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The Intracellular Uncoating of Poxvirus DNA

II. The Molecular Basis of the Uncoating Process

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The uncoating of poxvirus DNA is a two-step process. The first stage begins immediately after penetration of virus particles and is effected by enzymes present in the uninfected cell; the products of this stage are virus cores. Viral DNA within cores is not accessible to DNase. The second stage of uncoating results in the breakdown of the cores to release naked poxvirus DNA. This stage is inhibited by fluorophenylalanine and puromycin, inhibitors of protein synthesis, as well as by actinomycin D and irradiation with u.v. light, inhibitors of messenger RNA synthesis. It is concluded that in order for virus cores to be broken down a protein has to be synthesized after infection. This protein is synthesized only in response to infection with virus particles containing undenatured protein. Evidence is presented suggesting that the synthesis of this protein is under the control of the host-cell genome. A scheme is presented which is capable of explaining all known facts concerning poxvirus reactivation, initiation and uncoating. It postulates that a viral protein released during the first stage of uncoating causes derepression of a portion of host cell DNA, permitting the synthesis, through the mediation of messenger RNA formation, of a protein which is instrumental in degrading virus cores and thus releasing viral DNA in the free state.

1. Introduction

This report describes results obtained in a study of the mechanism of the uncoating of poxvirus DNA. Some preliminary data have already been discussed briefly (Joklik, 1962*a*); a detailed account of the experimental techniques used and the basic characteristics of the uncoating process has been presented in the preceding paper (Joklik, 1964). In short, poxvirus uncoating is a two-stage process. The first stage is carried out by enzymes pre-existing in the infected cell and affects virtually all particles immediately after they gain access to the interior of the cell; its product is a partially degraded form of the virus particle which has lost virtually all phospholipid and some of its protein. The second stage occurs after a lag of about one hour and results in the sensitization of viral DNA to the action of DNase, i.e. an uncoating. The fraction of virus particles completely uncoated is independent of the multiplicity and depends on the strain and metabolic state of the host cell. The lag of one hour before DNA is uncoated decreases with increasing multiplicity and is abolished in cells pre-exposed to virus, suggesting that during this lag some process occurs which results in the establishment of the uncoating mechanism operative during the second stage.

This paper deals with the molecular basis of the uncoating phenomenon. We have found that the establishment of the second stage of the uncoating mechanism is inhibited by puromycin and fluorophenylalanine as well as by actinomycin D,

suggesting a requirement for protein synthesis coded by messenger RNA. The protein (or proteins) once formed is stable. There is evidence that it is coded by the genome of the host cell. A model for poxvirus uncoating, reactivation and initiation is presented.

2. Materials and Methods

The cells and virus strains used, as well as the preparation of highly-purified and radioactively-labeled virus and the techniques used for the measurement of uncoating, have been described in the previous paper.

3. Results

(a) *Uncoating under conditions of protein synthesis inhibition*

(i) *FPA*†

Under the conditions employed (Eagle's medium (Eagle, 1959) lacking phenylalanine and supplemented with 5% freshly dialysed fetal calf serum) 2 mM-FPA inhibited the incorporation of [^{14}C]arginine into total HeLa S3 or L cell protein by 50% within 1 hour, 75% within 2 hours and 90% within 3 hours. The effect on the cells was fully reversible; cells exposed to 2 mM-FPA for up to 5 hours were, after removal of FPA and addition of phenylalanine, capable of attaching to glass and dividing with the same efficiency as untreated control cells. Pre-incubation with this amount of FPA for 3 hours before infection with virus was therefore adopted as routine procedure. When such cells were infected with RP virus labeled with ^{32}P, the amount of viral DNA uncoated was less than 15% compared with control experiment cells in complete medium (Fig. 1). The effect of FPA could be reversed by substituting phenylalanine (2 mM), the lag period before uncoating commenced being somewhat reduced. The same amount of acid-soluble radioactive material, which is derived from viral phospholipid (discussed in the previous paper), was produced in the absence and presence of FPA: 80 to 90% of the total phospholipid was rendered acid soluble. In both cases the acid-soluble radioactive label first appeared within the cell and later in the medium.

The effect of FPA on the breakdown of viral protein was also studied, using RP virus labeled with L-[^{14}C]leucine; the results have already been presented (Joklik, 1962*a*). It was found that for the first 2 hours after infection FPA has no effect on the release of viral protein from virus particles. Only after about 2 hours did FPA inhibit the progress of this dissociation. Since this is the time when uncoating of viral DNA is most rapid, this was interpreted as an inhibition of the breakdown of some protein structure closely associated with viral DNA, probably the core protein, which protects the DNA from the action of DNase.

It would appear that of the two separable reactions leading to the uncoating of RP DNA, the first, beginning immediately on penetration of the virus particles and leading to the solubilization of phospholipid and the formation of virus cores, is not arrested when protein synthesis is inhibited, suggesting that the enzymes concerned are pre-existent in the uninfected cell; but that for the second step in the uncoating of poxvirus DNA to proceed, the step which shows an initial lag, protein synthesis

† Abbreviations used: FPA = fluorophenylalanine; FU = fluorouracil; RP = rabbitpox virus; p.f.u. = plaque forming unit; S-RNA = soluble RNA.

is necessary. The slight reduction in the lag time on reversal of FPA inhibition is most probably attributable to the fact that the first stage is already complete. The protein synthesized is thus not essential for some function during penetration; it is essential for the breakdown of virus cores.

One may inquire whether the protein which is synthesized during the initial one-hour lag is stable. Cells were accordingly pre-infected with unlabeled RP virus at an added multiplicity of 4 p.f.u./cell and incubated for 3 hours. A sample of these cells was then super-infected with RP labeled with ^{32}P; this virus was uncoated without lag showing that the uncoating mechanism had been induced in these cells during

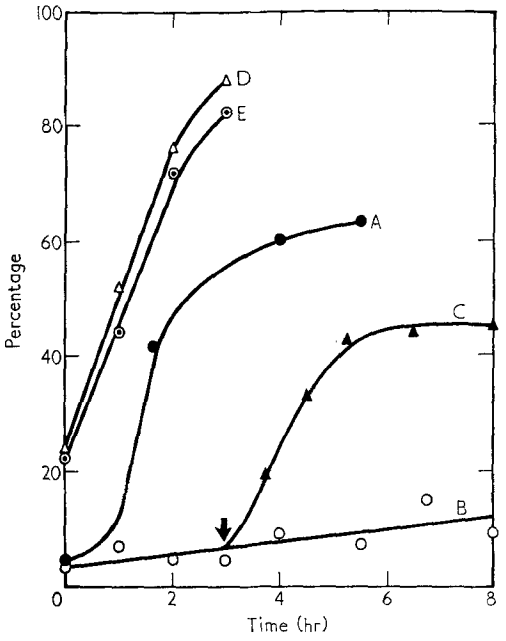

FIG. 1. The effect of 2 mM-FPA on the time-course of uncoating of RP virus. For details see text. Curve A: uncoating in normal HeLa S3 cells. Curve B: uncoating in cells continuously incubated in 2 mM-FPA since 3 hr before infection. Curve C: uncoating in FPA-treated cells reversed at 3 hr. Curves D and E: breakdown of phospholipid in normal and FPA-treated cells respectively. Multiplicity: 35 viral particles per cell. The ordinate is expressed as the percentage of viral DNA uncoated or viral phospholipid degraded to acid-soluble material.

the pre-infection (Table 1). The rest of the cells was transferred to medium containing FPA (2 mM) and incubated for 4 hours. They were then infected, still in the presence of FPA, with radioactively-labeled RP; and this virus was again uncoated without a lag in the continued presence of FPA. A control showed that 4-hour pre-treatment with FPA greatly diminished uncoating of labeled RP infecting in the presence of FPA. The protein synthesized during pre-infection is thus stable for at least 4 hours.

(ii) *Puromycin*

The effect of pre-treating cells with various concentrations of puromycin for various lengths of time on the incorporation of [^{14}C]arginine into total cell protein was measured. On the basis of the results, pre-treatment with 50 μg/ml. for 2 hours was

adopted as standard procedure. This treatment inhibited [^{14}C]arginine uptake by 90 to 95%. As in the case of FPA, the effect of this concentration of puromycin on the survival of cells was tested by plating after removal of puromycin. There was no diminution of the number of cells able to attach to glass, spread and divide.

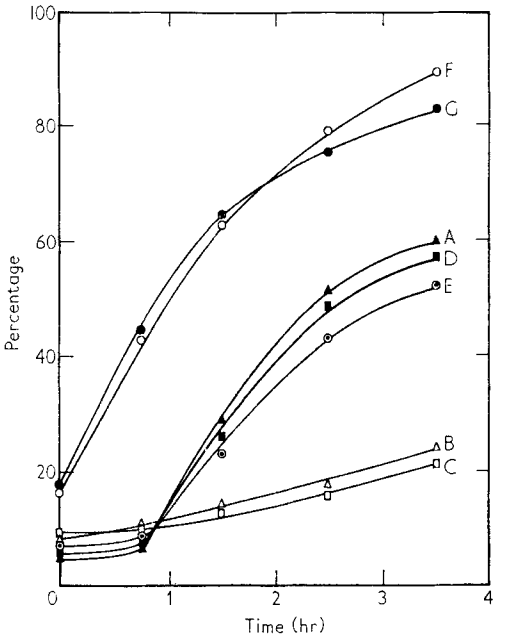

FIG. 2. The effect of puromycin (50 μg/ml.) on the breakdown of viral phospholipid and the uncoating of viral DNA. Curve A: uncoating in normal HeLa S3 cells. Curves B and C: uncoating in cells continuously incubated with puromycin since 2 or 5 hr respectively before infection. Curves D and E: uncoating in cells pre-treated with puromycin for 2 and 5 hr respectively before infection; puromycin removed immediately after adsorption of virus. Curves F and G: breakdown of viral phospholipid in normal cells and cells continuously incubated with puromycin since 2 or 5 hr before infection. The ordinate is expressed as in Fig. 1. Multiplicity: 30 virus particles per cell.

TABLE 1

Demonstration of the stability of the protein essential for uncoating

Treatment of cells	% DNA uncoated in period 0 to 45 min after infection	% DNA uncoated in period 0 to 210 min after infection
Infected with ^{32}P-labeled RP †(n = 35)	4	60
Pre-infected with unlabeled RP for 3 hours; superinfected with ^{32}P-labeled RP (n = 29)	31	49
Pre-treated with 2 mM-FPA for 4 hours; infected with ^{32}P-labeled RP (n = 31) in presence of 2 mM-FPA	3	10
Pre-infected with unlabeled RP for 3 hours; pre-treated with 2 mM-FPA for 4 hours; infected with ^{32}P-labeled RP (n = 25) in presence of 2 mM-FPA	24	37

† n = adsorbed particle multiplicity.

Pre-treatment of cells with 50 μg/ml. of puromycin decreased uncoating by 80 to 90% (Fig. 2). Just as in the case of FPA, phospholipid breakdown was not affected by the presence of puromycin. It is also seen that the effect of puromycin was reversible. In order to show this, cells were incubated in puromycin-containing medium (50 μg/ml.) for 2 or 5 hours; the cells were then infected with RP labeled with ^{32}P, the puromycin removed and the progress of uncoating followed. The reversed cells uncoated virus almost as rapidly as control cells.

An unexplained feature of the effect of puromycin, as well as of FPA, on the uncoating of poxvirus DNA was the fact that whereas arginine incorporation into total cell protein and thus protein synthesis was inhibited by about 90%, uncoating

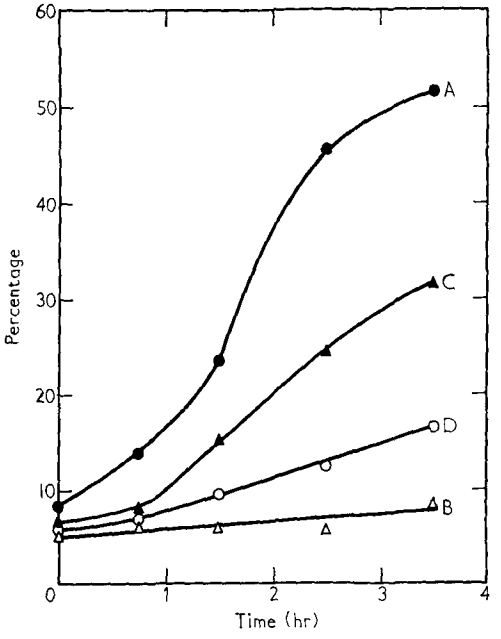

FIG. 3. The effect of actinomycin D (2 μg/ml.) on the time-course of uncoating RP virus DNA. Curve A: time-course of uncoating in normal L cells. Curve B: time-course of uncoating in cells continuously exposed to actinomycin D since 1 hr before infection. Curve C: Similar to B but actinomycin D removed immediately after infection. Curve D: similar to C but cells exposed to actinomycin D for 4 hr before infection. Ordinate: percentage of viral DNA uncoated. Multiplicity of infection: 42 virus particles per cell.

was sometimes inhibited by no more than 70%. If a small amount of the protein causing uncoating were indeed formed even in the presence of puromycin or FPA, then the presence of this protein should be recognizable by the absence of a lag in the uncoating of virus infecting cells pre-infected in the presence of puromycin or FPA. Cells were therefore infected in the presence of puromycin with unlabeled RP at a multiplicity of 20 to 30 particles per cell, and incubated for 3 hours to allow establishment of the uncoating mechanism. On superinfection with labeled RP in the continued presence of puromycin, no uncoating was observed. This indicates that the limited uncoating observed in the presence of 50 μg/ml. of puromycin is a nonspecific phenomenon and not due to uncoating by the mechanism which is under study.

It is difficult to predict the sequence of morphological transformations likely to be exhibited by particles during this non-specific uncoating. As viewed by means of the

electron microscope this sequence might differ but little from that exhibited by particles uncoated in the normal manner as described by Dales (1963), since presumably here too the core will be the intermediate.

(b) *Uncoating in the presence of actinomycin D*

Since protein synthesis is essential for pox virus DNA to be uncoated, it was to be expected that actinomycin D, as an inhibitor of messenger RNA formation, would also inhibit uncoating. This prediction was borne out. Treatment of cells with 2 μg/ml. of actinomycin D for one hour inhibited incorporation of [^{14}C]uridine into total cellular RNA by 90 to 95% and was routinely employed. This treatment killed a large proportion of cells. On removal of actinomycin D by washing generally not more than one-third of the cells and often less were capable of attaching to glass and

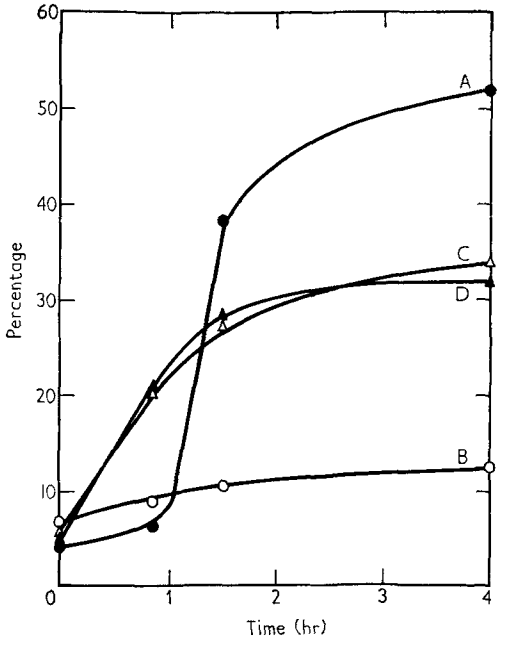

FIG. 4. Demonstration of the stability of the uncoating mechanism. For details see text. Curve A: progress of uncoating in normal L cells. Curve B: progress of uncoating in cells continuously exposed to actinomycin D since 1 hr before infection. Curve C: progress of uncoating in cells pre-infected with RP virus (added multiplicity 2 p.f.u./cell) for 3 hours. Curve D: progress of uncoating in cells pre-infected as in C, then exposed to actinomycin D for 1 hour before, during and after super-infection with ^{32}P-labeled RP virus. Multiplicity of labeled virus: 38 virus particles per cell. Ordinate: as in Fig. 3.

dividing. Figure 3 shows the effect of this amount of actinomycin D on the uncoating of RP DNA, when it was present for 1 hour before infection, during adsorption and throughout the subsequent incubation period. The drug inhibited uncoating by 85 to 95%. As in the case of FPA and puromycin, however, phospholipid breakdown and thus the first step in uncoating was not or relatively little affected.

The possibility was considered that actinomycin D did not prevent the formation of the protein necessary for uncoating, but rather that actinomycin D–viral DNA complexes were not digested by DNase, thereby giving the appearance that no

DNA had become sensitized to DNase. This was disproved in two ways. (a) Actinomycin D was added to a sonicated sample of infected control cells in which uncoating had taken place. The amount of DNA digested by DNase was the same in the presence and absence of actinomycin D. Actinomycin D thus has no effect on the susceptibility of viral DNA to DNase. (b) Cells were pre-infected with unlabeled RP virus for 3 hours. Actinomycin D was then added and incubation continued for 1 hour. The cells were then infected in the continued presence of actinomycin D with ^{32}P-labeled RP. This superinfecting virus was uncoated without lag (Fig. 4). This proved two points. First, in agreement with (a) above, it demonstrates that the inhibition of uncoating by actinomycin D is not due to its inhibition of DNase, but must rather be ascribed to the non-formation of the protein necessary for uncoating; second, just as in the case of FPA above, this experiment showed that pre-induced enzyme is stable and can, in the continued presence of actinomycin D, cause uncoating of subsequently infecting virus.

The action of actinomycin D was reversible within narrow time limits. L-cell suspensions were exposed to actinomycin D for 1 or 4 hours. They were then divided into 2 parts; actinomycin D was removed from one and left in in the other. The cells were then allowed to adsorb ^{32}P-labeled RP and the kinetics of uncoating measured (Fig. 3). The effect of actinomycin D could be extensively reversed if its removal was carried out within 1 hour. If left for 4 hours, its effect was virtually irreversible.

(c) *Other inhibitors*

(i) *Mitomycin C*

Mitomycin C was used at a concentration of 10 μg/ml. In cells pre-incubated in suspension culture with this drug for 26 hours, uncoating of RP DNA was inhibited by over 85%. However, the cells were then in poor condition. Adsorption was only 42% of that to control cells. In cells pre-incubated with the drug for 5 hours, which were then in good condition and adsorbing virus as well as control cells, uncoating was inhibited by 60%. In cells to which the drug was added at the time of infection, mitomycin C failed to affect the extent of uncoating.

(ii) *Colchicine*

A number of studies, notably those by Prescott & Bender (1962), have strongly suggested that metaphase is a period of low metabolic activity. In particular it appears that no RNA, and therefore no messenger RNA, is synthesized during this period. Cells arrested in metaphase with colchicine thus offer a tool for studying the uncoating of RP in cells in which host-cell messenger RNA synthesis does not occur and thus for determining whether host-cell messenger RNA synthesis is involved in the synthesis of the protein necessary for uncoating. The results suggested that this was indeed the case. HeLa S3 cells growing in spinner culture were treated for 24 hours with colchicine (5 μg/ml.). At this stage incorporation of [^{14}C]uridine into total HeLa cell RNA was only 19% of that into control cells. This culture was then washed and allowed to recover for 15 hours. A second culture of HeLa cells was incubated for 24 hours with colchicine and a third served as a control. Uncoating of RP virus was inhibited by over 80% in the culture inhibited by colchicine, and by less than 30% in the reversed culture (Fig. 5). It thus appears likely that host cell messenger RNA synthesis is indeed necessary for uncoating RP DNA.

(iii) *5-Fluorouracil*

The results so far have suggested that messenger RNA synthesis is essential for the formation of a protein necessary for uncoating poxvirus DNA. It was therefore of interest to determine whether 5-FU would interfere. 5-FU could inhibit by being incorporated into messenger RNA in place of uracil, leading to erroneous pairing with S-RNA, so that the uncoating protein molecules assembled would have a different amino-acid sequence from the normal molecule (Champe & Benzer, 1962). These might be functionally inactive molecules; the result would be absence of uncoating. An alternative possibility has been suggested by Dr. H. Marcovitch (Institut Pasteur): if the protein necessary for uncoating is coded by the genome of the host cell, then

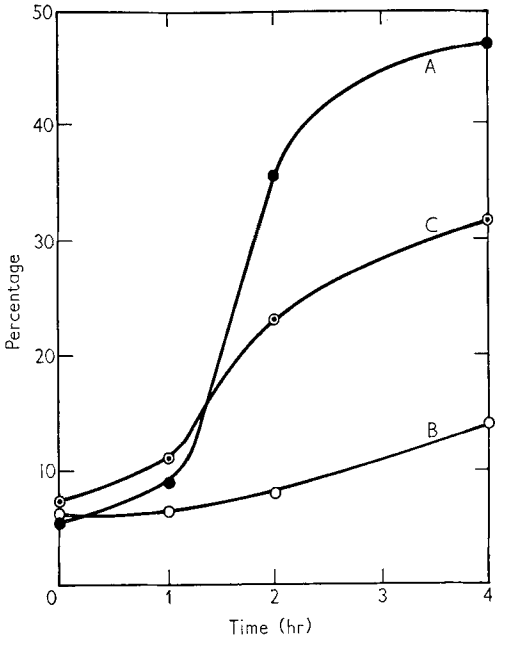

FIG. 5. Effect of colchicine (5 μg/ml.) on the uncoating of RP virus DNA. For details see text. Curve A: progress of uncoating in normal S3 cells. Curve B: progress of uncoating in cells exposed to colchicine for 24 hr. Curve C: progress of uncoating in cells exposed to colchicine for 24 hr, then reversed for 15 hr. Multiplicity of labeled RP virus: 32 virus particles per cell. Ordinate: as in Fig. 3.

since the protein is only synthesized as a response to infection, its formation is repressed in the normal uninfected cell. No matter whether the repressor is RNA or protein, in either case the synthesis of the repressor involves information transfer from DNA. In the presence of 5-FU faulty repressor may be made, resulting in derepression. If the faulty messenger RNA now made does not lead to the synthesis of inactive uncoating protein, this could result in cells which already contain, in the uninfected state, the protein which is ordinarily synthesized only in the infected cell after a lag. Such cells should be able to uncoat RP DNA immediately on infection. In order to test these two alternatives HeLa cells were suspended in 10^{-4} M 5-FU for 17 hours and then challenged with ^{32}P-labeled RP. Uncoating was inhibited by 40%; there was no evidence of a decreased lag before uncoating. This might indicate that some mistakes in coding are made in the presence of the base analogue. However, the effect was less than required for detailed analysis.

(d) *Effect on uncoating of irradiating cells with u.v. light*

The work presented so far has shown that protein and messenger RNA synthesis are essential for the uncoating of RP DNA. The question remains as to which DNA codes for this synthesis: that of the host cell or that of the virus. The experiments with colchicine are suggestive in this connection, since colchicine would be expected to prevent host-cell DNA from coding, but not viral DNA. The most convincing experiment would obviously be one in which host-cell messenger RNA synthesis was permanently inhibited by an agent which could be removed before the addition of virus. Ultraviolet light is such an agent. Table 2 shows the effect of u.v. irradiation

TABLE 2

Effect of irradiation with u.v. light on the ability of cells to incorporate [2-^{14}C]uridine into RNA, [^{14}C]arginine into protein and to uncoat viral DNA

Relative dose of u.v. light	Per cent inhibition of		
	incorporation of [2-^{14}C]uridine	incorporation of [^{14}C]arginine	ability to uncoat viral DNA
0	—	—	—
1	27	4	0
2	53	24	50
4	74	48	75
8	88	75	85

on the ability of HeLa cells to incorporate [^{14}C]uridine into RNA, to incorporate [^{14}C]arginine into protein and to uncoat RP DNA. Irradiation with u.v. light inhibited the synthesis of host cell RNA; and uncoating of RP DNA was inhibited to the same degree. The synthesis of host cell protein was much less sensitive. The irradiation of pre-infected cells failed to inhibit uncoating of subsequently infecting virus, demonstrating that adsorption and penetration of virus and any other events such as virus transport within the cell, etc., are not affected by u.v. light (and, further, that the protein essential for uncoating is not destroyed by u.v. light). Since there seems to be no reason why messenger RNA synthesis coded by vaccinia DNA should not proceed in cells irradiated with u.v. light, the conclusion is that host cell RNA synthesis, that is, messenger RNA synthesis, is essential for the uncoating protein to be formed.

(e) *Banding of partially and fully uncoated virus*

All the evidence suggests that the uncoating of poxviruses is a two-step process: virus particles are degraded to virus cores, and the virus cores are then broken down to release naked viral DNA. Intact virus, virus cores and naked DNA are readily separable in sucrose density gradients. Three sucrose gradients (25 to 40% in 1 mM-phosphate, pH 8) were prepared. The first was layered with intact ^{32}P-labeled RP. The second gradient was layered with a sonicate of cells infected with ^{32}P-labeled RP for 3 hours: in such cells uncoating had proceeded normally. The third was layered with a sonicated sample of cells infected for 3 hours with ^{32}P-labeled RP in the presence of 2 μg/ml. actinomycin D; in such cells virus had only been partially uncoated and virus cores had accumulated. The gradients were centrifuged at 15,000 rev./min (rotor SW25·1 of the Spinco model L centrifuge) for 60 minutes and the radioactivity profiles analysed (Fig. 6). Intact virus was found close to the bottom

of the gradient, while radioactivity in the second gradient was predominantly at the top, with a small amount of radioactivity near the center of the gradient representing cores not uncoated. Virus cores in the third gradient were located in the same position near the center; no intact virus was present in this gradient. Experiments on the nature of virus cores are under way.

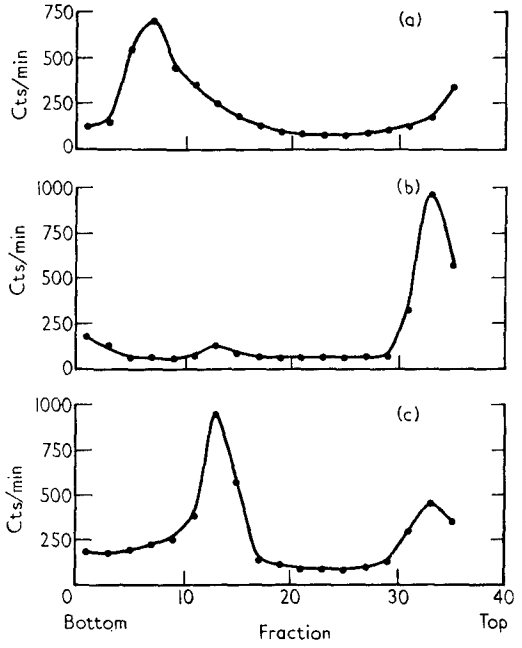

FIG. 6. Banding in sucrose density gradients of intact RP virus (a), uncoated RP virus DNA (b), and RP cores (c). The sonicated cell samples analysed in (b) and (c) were prepared by harvesting 5×10^7 cells infected with labeled RP virus in the absence and presence of actinomycin D respectively, resuspending in 1 ml. phosphate buffer (0·001 M) and sonicating for 15 sec. For gradient (a), purified labeled RP virus was added, immediately prior to layering on the gradient, to a sonicated sample of normal uninfected cells prepared in the same manner. For further details see text.

4. Discussion

The experiments described in this and the accompanying paper demonstrate that the uncoating of poxvirus DNA is a two-stage process. The first stage begins immediately after penetration of the adsorbed virus particle and results in the breakdown of all viral phospholipid and the dissociation of a considerable amount of viral protein. It is caused by enzymes pre-existing in the cell and takes place in the presence of puromycin and FPA, inhibitors of protein synthesis, in the presence of actinomycin D, and after irradiation of the cells with u.v. light, the latter two inhibitors of messenger RNA formation. The products of this initial phase of the attack on invading virus are probably identical with the cores which are discernible in electron micrographs of thin sections of recently infected cells (Dales, 1963). They can be isolated in sucrose density gradients in which they sediment appreciably more slowly than whole virus, but faster than completely uncoated DNA.

The second stage of uncoating, the uncoating of virus cores, also revealed by the electron microscopic study of Dales (1963), is characterized by its dependence on prior protein synthesis. This protein is formed as a response to infection by intact

virus particles. Heat-inactivated reactivable particles cannot initiate formation of the uncoating mechanism (Joklik, Woodroofe, Holmes & Fenner, 1960; Joklik, 1962*b*); it therefore seems that the effective inducer is undenatured viral protein. The fact that viral DNA is still coated while the protein in question is being synthesized, and, further, the demonstration of the inability of u.v.-irradiated cells to uncoat virus, suggest that the synthesis of the protein necessary for uncoating is under the control of host cell DNA. A viral protein could be visualized as acting as an inducing agent which derepresses that portion of the host-cell genome which carries the code for the protein which causes complete uncoating of viral DNA. Derepression is indicated since uninfected cells do not contain uncoating protein in any quantity as far as one can tell, and therefore are probably not synthesizing it.

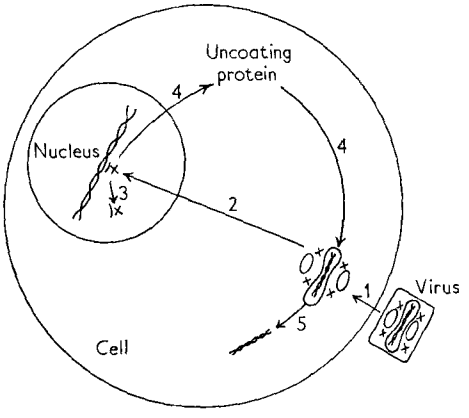

FIG. 7. Proposed mechanism of the uncoating of RP virus. For details see text.

A hypothesis for poxvirus uncoating is illustrated in Fig. 7. An intact virus particle enters the host cell (1). The initial step of uncoating releases (a) the viral inducer protein (marked X; this protein would be in an inactive form in heat-inactivated reactivable particles) and (b) virus cores within which the viral genome is still inaccessible to DNase. A number of viral protein inducer molecules are shown in the figure; however, it is not known at the moment how many of such molecules are in fact present in one virus particle. The viral inducer protein then derepresses the section of the host-cell genome coding for uncoating protein (2 and 3). Nothing is known concerning the mechanism of this derepression. It may be that, as pictured, the viral inducer protein itself moves to the nucleus where it combines with the repressor. However, it is clear that other mechanisms are possible. There is for instance no evidence that the viral inducer protein itself does in fact move to the nucleus; it may well be that viral inducer protein initiates a sequence of reactions and that it is some other molecule which actually functions in derepressing. Further, there is no evidence that only that portion of the host cell genome is derepressed which codes for uncoating protein. It may well be that one of the first effects of infection is a much more extensive, and possibly even complete, breakdown of repression in the host cell. However that may be, the evidence is that intact viral protein induces the synthesis of uncoating protein. This is postulated to proceed in the cytoplasm *via* messenger RNA formation (4). Once formed, the uncoating protein degrades viral cores, releasing naked poxvirus DNA (5).

This scheme accounts for all facts known at present about the phenomena of pox virus reactivation, poxvirus initiation (Cairns, 1960) and poxvirus DNA uncoating. One can only speculate as to the possible reasons for such an elaborate uncoating mechanism, the possible selective advantage conferred on cells possessing the piece of DNA coding for uncoating protein, and thus a possible dual role for uncoating protein. The validity of the scheme can be proved by isolation of the viral protein which causes the induction, and of the uncoating protein synthesized by infected cells. Work is in progress on these two projects.

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