and radioactivity profiles resembled those shown in Fig. 11. The amount of label present in the virion, sub-virion and free DNA regions was summed and the relationships shown in Table 6 established. The per-

TABLE 6

Withdrawal of viral DNA from the replicating pool

Fraction	$1\frac{3}{4}$ to 2 hr	Viral DNA labeled $3\frac{1}{2}$ to $3\frac{3}{4}$ hr	$4\frac{3}{4}$ to 5 hr
Virions	9·5	8·5	3·4
Subvirions resistant to DNase	4·1	3·5	1·5
Subvirions susceptible to DNase	0·8	0·9	1·0

The numbers in the body of the table represent the percentage of total DNA recovered in the gradients present in the indicated fraction.

centage of total radioactivity present in both the virion peak and the DNase-resistant sub-virion fraction was almost the same for the two early pulses, but much lower for the late one. In contrast, the fraction of counts in the sub-virion fraction sensitive to DNase was almost the same for all the three pulses. It follows that withdrawal from the pool of replicating DNA does not begin before about four hours after infection, and that DNase-susceptible forms of viral DNA associated with material,

probably protein in nature, which increase its sedimentation rate, are intermediates on the maturation pathway.

(2) Viral DNA replicating between two and two and a half hours after infection was labeled with [^{14}C]dT. Puromycin was then added at intervals and the amount of label banding as virions, and as DNase-resistant and DNase-sensitive sub-virions, was measured at seven hours after infection. This time was chosen as the one most likely to provide an insight into the flow of viral DNA through immature viral forms into virions. The distribution of radioactivity in the gradients is analysed in Table 7 and Fig. 12. In the absence of puromycin, 6·3% of the labeled DNA was located in

TABLE 7

Effect of inhibition of protein synthesis on incorporation of viral DNA into mature and immature viral progeny

Fraction	Puromycin added at 3 hr	4 hr	5 hr	6 hr	Not added
A—Virions	0	80	375	920	1,510
B—Subvirions resistant to DNase	0	240	580	905	1,530
C—Subvirions sensitive to DNase	0	360	575	1,045	580
D = A + B + C	0	680	1,530	2,870	3,620
Free DNA	27,500	26,000	27,000	26,000	24,500
A/D	—	0·12	0·24	0·32	0·42
B/D	—	0·35	0·38	0·32	0·42
C/D	—	0·53	0·38	0·36	0·16

Viral DNA was labeled between 2 and 2½ hours after infection (5 μc [^{14}C]dT, 2×10^8 cells). Puromycin: 50 μg/ml. Numbers in the body of the table represent the number of cts/min recovered in the indicated fraction, or the ratios indicated.

the virion band and 8·7% in the sub-virion region (of which 73% was resistant to DNase). Thus 12 to 13% of the DNA was "coated". When puromycin was added at three hours, none of the DNA labeled between two and two and a half hours was incorporated into either sub-virions or virions by the seventh hour: all of it remained at the top of the gradient, completely digestible by DNase. With puromycin added at four, five and six hours, intermediate values were obtained. Puromycin addition at four hours resulted in a 95% inhibition of incorporation of label into virions, relative to controls, and a 72% inhibition of incorporation into sub-virions. If added at five hours the values were 75 and 45%, respectively, and if added at six hours, 39 and 8%. The results are consistent with the hypothesis that label flows from free DNA into virions via the DNase-sensitive and then the DNase-resistant sub-virion stages. Further, they establish that only about one-quarter of the protein necessary for this flow (presumably structural viral protein) has been synthesized four hours after infection, and that only a quarter of the last of the proteins necessary for the formation of virions is synthesized before five hours. It should be emphasized that we are not dealing here with the total amount of the particular protein species that the infected cell synthesizes throughout the infection cycle: we are examining the yield at seven hours, which amounts to about one-quarter of the total.

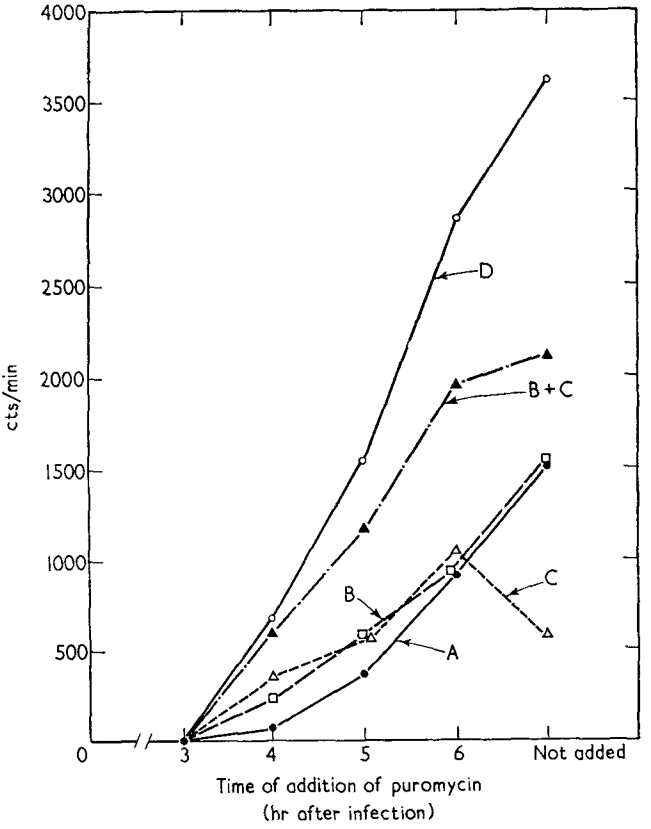

FIG. 12. The effect of inhibition of protein synthesis on incorporation of viral DNA into mature and immature viral progeny. The results presented in Table 7 are graphed. A, B, etc., have the same connotation as in that table.

4. Discussion

The experiments here described provide the first direct data on the time-course of synthesis of vaccinia virus DNA. In general, they confirm and greatly extend the findings of Salzman (1960) obtained by the indirect dFU inhibition technique.

Viral DNA replication commences at one to one and a half hours after infection. Its rate rises extremely rapidly; the maximum is attained at two to two and a half hours and it then diminishes almost equally rapidly. Over 90% of viral DNA is replicated between one and a half and four and a half hours.

Viral DNA replicates in the cytoplasm. The question accordingly arises as to the nature of the enzymes catalysing its replication. Magee (1962) and Green, Piña & Chagoya (1964) have reported that HeLa and KB cells infected with vaccinia virus contain up to three times greater DNA polymerase activity than normal cells; and we have recently observed much greater increases in the cell–virus system employed in the present study (Jungwirth & Joklik, unpublished results). The fact that puromycin added to cells one hour after infection, and after the synthesis of a certain amount of the protein necessary for uncoating, prevents DNA replication constitutes further evidence that a new protein is involved. This is probably a new specific enzyme catalysing the replication of viral DNA, coded for by the viral genome; and indeed, viral M-RNA is synthesized at this time (Becker & Joklik, 1964).

The maximum rate of viral DNA replication, about two and a half hours after infection, is several times greater than the normal rate of host cell DNA synthesis in logarithmically growing cells. This is not surprising, for the amount of viral DNA replicated is quite large. The amount of DNA in the final virus yield per cell (often 10,000 particles) is itself equivalent to about 15% of the host cell DNA complement, while the amount of viral DNA not incorporated into viral particles is usually three to four times greater. The total amount of viral DNA synthesized is thus equivalent to about half of the host cell DNA complement. Most of this DNA is synthesized in about one-twelfth of the generation time of the host cell, accounting for the exceedingly rapid rate of [^{14}C]dT incorporation in infected cells.

Host cell DNA replication is reduced by more than 50% within 30 minutes after infection. This decline continues until it is obscured by the tremendous burst of cytoplasmic replication of viral DNA. If the latter burst is prevented, e.g. by infecting cells with virus inactivated by u.v.-irradiation, which does not undergo the second stage of uncoating (Joklik, 1964*c*), then it is possible to observe that the decline in the replication rate of host cell DNA continues until, three hours after infection, this rate is only about 3% of that in uninfected cells. This inhibition occurs even in the presence of puromycin, and is therefore not due to protein synthesized after infection, but instead is triggered by the introduction into the cell of foreign protein (Joklik & Becker, manuscript to be published).

When cells are broken in medium containing appreciable amounts of magnesium (1·5 mM), the replicating viral DNA is found to be associated with very large structures which can be centrifuged into sucrose gradients far more readily than either virions or naked viral DNA. These aggregates have a number of curious properties. (1) They break down if the concentration of magnesium is lowered. Since one can only guess at the relevant local concentration of magnesium within the cell, it is not possible to establish beyond doubt that the aggregates isolated in buffers containing 1·5 mM-magnesium are actually the structures present within the cell. Lowering the concentration of magnesium fivefold already disperses them to a significant degree. We have not tried the effects of higher concentrations of magnesium. We believe that 1·5 mM-magnesium is a reasonable estimate of the intracellular magnesium concentration in the growth medium here used, and that the aggregates are indeed structures existing within the cell. (2) The viral DNA actually replicating, and not merely that newly replicated, is associated with the aggregates. Even after a one-minute pulse of [^{14}C]dT, all incorporated label is associated with aggregates. (3) As would be expected on this basis, parental DNA is found to be associated with the aggregates soon after it is uncoated. (4) On the other hand, since the aggregates are unlabeled when the label is [^{14}C]UR, they do not contain polyribosomes formed by viral M-RNA. Whether they contain RNA–DNA hybrids is an open question at the moment; experiments to test this are under way. (5) The aggregates increase in size as infection progresses. However, the fraction of viral DNA associated with aggregates decreases progressively, from a value very close to 100% for the first three hours to less than 50% at seven hours. (6) Coating of viral DNA takes place within the aggregates.

The aggregates we observe here may well correspond to the "factories" previously characterized in poxvirus-infected cells by autoradiographic, microscopic (Kato, Takahashi, Kameyama & Kamahora (1959)) and electromicroscopic studies. The following evidence can be adduced: (1) "factories" are the structures within which

viral DNA replicates (Cairns, 1960); replicating viral DNA is associated with the aggregates; (2) parental viral DNA associates with the "factories" (Dales, 1963); it is also associated with the aggregates; (3) "factories", like aggregates, contain no detectable amounts of rapidly labeled RNA (Cairns, 1960). (As discussed above, this should not be taken as implying that viral M-RNA is not coded in the aggregates; merely that neither "factories" nor aggregates contain polyribosomes formed by viral M-RNA); (4) "factories" are composed of fibrous material and are not bounded by a limiting membrane (Dales, 1963); the aggregates are not disrupted by sodium deoxycholate, implying the absence of a lipid-containing membrane (in distinction to the "virus synthesizing bodies" found in cells infected with poliovirus) (Becker *et al.*, 1963); (5) poxvirus matures within "factories" (Randall, Gafford & Arhelger, 1961; Dales, 1963); coating of viral DNA is associated with the aggregates.

It should be noted here that although inclusions are associated with most poxvirus infections, not all inclusions are of such a loose fibrillar character as those associated with vaccinia virus infection. Thus fowlpox virus inclusions are stable structures which may be isolated at the end of the infection cycle and used as a source of virus for further purification (Randall, Gafford & Darlington, 1962). Quite clearly the vaccinia aggregates do not correspond to such well-defined structures, and are far more labile.

There is no evidence as to what the material is within the aggregates which responds to magnesium. It may be replicating DNA itself (there is evidence that replicating phage DNA has unusual properties (Frankel, 1963)); or it may be a protein, acting as a "cement" which helps to circumscribe the space within which DNA replicates. However that may be, it is clear that after four to five hours, the fraction of viral DNA which is associated with aggregates decreases. The reasons for this are not clear. It is likely that the amount of the macromolecule responding to magnesium decreases at this time, perhaps because it is broken down or because its tertiary structure changes so that it no longer responds to magnesium to the same extent.

Coating of viral DNA begins at about five hours. This estimate is derived from three lines of evidence: (1) measuring the development of resistance to DNase of DNA replicated early during the infection cycle; (2) measuring withdrawal of DNA from the replicating pool into structures larger than DNA (the sub-virion and virion fractions); and (3) measuring the effect of puromycin added at various times after infection on the flow of DNA into these same larger structures. The composite picture which is obtained is: withdrawal of DNA from the replicating pool starts at about four hours. Protein synthesis is necessary for this withdrawal. The early protein(s) convert viral DNA to a form which is significantly larger than a naked molecule but which is still susceptible to DNase. Further proteins are then synthesized which cause the viral DNA to become resistant to DNase, that is, coated; however, the resulting structures are still smaller than whole virus. These structures band very diffusely, indicating that they are heterogeneous. Finally, DNA is incorporated into structures banding as virions. Little of the proteins necessary for this last step is produced before five hours.

Although the amount of viral DNA incorporated into virions increases steadily between $7\frac{1}{2}$ and 24 hours, the amount present as sub-virions remains constant, as if there were a constant pool of these incomplete virus particles through which DNA flows into virions. At all times about 20% of the sub-virions are sensitive to DNase.

Only 30 to 50% of the viral DNA is eventually incorporated into sub-virions or virions, perhaps because of topographic limitations.

A final word should be said about the association with viral DNA of protein coded for by the viral genome. Although Shatkin (1963) has observed that such protein is detectable within two hours of infection, from the data presented above it is unlikely that this is structural virus protein. It is more likely to be other antigenic protein coded for by the very large viral genome. Further, although Shatkin's observation that such early protein is associated with viral DNA agrees with the fact that viral antigens are detectable in the factories by staining with fluorescent antibody, our results, like those of Cairns (1960), indicate that the factories are not the site of the *synthesis* of this new protein. It would appear instead that proteins specified by the viral genome via M-RNA are synthesized in the space surrounding the factories and are then somehow attracted back to the factories or aggregates. Synthesis of structural viral protein commences at about four hours and it must be supposed that it too is attracted back to the aggregates, and that when its concentration reaches a critical value, coating of viral DNA commences. The synthesis of viral M-RNA declines at about the time that coating commences (Becker & Joklik, 1964): the reason for this cannot be that all template DNA is coated, since up to 70% of viral DNA is usually found in the free state, at the top of the gradients, at the end of the infection cycle. Whether the viral DNA which is not coated is complete is not clear at the moment; experiments to test this are under way.

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