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The Poxviruses[1]

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INTRODUCTION

During the past decade, our understanding of the multiplication of animal viruses has increased greatly. This has been due on the one hand to technical advances such as the advent of techniques for the in vitro culture of large amounts of homogeneous cell populations and the development of methods for characterizing protein and nucleic acid molecules, and on the other to the adoption of an experimental approach aimed at correlating macromolecular structure with function. In this review, an attempt will be made to review the considerable body of data which has

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TABLE 1. *The poxviruses*

Subgroup	Virus
Vaccinia	Vaccinia Cowpox Rabbitpox Ectromelia (mousepox) Variola (smallpox) Alastrim
Myxoma	Myxoma Fibroma Squirrel fibroma Hare fibroma
Birdpox	Fowlpox Canarypox Turkeypox Pigeonpox
Paravaccinia	Molluscum contagiosum Contagious pustular dermatitis (CPD, Orf) Goatpox Sheeppox Bovine lumpy skin disease (LSD) Milker's nodule virus (pseudo-cowpox) Bovine papular stomatitis (BPS)
Unclassified	Swinepox Fishpox Yaba monkey tumor virus

accumulated concerning events during the poxvirus infection cycle in terms of the synthesis and functioning of specific macromolecules.

STRUCTURE AND CHEMISTRY OF POXVIRUSES

Morphology

Assignment of virus strains to the poxvirus group has in the past depended almost entirely on morphology. Predilection for skin as the habitat and antigenic relationship, as well as evidence for deoxyribonucleic acid (DNA) as the genome, have served as additional criteria (37).

More recently, it has become apparent that ability to reactivate other members of the group when heat-inactivated (41) and possession of the common internal antigen (186) are more decisive taxonomic criteria.

Table 1 lists the viruses which at present may reasonably be regarded as being poxviruses (37, 38, 44, 56*a*, 57*a*, 126, 129, 130, 131*a*, 136, 147, 152, 186*a*). Members of the first three subgroups are indistinguishable on morphological grounds, as are the members of the paravaccinia subgroup, from which they are readily differentiated. Members of the vaccinia subgroup are closely related antigenically, although the new sensitive immunogel diffusion technique has revealed minor differences in antigenic constitution (159). The same applies to members of the myxoma subgroup (157), antisera to which do not react with viruses of the vaccinia subgroup unless special measures are adopted (see below, *Proteins of poxviruses*). Within the paravaccinia group, contagious pustular dermatitis of sheep (CPD) and goatpox (37), and bovine lumpy skin disease (LSD) and sheeppox (152), appear to be related antigenically.

Many excellent electron microscopic studies on the structure of poxviruses have been carried out. The techniques most commonly used have been negative staining with phosphotungstate (20, 25, 36*a*, 68, 76, 129, 135, 183), thin sectioning with and without enzymatic digestion (25, 127, 143, 145, 146), and enzymatic digestion after fixation (143, 144). A virus particle of any of the strains in the vaccinia, myxoma, and birdpox subgroups is probably best described in terms of the model described by Westwood et al. (183), which is consistent with the studies of other workers. Most of the virus particles (the M particles) exhibit a highly characteristic beaded surface which has been likened to that of a mulberry, the beading being formed by loops of threadlike structures 90 A wide, which are themselves double helices formed from two 30 A strands coiled to a 120 A pitch. The material of this beading is most probably protein. The dimensions of the particle seen thus are 2,000 to 2,500 A by 2,500 to 3,200 A: electron microscopic size determinations are strongly influenced by preparation conditions (127). In aged preparations, virus particles often seem to "unwind," resulting in twisted ropelike structures with many adherent virus particles (76); the formation of these structures is most probably responsible for the clumping commonly encountered in aged preparations. The unwinding is facilitated by treatment with trypsin, which allows further examination of the fine structure of the uncoiled material.

There is some evidence for the existence of an outer "envelope" surrounding the particle (183), the space between it and the beaded surface being occupied by "soluble protein antigens." Further, it seems that these poxvirus particles can exist not only in the M form which is not penetrated by phosphotungstic acid (PTA), but also in a C form which is (183). The two forms are interconvertible during preparation of the specimen, but the basis of the interconversion is not well under-

stood. Its existence emphasizes the need for careful standardization of procedures used to examine these complex particles.

The layer with the beaded surface surrounds a "nucleoid," which often appears flattened and rectangular in the wide, and bi-concave in the narrow, aspect. The thickness of the layer is therefore not uniform, but is greater in two regions known as the "lateral bodies." The nucleoid itself measures about 1,000 by 2,000 A in lateral cross section, and is surrounded by a coat about 90 A thick from which regularly spaced projections 40 to 45 A long emanate. The nucleoid is filled with dense filamentous material (fibers 15 to 40 A wide) which, in appropriate section, often appears confined to three circular areas, suggesting a tight S-shaped helical arrangement. This material is undoubtedly composed of DNA-protein, and represents the viral genome. Various authors (76, 145) have shown that after enzyme treatment of fixed virus, or after mild treatment with sodium dodecyl sulfate (SDS) of intact virions, strands of DNA-containing material uncoil from this region.

Controlled degradation of vaccinia virus has also been achieved by treatment with 2-mercaptoethanol, the nonionic detergent Nonidet P40, and trypsin (36*a*; *personal communication*). The outer coat of the virus is removed first, yielding structures composed of cores to which the two lateral bodies are still attached. These are then dissociated by trypsin to yield naked cores, the walls of which are composed of an outer layer of cylindrical subunits 100 A long and 50 A in diameter and a smooth inner layer 50 A thick. The cores rupture under stress in the middle of the side faces, releasing long threads of DNA. It is conceivable that viral DNA exists in a narrow region immediately inside the double-layered core wall, possibly wound around a "spacer" (Easterbrook, *personal communication*).

In its essentials then, the typical virion of the first three subgroups may be regarded as composed of an "outer coat" consisting of (possibly) an envelope and a layer of more or less regularly arranged protein subunits which includes the two "lateral bodies," all of which surrounds the genome-containing nucleoid or "core" which is bounded by its own coat.

The appearance of particles of the paravaccinia subgroup is different. Not only are the particles smaller (1,600 by 2,600 A) and more ovoid, but their surface structure is different and highly characteristic (18, 129, 130, 131, 147). Instead of the beaded "mulberrylike" type of arrangement seen on particles of the first three subgroups, one observes here a crisscross pattern produced by the crossing of two sets of parallel threads. This is thought to be due to super-imposition of the images of threads which run at different angles on the upper and lower surfaces. The winding follows a spiral anticlockwise (i.e., left-hand screw) pattern, and 12 to 15 turns can generally be distinguished. The threads are about 100 to 120 A wide and 8,000 to 10,000 A long, and occasionally exhibit a periodicity of 40 to 45 A. They are protein in nature. The internal structure of paravaccinia viruses seems to be very similar to that of the viruses of the first three subgroups. Models have been proposed (18, 131).

Chemical Composition

Adequate chemical analysis has been carried out only on members of the vaccinia subgroup (71–75, 82, 174, 189). About 5% DNA, 2% lipid, and 2% phospholipid are found; the rest is protein. Practically all carbohydrate can be accounted for as deoxyribose in DNA. Certain trace components, notably riboflavine and copper, are most probably not integral viral components (73, 74, 82, 189). Small amounts of protein [as shown for the enzyme alkaline phosphatase (75)] and nucleic acid (83) are often adsorbed to the virus particles. The weight of a vaccinia virus particle is about 5.5×10^{-15} g (82, 173) and of a typical paravaccinia virus particle about 3.7×10^{-15} g (131). The amount of DNA is the same in the two types of virus particle [about 2.75×10^{-16} g (3, 62, 131)].

Several methods for the purification of poxviruses have been described; the best are those described in references 82, 83, 118, 131, 151, 156, 173, 175, and 188.

Viruses are sometimes classified as ether-sensitive or ether-resistant. When poxvirus strains are tested for sensitivity to ether under defined conditions, survival rates range from $10^{-1.5}$ for vaccinia and CPD to 10^{-4} for sheeppox and myxoma (152). Thus, all virus strains are inactivated by at least 95%, and none more than 99.99%. As pointed out above, vaccinia and myxoma viruses, at the two extremes of the sensitivity spectrum, are indistinguishable morphologically. The basis of the difference in sensitivity to ether, which is not large, is not known.

Proteins of poxviruses. As is to be expected from the complexity of its structure, the protein complement of the poxvirus particle is made up of a large number of molecular species. This has been demonstrated in two ways. First, Joklik and Maizel (*unpublished results*) dissolved highly purified preparations of cowpox virus in a mixture of

SDS, urea, and mercaptoethanol, and subjected the mixture to polyacrylamide gel electrophoresis. Patterns with as many as 15 bands were observed. Further, Zwartouw et al. (190) extracted highly purified preparations of vaccinia virus with alkaline buffers and detected by means of the immunodiffusion technique eight components which reacted with hyperimmune antisera. Similarly, mechanical disintegration splits off from the virion eight precipitinogens, some of which are derived from the peripheral protein layer, others from the viral nucleoid (119).

Second, proteins which react with hyperimmune antisera are found in homogenates of cells infected with poxviruses. These proteins have been analyzed by the immunodiffusion technique. Extracts of rabbit skin infected with vaccinia virus yielded up to 17 precipitin lines, 7 of which were identical with lines obtained by extracting highly purified virus (184; *see also* 119). Similarly, in extracts of HeLa cells infected with rabbitpox virus over 20 separate antigens can be detected (4, 5). One of these antigens is no doubt that which combines with neutralizing antibody; this antigen has been partially purified and has been found to have a molecular weight of between 100,000 and 200,000 and to elicit on injection the formation of neutralizing antibody (6, 7). This type of analysis has also revealed slight differences in the composition of the mixture of antigens in extracts of cells infected with closely related virus strains; thus, cells infected with cowpox (CP u^+) reveal a line which is absent in cells infected with CP u (a white mutant; see below, *Genetic Recombination*) and only just detectable in cells infected with vaccinia virus, which, on the other hand, contain a line not exhibited by extracts of cells infected with CP u^+ (159). Similar differences have been found between various strains of myxoma virus, and fibroma virus (157).

Since extracts of infected cells reveal more components capable of reacting with antiserum than do extracts of highly purified virus, it has been suggested that the extra components may represent nonstructural viral proteins (184). However, this is not justified, since not only did alkaline extraction of pure virus result in the solubilization of only 20% of the viral material (190), but the complete solubilization of pure virus with urea, SDS, and mercaptoethanol yielded up to 15 bands on gel electrophoresis (Joklik and Maizel, *unpublished data*).

Finally, although, as has been pointed out above, there is no demonstrable serological cross-reaction among members of the various poxvirus subgroups when extracts of infected cells containing virions and soluble antigens are tested against heterologous antisera, alkaline digestion of all poxviruses yields a fraction, the so-called "NP" antigen, which does react with antisera against all poxvirus strains tested (186). It might well be proposed that possession of this universal internal poxvirus antigen be regarded as the fundamental criterion for classification of any new virus as a poxvirus.

DNA of poxviruses. The DNA of a number of different poxvirus strains has been examined. In general, poxvirus DNA is not easily liberated from the virus particles; although examination in the electron microscope shows that treatment with SDS alone causes DNA strands to separate from virus particles (76) and although preparations of viruses of the vaccinia subgroup become highly viscous on the addition of SDS, treatment of the mixtures with phenol does not lead to extraction into the aqueous phase of more than a few per cent of the total amount of DNA present (85). The DNA genome of poxviruses is evidently associated much more intimately with protein than that of the T phages (117) or of the papova viruses (182). For the complete separation of DNA and protein, treatment with SDS as well as with a protease [papain (85) or pronase (148)] is required.

When free, the extremely long (83 μ) DNA molecules are very easily sheared (29), but this occurrence can, however, be minimized by carrying out all manipulations very carefully and by ensuring a high DNA concentration throughout (69). In spite of all precautions, the molecular weight of isolated cowpox virus DNA is only about one-half of that expected, namely about 80×10^6 (85). By all criteria applied, it is double-stranded. It contains 36% guanine plus cytosine (GC) as determined by chemical analysis (A equals T and G equals C); its T_m is 87 C, equivalent to 36% GC; and its density in CsCl is 1.695, equivalent to 37% GC (85).

The DNA of the Levaditi strain of vaccinia virus has also been examined (149); 5 to 10% of the DNA was single-stranded, and the rest was double-stranded. The single-stranded component was derived not from every virus particle, but from only about 20%, which were lower in density than the rest and had a lower specific infectivity. These findings are difficult to understand, and the most likely explanation is that the "single-stranded" DNA is either an isolation artifact or an impurity adsorbed to certain particles.

The DNA of fowlpox virus has also been examined (177). It was extracted by lysing the virions with 3% SDS, followed by deproteinization with chloroform-butanol and phenol. No single-stranded component was detectable. The T_m in 7.2 M $NaClO_4$ was 39.1 C (34.6% GC),

and the density in CsCl was 1.6945 (35% GC), which was slightly lower than that of rabbitpox virus DNA (1.6959) (177). About 15% of partially sheared fowlpox DNA renatured even after rapid cooling, suggesting either that the DNA is cross-linked once per intact molecule or that there are relatively long sequences of AT in certain parts of the molecule which act as nuclei for spontaneous renaturation of the two strands (177).

None of these DNA preparations has shown any infectivity. This is no doubt due to the difficulty experienced in isolating intact DNA molecules from the virus. It has been recently reported (2) that infectious poxvirus DNA preparations may be obtained by uncoating virus in vitro with "uncoating protein" (88). This will be discussed further below.

The amount of ribonucleic acid (RNA) in poxviruses is at most 1% of the amount of DNA (83, 151, 189); in fact, it is most likely that poxviruses contain no RNA at all. However, the presence of an RNA molecule with a molecular weight of the order of 200,000 has not been ruled out.

Phospholipid of poxviruses. Poxviruses also contain phospholipid (2.2%), neutral lipid (2%), and cholesterol (1.3%) (71, 189). No extensive work on these constituents has been carried out. From the high specific activity of phospholipid phosphorus in virus labeled with P^{32}, one may conclude that most of the phospholipid molecules incorporated into mature virus particles derive their phosphorus from phosphorus in the acid-soluble pool at or after the time of infection. There is no evidence that the viral genome directs the synthesis of viral phospholipid. It is likely that viral phospholipid is derived from newly formed host cell phospholipid and that its composition is therefore characteristic of that of the host cell, as has been demonstrated for myxoviruses (98).

Poxvirus Hemagglutinin

Many poxviruses cause formation of a hemagglutinin (38) which first becomes detectable when 20 to 40% of mature virions have accumulated (111, 137). Virions do not hemagglutinate; the hemagglutinin is not part of the virion and is readily separated from virions by density gradient centrifugation. It agglutinates red cells from about 15% of fowls only (38); the genetics involved have not been investigated. It is a very pleomorphic particle about 50 mμ in diameter (132) and is rich in lipid [74% of the total lipid is neutral lipid, the rest phospholipid (53)]. Soon after its formation, the hemagglutinin is detectable at the surface of the cell. This circumstance has been utilized in the development of a plaque-hemadsorption technique (138).

It is uncertain how closely the synthesis of the hemagglutinin is under the direction of the viral genome. Although neither rabbitpox virus (RP u^+) nor a large number of RP u mutants (see below, *Genetic Recombination*) cause formation of a hemagglutinin, RP u8 does so (55). In crosses between vaccinia virus strain Lederle 7N and rabbitpox virus RP u^+, which differ in many characteristics, including ability to produce hemagglutinin, there are recovered among the progeny all classes of hemagglutinin-positive and hemagglutinin - negative recombinants (139). These results suggest that the information to form the hemagglutinin resides in the viral genome. Attempts with the use of inhibitors to determine where the code for hemagglutinin resides have been unsuccessful. As would be expected, mitomycin C does not inhibit its formation (138) but actinomycin D does, although to a somewhat smaller degree than it inhibits production of virions (145). The finding that the densities of the hemagglutinins produced by the same virus strain in different tissues differ (132) suggests that although the viral genome contains information to *cause* the synthesis of the hemagglutinin, the information necessary for its *actual* synthesis resides in the genome of the host cell.

Elementary Body-Infectious Unit Ratio (EB/IU)

The hosts most commonly used for measuring infectious units of viruses of the vaccinia subgroup are primary chick embryo fibroblasts and certain continuous cell lines (plaque formation), the chorioallantoic membrane of the developing chick embryo (pock formation), and the rabbit's back (formation of local lesions). The number of infectious units exhibited by a particular poxvirus preparation may vary greatly, depending on the nature of the host cell. For instance, the titers exhibited by cowpox virus on the chorioallantoic membrane are over an order of magnitude lower than those exhibited on the rabbit's back, whereas for other closely related virus strains the titers are almost equivalent, and the number of plaques on an established cell strain may be greater or less than the number of pocks on the chorioallantoic membrane.

Under optimal conditions, the ratio EB/IU is 5 to 10 for viruses of the vaccinia subgroup (173, 82); often it is 20 to 40. The most economical hypothesis is to assume that all virus particles are infectious, but that the EB/IU ratio reflects the probability each virus particle has of initiating

productive infection in the particular assay system used. As shown below, under optimal conditions most poxvirus particles can adsorb, most of them are taken up by the cell, most of them are uncoated, and there is no evidence that the DNA of a large proportion of a virus population is defective.

Evidence has been presented that under certain conditions the ratio EB/IU changes on repeated passage (167); this has been referred to as a change in "quality" (47). It was reported that, whereas the yield in terms of the total number of particles increased rapidly during the first passages in a new host, the specific infectivity increased much more slowly. This effect plainly depends on the characteristics of the starting virus preparation; in my experience (Joklik, *unpublished data*) a preparation of very highly purified WR virus grown on HeLa cells exhibited an EB/IU ratio of about 20 in the first passage in L cells, and this could not be decreased during 15 subsequent passages. It was also reported that the first virus particles to be formed in a single growth cycle have a lower "quality" than later ones (47). This is not so in HeLa cells. Joklik and Becker (90) labeled viral DNA for a brief period and then followed its incorporation into virions by using density gradient centrifugation as a sensitive tool for separating the various structures into which the labeled DNA has been incorporated. The very first virions to be formed had exactly the same specific infectivity as the total yield. Thus, Galasso and Sharp relied on morphology for identification of virions; Joklik and Becker, on physical properties. The gradual increase in quality found by Galasso and Sharp could well be due to their identification of immature virus particles as virions.

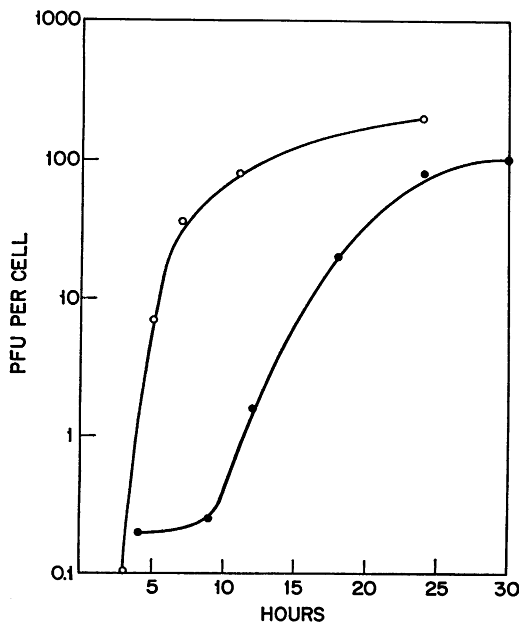

FIG. 1. *Single-step growth curve of vaccinia virus strain WR in HeLa S3 cells in suspension culture (open circles) (187) and of vaccinia virus strain V-MH in HeLa S3 monolayers (closed circles) (78).*

Galasso and Sharp (48) found that the nature of the horse serum used in the medium markedly affected the "quality" of the yield. The yield in terms of number of particles was always the same, but the specific infectivity (quality) varied over a 16-fold range with different batches of serum. If the sera were heated at 56 C for 30 min before use, these differences were largely eliminated. The horse sera themselves did not inactivate the virus. On the evidence presented, one must accept the authors' explanation that some serum component exerts an indirect influence on the quality of the progeny through the agency of the cell, but one cannot help suspecting that the true explanation may be less complicated.

BIOLOGICAL CHARACTERISTICS OF THE POXVIRUS GROWTH CYCLE

One-Step Growth Curve

A typical one-step growth curve of vaccinia virus strain WR in HeLa S3 cells growing in suspension culture is depicted in Fig. 1 (187). Also in that figure is a growth curve of vaccinia virus strain V-MH growing in HeLa cell monolayers (78). The comparison is made to illustrate the extent to which the growth curves of two very closely related virus strains, growing in genetically similar or possibly even identical cells, but under different conditions, can differ. Since culture conditions differ notoriously from laboratory to laboratory, and since even closely related virus strains differ in minor but significant characteristics, it is clearly unwise to apply kinetic data from any one virus-cell system to others.

As recently as 5 or 6 years ago there was argument as to whether poxviruses underwent "eclipse" (116). Such arguments have now been laid to rest. Not only is it possible to show that during the first hours after infection the number of cells capable of going on to produce virus greatly exceeds the number of demonstrable infectious viral particles (Joklik, *unpublished data*; 32), but the uncoating experiments (87, 88; see below) also show conclusively that the genome of poxviruses is separated from its coat. Since uncoating of the genome is the unique attribute of viruses, poxviruses are therefore typical viruses.

Poxviruses in general are not liberated readily from the cells in which they multiply. For viruses of the vaccinia subgroup, intracellular titers exceed extracellular titers by at least 10-fold for as long as 96 hr after completion of maturation. However, there is evidence that in confluent monolayers cell-to-cell spread of virus occurs very early during the infection cycle, possibly as early as 7 hr after infection (133, 134). This is no doubt the reason why plaque titrations can be read within 48 hr. This early cell-to-cell spread is somewhat delayed, but by no means abolished, by the presence of antibody to virus. Comparison of the number of infected cells with the number of IU of virus during the first 10 hr after inoculation similarly led to the conclusion that infection of contiguous cells occurs very early. It is not clear whether this is due to the earliest progeny virions or to an incomplete form of the virus, possibly naked virus DNA.

Visualization of Viral Inclusions or "Factories": Light Microscopy and Autoradiography

The development, nature, and staining properties of the viral inclusions in infected cells have been studied in minute detail with the light microscope. This type of work has been carried out with most refinement by the Japanese school of workers (for a summary, *see* 99). This approach is purely descriptive and is inherently limited in the information it can yield. Autoradiography (incorporation of thymidine-H^3) and staining with antiviral antibody coupled with fluorescein has also been carried out; this approach confirmed that the inclusions visible by light microscopy were the sites of synthesis of viral DNA and protein, or at least the sites where such molecules accumulated in significant amounts. Viral inclusions are found not only in the perinuclear region, but throughout the cell (19).

There have been two studies in which these techniques of light microscopy, autoradiography, and staining with fluorescent antibody have been used in a sufficiently quantitative experimental design so as to significantly advance our knowledge concerning the infectious cycle of poxviruses. The first was that of Cairns (19). It was found that the number of inclusions per cell was directly proportional to the multiplicity of infection. In cells infected at a multiplicity of less than one, there was considerable asynchrony with respect to the time at which the inclusions or "factories" became apparent; in the cell-virus system used (HeLa cell monolayers and vaccinia V-MH), this time varied from 3 to 6 hr. When the multiplicity was more than 1, say 10, so that 10 factories on the average developed in each cell, then asynchrony was markedly reduced and the median time of appearance coincided with the earliest time at which factories could be detected in singly infected cells. This suggested that the development of a factory depends on some critical event, initiation, caused by each virus particle with constant probability in each time interval during the early part of the infection cycle. The higher the multiplicity of infection, the higher is the probability that this event will occur early, and once this event has occurred, *all* virus particles capable of causing development of a factory are stimulated, or enabled, to do so.

Factories were observed in three ways (19): (i) they incorporated thymidine-H^3; (ii) they could be stained with fluorescent antibody; (iii) they represented the *unlabeled* areas when precursors of RNA such as uridine-H^3 were used. There was no correlation between the stage of the mitotic cycle at the time of infection and the development of factories; for instance, there were factories in cells which were or were not in the S phase.

The second study is that of Kato et al. (100), who applied quantitative autoradiography to vaccinia-infected cells. The conclusions were: viral DNA synthesis commenced at 2 hr after infection; the maximal rate of viral DNA synthesis occurred about 1 hr later and greatly exceeded the rate of host cell DNA synthesis in uninfected cells; and infection very quickly markedly inhibited host cell DNA synthesis. It will be seen below that in all respects these findings tally with those obtained simultaneously by biochemical techniques (90).

Since factories become labeled after very short as well as long exposures to thymidine-H^3, it is likely that viral DNA is actually being synthesized in them. Since factories cannot be labeled with precursors of RNA, it is clear that viral messenger RNA (mRNA; which must be synthesized in them because they contain the template DNA) does not function there, and therefore, that viral structural proteins are not synthesized there. However, since they can be stained with fluorescent antibody, they are the sites where viral antigens first accumulate in significant amounts. As the infection cycle proceeds, the factories enlarge until the whole cell may be regarded as a large factory and the whole cell stains with fluorescent antibody (19). Whereas the inclusions formed by viruses of the vaccinia subgroup are not bounded by a membrane, and enlarge and merge as the infection cycle proceeds, inclusions formed by fowlpox virus in chick embryo cells are bounded by a stable membrane, and virus maturation proceeds within them; in fact, they may be isolated at the end of the infection cycle

and form an excellent starting material for the purification of fowlpox virus (155, 156). They may indeed represent integrated factories for virus synthesis. A review of the electron microscopic studies concerning the events involved in the maturation of poxviruses is outside the scope of this review (for a description, *see* 22).

Cytopathic Effects (CPE)

Poxviruses cause CPE in the cells they infect, which sometimes are still evident even though the virus does not multiply; they are then said to be "toxic" effects. The factor responsible is the poxvirus particle itself, not a separate, "soluble" toxin. Viruses of the vaccinia subgroup produce two types of CPE. The first, often detectable within 1.5 to 2 hr after infection, consists of cell rounding, and is also produced by infection with virus inactivated by heat and ultraviolet irradiation (4, 66), and in the presence of inhibitors of cell metabolism, such as azide, or inhibitors of virus multiplication, such as isatin-β-thiosemicarbazone (IBT; 4). Cell rounding is thus best thought of as a cellular response to the ingestion of virus particles for which no information encoded in the viral genome is required. The second type of CPE commences at about the time when viral DNA replicates, and consists of cell fusion; sometimes a cell sheet infected for 24 hr is composed of a small number of giant syncytia (4, 96). It has been argued that information transcribed from the viral genome is required for this type of CPE, since (i) it does not occur when viral DNA replication does not proceed, either due to the presence of inhibitors or because inactivated virus caused the infection and (ii) because this type of effect is strain-specific; thus, vaccinia virus strain IHD has been shown to consist of three variants, two of which cause cell fusion, while the third does not (96), and RP u^+ causes it, while many closely related vaccinia virus strains do not. However, IHD strains inactivated by ultraviolet irradiation still cause cell fusion (97). The degree of involvement of the viral genome in the causation of this type of CPE is therefore still uncertain.

Effect of Poxviruses on Cell Proliferation

Poxviruses are often thought to stimulate cell proliferation. Thus, the pocks on the chorioallantoic membrane appear as raised areas, suggesting an increase in the number of cells (38); viruses of the vaccinia subgroup as well as myxoma viruses form raised nodules with or without hemorrhagic lesions in the skin of a rabbit (38); fibroma virus forms benign tumors in rabbits. There is no evidence, however, that a cell infected with any poxvirus is capable of undergoing cell division (77, 179); the evidence for proliferating fibroma-infected rabbit cells is unconvincing (70). Studies with whole animals would be difficult to interpret, and have not been carried out. Tumor formation by poxviruses appears to be due to invasion by neighboring cells, increase in fluid content, and possibly stimulation of proliferation of uninfected cells by neighboring infected cells by either nutritional mechanisms or breakdown of contact inhibition, rather than to a direct effect of poxvirus particles or their genomes.

BIOCHEMICAL ANALYSIS OF THE POXVIRUS GROWTH CYCLE

In this section, I shall examine the uncoating of the viral genome, the synthesis and functioning of viral mRNA, the synthesis of the various classes of proteins specified by the viral genome, the replication of the viral genome, and its coating and incorporation into progeny virions.

Uncoating

It is obvious that the viral genome has to be freed from its protein coat so that it can replicate, and so that the information stored in its base sequence can be transcribed into mRNA. This uncoating process has been followed by inquiring into the fate after infection of highly purified virus labeled with radioactive isotopes (83, 84, 87, 88). Virus labeled in each of the three major constituents of the poxvirus particle has been used: DNA labeled with thymidine-C^{14}, phospholipid labeled with P^{32} (which also labels DNA), and protein labeled with C^{14}- amino acids.

Most of the work was carried out with RP u^+ and vaccinia strain WR, but applies also to several u mutants of RP, to CP u^+, and to vaccinia strains V-MH, Lederle 7N, and CL; there is no reason for doubting that it represents the uncoating process for all poxviruses.

In virus preparations labeled with thymidine-C^{14}, practically all the label is resistant to deoxyribonuclease, since the viral genome is protected by the protein and phospholipid-containing coat. The criterion for uncoating is the development of susceptibility to attack by deoxyribonuclease; a genome susceptible to deoxyribonuclease is regarded as being uncoated. When the adsorbed multiplicity of infection is of the order of 40 particles [1 to 2 plaque-forming units (PFU)] per cell, no DNA is uncoated for the first hour. After this lag period, uncoating commences, and is complete by 3 hr after infection; that is, by that time all the genomes that will be uncoated have been uncoated. The extent of uncoating depends on the cell strain, and most probably also on the

metabolic condition of the cells; the greatest extent found so far is about 70% (87). The reason that not every virus particle is uncoated is probably that certain virus particles are not taken up into the proper phagocytic vesicles within which uncoating is initiated (see below); there is no evidence whatever that these particles differ in any way from those that are uncoated. However, the fact that the efficiency of uncoating is less than 100% does immediately, at least in part, account for the fact that not every virion causes productive infection. Once the DNA is uncoated, it is *stable* for at least 7 hr (stability being defined as nonappearance of acid-soluble breakdown products).

The extent of uncoating is *unrelated* to the multiplicity of infection; it is the same whether the multiplicity is 500 or 0.2 particles per cell (87). This indicates that all particles (or at least 70% of them) are capable of being uncoated, and similarly that every virus particle is capable of causing the reactions leading to the initiation of uncoating; for if there were certain *initiator* particles, then the extent of uncoating would be expected to decrease with the probability of each cell receiving at least one of them.

The length of the lag period before any viral DNA is uncoated depends on the multiplicity of infection. As the multiplicity is increased up to 500 to 1,000 particles per cell, the lag period decreases to little over 0.5 hr, and the time for 50% uncoating is reached by about 1.25 hr after infection; when the multiplicity is low, about 0.2 particles per cell, the lag period is about 2 hr, and the time for 50% uncoating is not reached before about 2.5 hr (87). This result may be related to the results of Cairns (19) discussed above; it appears that a certain essential reaction has to proceed for uncoating to be initiated, and that the higher the multiplicity of infection the earlier this reaction occurs. One may anticipate that, if poxvirus is caused to infect a cell in which this reaction has already taken place, the genome of the superinfecting virus should be uncoated without delay. This is indeed found (87). Asynchrony in the development of viral inclusions is thus clearly due in large part to asynchrony of uncoating.

Further information concerning the uncoating process is provided by using virus labeled with P^{32} (84, 87). In such virus particles, phospholipid as well as DNA is labeled; 70% of the label is in DNA and the rest is phospholipid (apart from a small amount in a fraction thought to be phosphoprotein). Comparison of the fate of virus labeled with thymidine-C^{14} and P^{32} allows one to discern the fate of the phospholipid moiety of the invading poxvirus particle. If P^{32}-labeled virus is used as the infective agent, acid-soluble radioactive label is found first within the cell and subsequently in the medium. Since uncoated DNA is not degraded to acid-soluble substances, its source is plainly the phospholipid. By 3 hr after infection, the amount of P^{32} rendered acid-soluble corresponds exactly to that expected to be in phospholipid; after this time, no more becomes acid-soluble. Since the first acid-soluble label to be found is intracellular, the initial attack on the virus occurs within the cell. There is no lag in the breakdown of phospholipid; it commences immediately on infection. No matter what the extent of uncoating of viral DNA, *all* phospholipid of *all* virus particles is always converted to acid-soluble substances (mainly glycerophosphate), and the percentage of particles which have been deprived of their phospholipid always exceeds the percentage of particles whose DNA has been uncoated.

Finally, the fate of the protein of infecting virus particles can be determined by using virus labeled with an amino acid, say leucine-C^{14} (84). In this case, one may inquire whether any viral protein is completely degraded to acid-soluble material, and whether any protein subunits of the viral coat are split off from the particles. It is found that very little viral protein is degraded completely and that this degradation occurs at the cell surface, since the acid-soluble products appear in the medium rather than within the cell. However, up to 50% of the viral protein is split off from the virus particles and appears in a form in which it can no longer be deposited in centrifugal fields capable of sedimenting intact virus particles, but in which the label is still precipitable by acid and therefore macromolecular. This dissociated viral protein appears first within the cell; the dissociation process therefore occurs inside the cell. This process also commences without a lag immediately on infection, reaches 50% of completion at about 2 hr, but then continues slowly until 5 to 6 hr after infection. The release of viral protein from virions is to be interpreted as reflecting the breakdown of the outer coat of the virus before and during the uncoating of the viral genome. It is clear that the presence within the cell of viral coat protein molecules could have pronounced effects on the pattern of host cell macromolecular synthesis.

The uncoating of the poxvirus genome thus seems to be a two-stage process. The first stage proceeds immediately on infection and results in the stripping off of a large proportion of coat protein and the breakdown of phospholipid. The second stage occurs only after a lag, the length of which is dependent on the multiplicity of infection, and results in the final uncoating of the viral genome.

The question then arises as to the nature of the reactions which proceed during the lag phase. Inhibitors of protein synthesis such as *p*-fluorophenylalanine (FPA) and puromycin reversibly inhibit the second stage of uncoating but do not affect the first (88). Thus, protein synthesis is *not* necessary for the first stage of uncoating, but is necessary for the second stage, the actual release of viral DNA. The necessary protein, the "uncoating protein," is readily shown to be stable. If cells are infected with unlabeled virus, then 3 hr later puromycin is added so as to inhibit all further protein synthesis, and 4 hr later the cells are superinfected with labeled virus in the continuing presence of puromycin; this superinfecting virus is uncoated without lag. Uncoating protein is thus functional for at least 4 hr after its formation (88).

As would be expected, mRNA synthesis is involved in the synthesis of uncoating protein; actinomycin D completely inhibits the second stage of uncoating while in no way affecting the first (88).

Two questions now arise: (i) what causes the synthesis of uncoating protein, and (ii) is it translated from mRNA transcribed from the host cell genome or from the viral genome? The evidence is as follows.

(i) It is known that poxvirus heated at 55 C for 120 min or 60 C for 12 min is incapable of multiplication in normal cells (79). Such virus, however, is capable of multiplying in cells infected with unheated, infectious poxvirus. This is the phenomenon of poxvirus reactivation (see below; 40). The reason why the heated virus does not multiply is not inability to adsorb nor damage to the genome: the reason is that it is not uncoated (86). However, heated poxvirus *is* uncoated in cells infected with infectious poxvirus (86). Further, it can be shown, by determining whether superinfecting virus is uncoated with or without a lag, that no uncoating protein is present in cells infected with heated virus (89). It is thus apparent that uncoating protein is synthesized as a result of the introduction into the cell of undenatured viral protein. This protein is called the "viral inducer protein" (88).

(ii) The information for the synthesis of uncoating protein is *a priori* unlikely to reside in the viral genome, since it is synthesized at a time when viral DNA is inaccessible to the action of deoxyribonuclease, i.e., before it is uncoated. It is more likely that the viral inducer protein derepresses a section of the host cell genome which contains the information for the synthesis of the uncoating protein. Indirect evidence for this view has been obtained (88). Virus is not uncoated in cells treated with colchicine, which causes the host cell growth cycle to be arrested in metaphase when the chromosomal material is in a condensed state, not permitting mRNA transcription (153). Nor is virus uncoated in cells irradiated with ultraviolet light. The dose response curve for RNA synthesis inhibition is almost exactly the same as that for uncoating inhibition, and both are much steeper than that for protein synthesis inhibition. Since ultraviolet irradiation prior to infection would not be expected to inhibit transcription of mRNA from the viral genome, the implication again is that synthesis of uncoating protein depends on host cell mRNA synthesis. Controls were carried out to determine whether other functions essential to uncoating were inhibited by ultraviolet irradiation. These took the form of irradiating with ultraviolet light cells infected with unlabeled virus for 3 hr, and therefore containing the uncoating protein; in such ultraviolet-irradiated cells, superinfecting virus was uncoated normally, demonstrating not only that no other uptake function was inhibited by ultraviolet irradiation, but also that uncoating protein is stable to ultraviolet irradiation (88).

Experiments have been carried out with heterologous preinfection. CP u^+ and three strains of vaccinia virus (V-MH, CL, and WR) were all able to induce the formation of uncoating protein acting on superinfecting RP u^+ [although the heterologous was slightly less efficient than the homologous effect (87)]. This result is of importance for the phenomenon of poxvirus reactivation (see below).

At the same time as the above-mentioned studies were carried out, Dales and his co-workers (23, 25, 26, 27) investigated the early fate of infecting poxvirus particles by means of electron microscopy. Like all other viruses so far investigated, poxvirus particles first establish intimate contact with the surface of the host cell and are then taken up into phagocytic vesicles by a process of invagination. At first the particles are clearly visible within these vesicles; then after 30 to 60 min both the outer coat of the particles as well as the walls of the vesicle fade and characteristic doughnut shaped "cores" are released into the cytoplasm. During the next hour or so, these then also disappear from the view of the electron microscope. These results can be correlated well with the biochemical experiments described above. The first biochemical stage of the uncoating process, the liberation of viral protein subunits and the degradation of viral phospholipid, can be equated with events which proceed immediately upon infection within the phagocytic vesicles. The second biochemical stage, the liberation of viral DNA, then corresponds to the breakdown of viral cores, which proceeds only after the first hour. It is possible to detect by means of

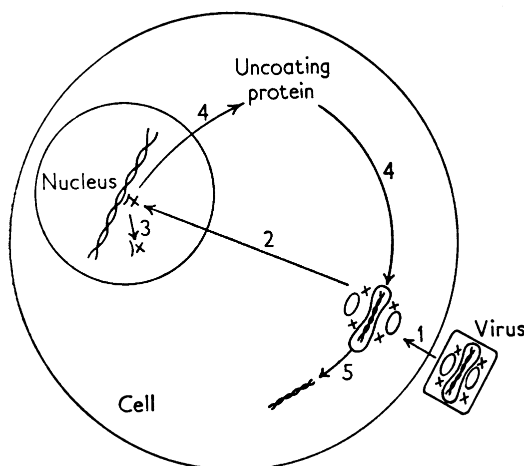

FIG. 2. *Scheme illustrating the hypothesis concerning the mechanism of poxvirus uncoating (88). (Reproduced by courtesy of Academic Press, Inc., New York.)*

density gradient centrifugation viral cores in cells infected in the presence of puromycin, in which only the first stage of uncoating occurs (88).

A model which can account for all evidence concerning the phenomena of poxvirus initiation (see above), poxvirus uncoating, and poxvirus reactivation (see below) is depicted schematically in Fig. 2 (88, 91).

The model supposes that a certain poxvirus protein molecule (the viral inducer protein) causes the host cell genome to code for a protein (the uncoating protein) which liberates the viral genome from the viral core. There is as yet no direct evidence for the existence of either protein in the sense of their having been isolated and characterized, or for the involvement of host cell DNA. The experiments of Abel (2; see below) provide independent functional evidence for the existence of an uncoating protein. The detection of uncoating protein presents no difficulty either intracellularly or extracellularly. The test for intracellular uncoating protein is to infect cells with labeled poxvirus and to test for the presence or absence of the lag period before uncoating commences. The test for extracellular uncoating protein is to add extracts of infected cells to poxvirus preparations as described by Abel (2). It has not yet proved possible to detect the action of uncoating protein in vitro by chemical means; that is, if an extract of cells infected for 3 hr is added to labeled virus in vitro, no sensitization of viral DNA to deoxyribonuclease has yet been observed, although the first step of uncoating probably proceeds to a small extent (Joklik, *unpublished data*). Nothing is known concerning the nature of the uncoating protein. It might, however, be pointed out that, whereas intact poxvirus virions are resistant to small amounts of trypsin, cores are uncoated by trypsin in vitro (Joklik, *unpublished data*).

Nothing is known concerning the nature of the viral inducer protein. The only evidence that it is a protein is its inactivation by low temperatures (55 C). It has the rather strange property of being abnormally sensitive to ultraviolet irradiation (89): indeed, inactivation of poxviruses by ultraviolet irradiation can be accounted for almost entirely as inactivation of the viral inducer protein. Virus inactivated by ultraviolet irradiation undergoes the first stage of the uncoating process but not the second (89). This is confirmed by electron microscopic studies (95). Further, cells infected with ultraviolet-inactivated virus contain no uncoating protein (89). An unusual feature of the inactivation of viral inducer protein by ultraviolet-irradiation is the fact that it follows single-hit kinetics. Each virus particle, therefore, only contains one molecule of uncoating protein or one unit with inducer function. On the other hand, viral inducer protein is not inactivated by nitrogen mustard, but is inactivated photodynamically and by alkylation. Viral inducer protein is readily assayed by determining whether its introduction into a cell is followed by synthesis of uncoating protein (89).

As discussed above, the evidence for the involvement of host cell DNA in the synthesis of uncoating protein is still circumstantial. If uncoating protein is indeed coded for by the host cell genome, it is probably a protein which has some other function besides that of causing uncoating of poxviruses, for possession of a protein with only this function could not confer any survival advantage onto the cell, and the corresponding gene would soon be eliminated. Since uninfected HeLa cells (or KB or L cells) do not contain uncoating protein, its synthesis in uninfected cells is presumably repressed. Possibly, the gene expresses itself in some other human or mouse cell, for instance during development. The derepression process may either be specific, affecting that gene only, or it may be nonspecific, introduction of the viral inducer protein into the cell causing more or less complete breakdown of the entire repression mechanism.

The technique of using radioactively labeled virus has proved of value in elucidating the fate of virus-antibody complexes (89). Such complexes adsorb to cells at almost the same rate as intact virions, but the majority elute. Those that are taken up into cells are degraded to cores which are not uncoated. Cells infected with virus-antibody complexes do not uncoat superinfecting normal virus in the usual manner; most of the

superinfecting virus elutes after loss of phospholipid (89). Electron microscopic studies by Dales have yielded results consistent with these (24, 26). They show that uncoating of virus-antibody complexes is abnormal and incomplete. It would be interesting to determine what effect variation of the number of antibody molecules per virus particle has on the fate of the complex.

Although the biochemical studies and the parallel electron microscopic investigation have yielded results which are consistent on most of the major issues, such as the two-stage nature of the uncoating process, its kinetics, the existence of cores, and the fate of virus irradiated with ultraviolet light and combined with antibody, there is one apparent major difference. The biochemical studies show that in HeLa cells there is *no* uncoating in the presence of 2 μg/ml of actinomycin D (88), but the electron microscopic studies show that uncoating does occur in L cells in the presence of 0.2 μg/ml of actinomycin D which "inhibits RNA synthesis by 90%" (95). From this, it is concluded that mRNA synthesis is *not* necessary for uncoating to occur (95). This conclusion, however, is not justified. There is ample evidence that ribosomal RNA synthesis is much more sensitive to actinomycin D than mRNA [or 4*S* transfer RNA (tRNA)] synthesis (56, 67, 142). Since the bulk of RNA synthesized in the HeLa or L cell is ribosomal RNA (166), it is quite possible that, at actinomycin D concentrations inhibiting *total* RNA synthesis by 90%, ribosomal RNA synthesis is arrested almost completely while mRNA synthesis, which, of course, is the crucial RNA under consideration here, is hardly affected. It is therefore not enough to show that "90% of RNA synthesis" is inhibited; an inhibitor is only useful if it is known that at the concentration used it is effective for the purpose at hand. The objection that higher concentrations of actinomycin D may have unknown additional effects is ruled out by control experiments which show that when 2 μg/ml of actinomycin D is added to cells preinfected for 3 hr and therefore containing the uncoating protein, superinfecting virus is uncoated without lag. This indicates that at a concentration of 2 μg/ml of actinomycin D none of the other processes involved in uncoating, such as uptake, etc., is inhibited. The conclusion is that both mRNA and protein synthesis *after* infection are essential for the uncoating of poxvirus DNA.

Synthesis and Fate of Viral mRNA

Very soon after the viral genome is uncoated, mRNA is transcribed from it and can be detected readily by pulse-labeling. The circumstances which make this possible are as follows. In the normal uninfected animal cell in tissue culture, such as the HeLa cell, all RNA is synthesized in the nucleus (166). Three types of RNA are known to be transported to the cytoplasm: ribosomal RNA, which in HeLa cells comprises two molecular species of 28*S* and 16*S* [molecular weights, 1.3 $\times 10^6$, and 0.6 $\times 10^6$, respectively (160)], mRNA, which is heterogeneous in size with sedimentation coefficients ranging from about 25 to 8*S* (57), and 4*S* tRNA. These RNA molecules are transported to the cytoplasm at different rates: 4*S* tRNA begins to appear in the cytoplasm within 2 to 3 min after it is formed; mRNA, within some 7 to 8 min if label of very high specific activity is used (uridine-H^3, 10 c/mmole) but not before about 20 min if uridine-C^{14} (20 mc/mmole) is used; and 16*S* ribosomal RNA appears in the cytoplasm within 25 to 30 min and 28*S* ribosomal RNA only after 30 to 60 min (57, 92, 93). Therefore, if uridine-C^{14} is used to detect newly formed RNA molecules and the cytoplasm is examined 10 min after addition of the label, the only label to be found in the cytoplasm is 4*S* tRNA. This is readily shown either by means of SDS-sucrose density gradients in which the sedimentation coefficients of the free RNA species are measured, or in sucrose density gradients in which the size of the particles or structures with which RNA is associated can be measured. Such gradients show that 10 min after exposure to uridine-C^{14} the polyribosomes in uninfected cells are still devoid of label, which indicates that they do not contain newly formed mRNA or ribosomal RNA (13). This type of analysis depends on the availability of cytoplasm free from nuclear contamination. This is readily obtained by controlled breakage of cells in a Dounce homogenizer (141); by use of the correct number of strokes (determined by direct experiment), it is possible to achieve over 95% cell breakage (determined by counting or plating) and less than 1% breakage of nuclei [determined by homogenizing cells containing labeled DNA and measuring the amount of label in the cytoplasmic fraction, i.e., the supernatant fluid after centrifuging the cell homogenate at 200 $\times$ *g* for 2 min (90)].

Whereas in the uninfected cell no RNA larger than 4*S* appears in the cytoplasm within 15 to 20 min after addition of uridine-C^{14}, in the cell infected with a poxvirus labeled RNA heavier than 4*S* can be detected in the cytoplasm within 2 to 3 min after adding uridine-C^{14}, and even earlier after adding uridine-H^3 (13, 93). This material is mRNA, since it is associated with polyribosomes; and it is transcribed from the viral genome since (i) its base composition is similar to that of viral DNA and quite different from that of HeLa cell DNA (13, 162) and (ii) it hybridizes with viral

DNA and not with HeLa cell DNA (13). Its size is relatively small early in the infection cycle (1 to 2 hr after infection, median $S = 12S$, 80% less than 16S) and larger later in the infection cycle (5 hr after infection, median $S = 20S$, 80% more than 16S). It is first detectable at about 1 to 1.5 hr after infection, that is, as soon as the viral genome is uncoated, is synthesized at the maximal rate at about 4 hr after infection, and very little more is synthesized after 7 hr (13; Fig. 3).

Poxvirus mRNA provides a unique opportunity for studying the functioning of mRNA, i.e., for examining how mRNA is incorporated into polyribosomes. This is very difficult to study in normal cells where mRNA is synthesized in the nucleus and has to pass into the cytoplasm before it is incorporated into polyribosomes; it is also known that ribosomes, which are also synthesized in the nucleus, do not reach the cytoplasm as such, but in the form of ribosomal subunits (92). Since, as mentioned above, it takes at least 7 to 8 min for even the first mRNA molecules to be detectable in the cytoplasm, it is impossible to follow their incorporation into polyribosome form. Transport of mRNA to the cytoplasm is a slow process, whereas its incorporation into polyribosome form is a rapid one, so that intermediates between free mRNA molecules on the one hand and normal-size polyribosomes on the other are unlikely to be detectable.

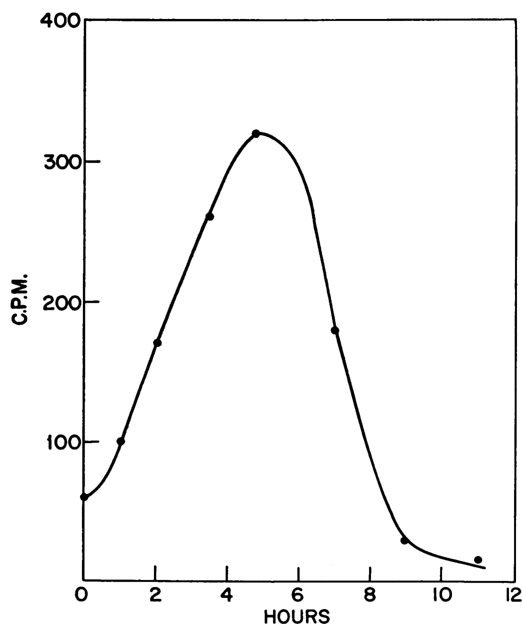

FIG. 3. *Time course of synthesis of vaccinia virus strain WR mRNA in HeLa S3 cells in suspension culture (13).*

The situation is quite different in the case of vaccinia mRNA. Not only is it synthesized in the cytoplasm, but the rate of its synthesis during the period 3 to 5 hr after infection is considerably more rapid (possibly three- to fivefold) than the rate of host cell mRNA synthesis in uninfected cells (13). There is thus a source of rapid supply of mRNA molecules very close to the site of formation of polyribosomes, and the chance of being able to detect intermediates in this process is very much better than in the case of uninfected animal cells.

To detect such intermediates, it is necessery first to examine the various states of ribosomal material in the cytoplasm. Ribosomal material exists in the cytoplasm of HeLa cells in four forms. Not only are there ribosomes attached to mRNA and single ribosomes, but there are also the two classes of subribosomal particles: 40S subunits containing 16S RNA, and the 60S subunits containing 28S RNA (92). One 40S subunit plus one 60S subunit make up a ribosome. These two particles are always present in cells in equivalent amounts, and generally account for 5 to 10% of the total ribosomal material in the cytoplasm. The total number of single ribosomes fluctuates markedly, and presumably reflects the metabolic state of the cell; the faster protein is being synthesized, the more ribosomes are in polyribome form and the fewer are present in the 74S monomer form (92).

The first reaction undergone by a newly formed vaccinia mRNA molecule is to combine with a 40S subribosomal particle (93); within 1 to 2 min of formation, about half the mRNA molecules are in this state. These complexes are then incorporated into polyribosomes, their free half-life being of the order of 2 min as determined by chase experiments. The finding of these complexes of mRNA molecules with 40S subribosomal particles has far-reaching implications. As mentioned above, 74S ribosomes of HeLa cells do not enter the cytoplasm from the nucleus as such. Instead, the 40S ribosomal subunit enters the cytoplasm first, followed some 30 min later by the 60S subunit (92). The combination of these two components to form a complete 74S ribosome takes place in such a manner that the complete ribosome appears first as part of a polyribosome rather than as a free ribosomal monomer (92). The most likely explanation, in view of the finding that vaccinia mRNA combines with a 40S subribosomal particle very soon after its formation, is that in the uninfected cell also the nascent mRNA molecules combine with 40S subribosomal particles (this union occurring in the nucleus where both are synthesized) and that mRNA is transported from the nucleus to the cytoplasm

while attached to a 40*S* subribosomal particle. In the cytoplasm, this complex then presumably combines with a 60*S* subribosomal particle yielding a complete ribosome attached to an mRNA molecule, and then with further ribosomal particles to become a polyribosome. It is thus likely that complex formation between an mRNA molecule and a 40*S* subribosomal particle is the first step in the formation of both ribosomes and polyribosomes.

With any polycistronic system, the question of regulation arises. Poxvirus DNA is capable of coding for a very large number of proteins, probably of the order of 500, but not all are made at the same time. To take the most obvious example, the so-called "early" enzymes are all synthesized in the period from 1 to 4 hr after infection, but most structural viral proteins are only synthesized after 4 hr (90). It would appear, therefore, that different parts of the viral genome function as templates at different stages of the infection cycle. A reflection of this may be the fact that "early" viral mRNA molecules appear to be relatively small, whereas "later" ones appear to be larger. It may be that the early ones, coding mainly for enzymes and proteins adjusting the synthetic functions of the host cell for the process of viral synthesis, are monocistronic messages, whereas later ones, coding for structural viral proteins, are polycistronic, thus ensuring some degree of proportionality in the relative number of the various protein subunit molecules synthesized. Direct tests to establish whether some mRNA molecules synthesized at one time are not synthesized at another time are now being carried out (Joklik, *unpublished data*). There is some evidence that certain portions of the parental genome cannot act as template; this will be considered below when the various proteins synthesized are considered.

It is currently postulated that mRNA molecules are more or less unstable, and that this instability results in a flexible metabolic pattern. It is not known whether all mRNA molecules are unstable or only some, or whether all unstable ones are equally unstable or whether there is a wide spectrum of instability; if there is a spectrum, it is not known what the basis of selection is, or how mRNA molecules are degraded. Bacterial mRNA molecules decay with a half-life of the order of 2 min (110). However, sometimes there is limited agreement only between various methods used to determine the half-life: for instance, as measured by loss of label, the half-life of *Bacillus megaterium* mRNA is less than 1 min; as measured by decrease in the number of polyribosomes, it is 3 to 5 min, a considerable descrepancy (164). Few studies have been carried out on the half-life of mRNA in animal cells. Darnell and his associates (141) measured the time course of decrease in the number of polyribosomes, and the decrease in the rate of overall protein synthesis in HeLa cells after administration of actinomycin D to inhibit formation of further mRNA; they deduced a half-life of about 3 hr. Similarly, the *functional* half-lives of the mRNA molecules coding for the specific proteins synthesizing acid mucopolysaccharides and collagen in established cultured cell lines were of the order of 3 hr (16, 28). On the other hand, the half-life of rat liver mRNA, as determined by the rate of reformation of polyribosomes after reversal of the inhibition of mRNA synthesis caused by ethionine, is 50 min (180); as determined by the rate of decrease in the number of polyribosomes after administration of actinomycin D it is 4 to 8 hr (176), and as determined by the rate of decrease in the ability to incorporate C^{14}-labeled amino acids into protein after administration of actinomycin D it is over 40 hr (158). It is clear therefore, that the complex problem of the stability of mRNA in animal cells is largely unresolved.

One can use three methods for estimating the half-life of vaccinia mRNA. All depend on arresting further mRNA synthesis with actinomycin D. (i) One can determine for how long a specific protein is synthesized. This has been done for the virus-induced DNA polymerase (see below, *Early enzymes*), and it is found that the enzyme continues to be synthesized at a virtually undiminished rate for at least 5 hr (94). The "half-life" of the corresponding mRNA is thus very long, considerably in excess of 5 hr. The same has been found to be true for the mRNA which codes for thymidine kinase in cells infected with vaccinia virus (94, 122). Thus, the mRNA molecules coding for the early enzymes appear to be very stable; it is, therefore, surprising that under normal conditions of infection they do not express themselves after the first 4 hr or so of the infection cycle (see below). However, mRNA molecules coding for structural viral proteins later on during the infection cycle (at about 5 hr after infection) function for no more than 1 hr (170). These mRNA molecules are *unstable*, with a half-life of rather less than 0.5 hr. There is, thus, a large difference between the functional stability of early mRNA coding for enzymes and late mRNA coding for structural viral proteins. The reason for this difference is not known. (ii) Assuming that breakdown of mRNA will result in the release of ribosomal monomers from polyribosome form, one can obtain an estimate for the half-life of mRNA by measuring the rate of this release. It is found that at 4 hr after infection the kinetics of the release of ribosomes is biphasic; about 50% of the

total number of ribosomes in polyribosome form are released within 30 min, but further release is slow (Woodson and Joklik, *unpublished data*). This suggests that at this time there are both unstable and stable mRNA molecules. (iii) A similar conclusion is reached as a result of experiments measuring the breakdown of viral mRNA by release of label. This type of analysis is uniquely possible in cells infected with vaccinia virus, since, as discussed below, from about 2 hr after infection on, no RNA labeled in the nucleus, either messenger or ribosomal, is transported to the cytoplasm (13, 162). It is thus possible to label viral mRNA, to block further synthesis with actinomycin D, and then to measure as a function of time the amount of label remaining in mRNA. It is again found that there is a rapid initial loss of label during the first 30 min, but that during the next 3 hr the amount of label in mRNA decreases only very slowly (Joklik and Woodson, *unpublished data*). Similar results have been reported by Shatkin (171).

In summary, it is found that when mRNA is measured by its ability to code for specific proteins, there are two types of vaccinia mRNA, "early" stable ones and "late" labile ones, with half-lives in excess of 5 hr and less than 0.5 hr, respectively; and, when mRNA is measured by the number of ribosomes bound to it or by loss of radioactive label, then both labile and stable mRNA molecules are detectable during the period from 4 to 6 hr after infection. Clearly, a great deal of work remains to be done.

Synthesis of Virus-Coded Proteins

The poxvirus genome, of molecular weight 160×10^6, is capable of coding for a very large number of proteins. Only few of these are known. They can be divided into two classes: (i) enzymes on the pathway of nucleic acid metabolism which are synthesized early in the infection cycle, and (ii) structural viral proteins which are synthesized mainly between 3 and 8 hr after infection.

Early enzymes. Hanafusa (65) found that extracts of L cells infected with vaccinia virus strain IHD exhibited an increased ability to incorporate thymidine into DNA over that of uninfected cells. This is due to increases in the amount of thymidine kinase (58, 102, 120), which is also a deoxyuridine kinase (30, 120), and DNA polymerase (58, 59, 94). None of the other kinases acting on either RNA or DNA precursors, nor any of several nucleoside or deoxynucleoside deaminases, are present in increased amount after infection. However, the level of deoxyribonucleases is also increased after infection; Hanafusa found the amount of deoxyribonuclease acting on double-stranded DNA to be elevated in L cells infected with IHD (65), Jungwirth and Joklik (94) found a deoxyribonuclease acting on single-stranded DNA in HeLa cells infected with vaccinia virus strain WR, and McAuslan (123, 123*a*) reported that deoxyribonucleases acting on both single-stranded and double-stranded DNA were present in increased amounts in HeLa cells infected with CP u+.

The time courses of the increase of all the three enzymes which have been studied extensively [thymidine kinase, DNA polymerase, and the deoxyribonuclease (single-stranded DNA)] are identical: the rise period extends from 1 to 4 hr after infection (94, 121). [The time course depends on the system used, and the numbers given here refer to the system HeLa S3 cells in suspension culture infected with vaccinia virus strain WR (94).] The increases in enzyme activity represent *de novo* synthesis of enzyme molecules since in all cases the increases cease immediately on addition of puromycin (94, 105). Heat-inactivated virus, which is not uncoated, fails to induce enzyme synthesis (120).

These observations give rise to a number of questions. The first is whether the enzyme molecules synthesized after infection are different from the pre-existing ones. The evidence is not complete as yet, but it is known that the host and virus-induced DNA polymerases have different primer affinities and *p*H activity profiles (94), and that the two thymidine kinases differ in heat stability and Michaelis constants (108, 122), as well as immunologically (108). However, it must be pointed out that "DNA polymerase" activity as measured by the incorporation of thymidine triphosphate into added single-stranded primer DNA in the presence of the three other deoxynucleoside triphosphates is a complex reaction, and that the participation in that reaction of certain nucleases is not ruled out; also, although the thymidine kinase reaction is probably simpler, the enzyme is known to be profoundly influenced by allosteric mechanisms (17). Conclusive proof that the host and virus-induced enzyme proteins are different will therefore have to await isolation, peptide map analysis, and amino acid sequence determination. No information whatever is available concerning the deoxyribonucleases.

The second question concerns the reason for the abrupt cessation of the synthesis of all three enzymes at about 4 hr after infection (121). In the presence of actinomycin D to inhibit further mRNA synthesis, DNA polymerase and thymidine kinase continue to be formed until at least 8 hr after infection (94, 122); the mRNA molecules coding for both these enzymes are therefore stable. The series of reactions causing synthesis of enzymes to stop prematurely has

become known as the "switchoff" phenomenon, and has been studied in particular as it applies to thymidine kinase (122).

Thymidine kinase formation is induced not only by infection with intact virions, but also by infection with virions inactivated to a survival of 10^{-5} to 10^{-6} by irradiation with ultraviolet light (121). No viral DNA replication occurs in such cells. There is no switchoff of the synthesis of thymidine kinase in cells infected with ultraviolet-inactivated virus; synthesis of the enzyme is said to be uncontrolled. In cells infected with both intact and inactivated virions, viral DNA replication proceeds, as does switchoff of thymidine kinase synthesis (121). The presence of active virions is thus dominant; they most probably express themselves through cytoplasmic factors. When viral genome replication is inhibited with aminopterin, switchoff is also abolished (121) and the synthesis of mRNA, as well as that of a certain amount of protein, is essential for switchoff to occur (122).

DNA polymerase and the deoxyribonuclease (single-stranded DNA) are subject to similar control mechanisms; in both cases, there is *no* switchoff if viral genome replication is inhibited by either aminopterin or 5-fluorodeoxyuridine (94). Two explanations of the switchoff mechanism can be entertained profitably at this time. (i) There is synthesized from progeny viral genomes but not from parental viral genomes an mRNA and thence a protein which specifically destroys certain mRNA molecules. There must be active destruction or some other prevention of function since, as pointed out above, the mRNA molecules in question are stable. There is no explanation why only some mRNA molecules are affected and not others; at the time of switchoff, synthesis of structural viral proteins proceeds at a rapid rate. Nor is there any clue as to whether the active protein is a specific protein or a structural viral protein which incidentally causes switchoff, or what the mechanism of the neutralization of the mRNA is. (ii) The phenomenon could be explained, though possibly with less plausibility, as a competition effect. It could be argued that the time when switchoff becomes effective coincides with the onset of the assumption of template function by progeny genomes (see below for the time course of viral DNA replication) and that the mRNA molecules transcribed from the large number of progeny genomes compete successfully for ribosomes with the relatively few molecules of mRNA coding for the early enzymes. When viral genome replication is inhibited, there is of course no such synthesis of mRNA from progeny genomes, and the competition does not occur—hence, no switchoff. Similarly, arresting the formation of late mRNA would eliminate competition. The reversible inhibition of switchoff by puromycin could be due to the inhibition of mRNA synthesis by puromycin; such inhibition has been found to be both severe and largely reversible (93).

The third question concerns the problem of the source of the genetic information necessary for the synthesis of the new enzyme molecules which are formed after infection. The evidence that there is such synthesis is sound, but it does not necessarily follow that the source of the information is the viral genome. After all, a great proportion of the normal HeLa cell genome, which is thought to contain the complete human genome, is prevented from functioning as template. In addition, a case has been made that uncoating protein is a protein the synthesis of which is derepressed after infection (88), and evidence is mounting that interferon also is such a protein (181). It is at least conceivable that the early enzymes also fall into this class. The evidence for the three enzymes under discussion here is as follows: it has been mentioned above that the synthesis of thymidine kinase is induced by ultraviolet-inactivated vaccinia virus which is uncoated very poorly, if at all (89, 95). Even if there is a small amount of uncoating, it is unexpected that a small number of uncoated viral genomes causes almost as rapid a synthesis of thymidine kinase as that caused by the much larger number of genomes which would be available from an intact virus inoculum. Further, it has now been found that ultraviolet-irradiated virus preparations which induce the synthesis of thymidine kinase are incapable of inducing the synthesis of DNA polymerase and of deoxyribonuclease (single-stranded DNA; 94). In addition, it has been reported that although 8-azaguanine inhibits the elevation in the incorporation of thymidine monophosphate into DNA after infection, and therefore most probably the induction by virus of the synthesis of DNA polymerase, it does *not* inhibit the induction of the synthesis of thymidine kinase which occurs after infection (20*a*). One could account for these differences in two ways. (i) If the code for all three enzymes is present in the viral genome, then that for thymidine kinase must be accessible in a different manner from that for the other two enzymes. Perhaps a very small part of the viral genome of ultraviolet-inactivated virions containing the code for thymidine kinase *is* uncoated, and an ultraviolet hit prevents further uncoating or further transcription, or both; alternatively, the cistron for thymidine kinase may be peculiarly resistant to ultraviolet damage. There are diffi-

culties in the way of all of these explanations, but they cannot be ruled out. (ii) The alternative is that although the code for DNA polymerase and deoxyribonuclease (single-stranded DNA) really does reside in the viral genome, that for thymidine kinase does not. On this theory, the virus-induced thymidine kinase would be coded for by the host cell genome, and the induction of its synthesis would be due to derepression by a viral protein component, as postulated for uncoating protein. A difficulty of this explanation is that there would have to be two viral inducer protein functions, since the synthesis of uncoating protein is not induced by ultraviolet inactivated virus, whereas that of thymidine kinase is. According to this hypothesis, the host cell genome would have two genes coding for thymidine kinase, one that is normally expressed and one that is normally repressed and is derepressed by infection.

Further evidence concerning this problem is supplied by the work of Kit and his collaborators. By prolonged cultivation in the presence of gradually increasing amounts of 5-bromodeoxyuridine, a strain of L cells has been selected which lacks the ability to synthesize thymidine kinase (TK^- cells; 106). By use of these cells and the same technique of 5-bromodeoxyuridine administration to cause the synthesis of defective viral DNA, mutant strains of vaccinia virus have been isolated which lack the ability to induce the formation of thymidine kinase (30). These TK^- virus strains grow well in TK^- L cells, demonstrating that the induction of thymidine kinase is "gratuitous." Some of the mutants have high reversion rates, but others are stable. Neither complementation nor recombination has yet been demonstrated.

The fact that there exist TK^- mutants of vaccinia virus has been adduced as strong evidence that the virus-induced thymidine kinase is coded by the viral genome (30). It is, however, possible to argue that TK^- mutants lack the proper derepressor for the normally repressed host cell gene coding for the new thymidine kinase, for the absence of which subculturing in 5-bromodeoxyuridine did not of course select. An interesting situation arises when cells are mixedly infected with TK^+ and TK^- virus strains; the amount of enzyme synthesized is then dependent on the ratio of the two multiplicities, not on the absolute multiplicity of either (128). TK^- virions can thus prevent TK^+ virions from initiating thymidine kinase synthesis, suggesting competition at some level. As suggested (128), competition could occur during polypeptide chain synthesis or during enzyme subunit combination should thymidine kinase prove to be composed of subunits (125). However, there may be competition between derepressors, those of TK^- strains being able to combine with but not effectively neutralize the repressor. The strongest argument in favor of the viral genome as the source of the code of the virus-induced thymidine kinase is the fact that herpesvirus also induces the synthesis of a thymidine kinase which, according to preliminary studies, differs from both host cell and vaccinia virus-induced enzymes (108); it is unlikely that herpesvirus derepresses yet another normally repressed gene in the host cell genome for coding yet another thymidine kinase.

If the viral genome does code for thymidine kinase, there must be a survival advantage associated with possession of this gene, as otherwise it would soon be lost. Perhaps this survival advantage takes the form of a slightly larger burst size which, over a number of multiplication cycles, would ensure that TK^+ strains would evolve as the dominant population components.

To summarize, the strongest evidence against the view that the virus-induced thymidine kinase is coded by the viral genome is that the synthesis of this enzyme, in contrast to that of the DNA polymerase and the deoxyribonuclease (single-stranded DNA), is induced by virus irradiated with ultraviolet light which is known to be poorly, if at all, uncoated. The strongest evidence for the opposite view is that the host enzyme, the vaccinia-induced enzyme, and the herpes-induced enzyme all appear to be different. A great deal of further work will be necessary to clarify this problem.

Structural viral proteins. The synthesis of structural viral proteins has been studied by three techniques: (i) immunodiffusion analysis with the use of hyperimmune antiserum (9); (ii) analysis of labeled proteins precipitable with hyperimmune antiserum (170); and (iii) analysis of the proteins necessary for the incorporation of naked progeny viral DNA into mature virions (90).

The first of these methods has great resolution, and allows enumeration of the molecular species of protein capable of reacting with antiserum, but there is no guarantee that only structural viral proteins react; early enzymes could react in this test if produced in large amounts and potent antigenically. The second method lacks resolution and again could also encompass nonstructural viral proteins, but provides a rapid technique for determining the time course of synthesis, the effect of inhibitors, etc. The third method is specific for structural viral proteins, but has not been exploited fully; it will be discussed below.

In addition to these three methods, one may

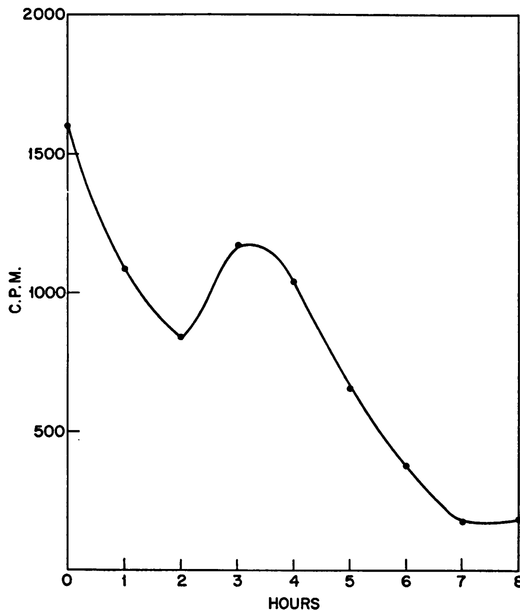

FIG. 4. *Time course of the incorporation of labeled amino acids into the cytoplasmic fraction of HeLa S3 cells in suspension culture infected with vaccinia virus strain WR (187).*

also measure the overall capacity of the infected cell to incorporate radioactively labeled amino acids. This method will measure synthesis of virus-specified protein almost exclusively since (i) the ability to synthesize cellular protein is markedly inhibited soon after infection (170), (ii) no host cell mRNA reaches the cytoplasm after about 2 hr after infection (13, 162), and (iii) studies with IBT (see below) strongly suggest that from 3 hr after infection on no more than a very small proportion of the total protein synthesized is host cell protein (187). All the evidence suggests that from 2 to 3 hr after infection on most of the protein synthesized in the infected cell is virus-specified protein.

The overall rate of protein synthesis in the infected cell is shown in Fig. 4. This figure should be related to Fig. 1, 3, and 5, showing the viral growth curve, and the time course of synthesis of viral mRNA and viral DNA, respectively. The period from 3 and 5 hr in Fig. 4, when a maximum in the rate of protein synthesis occurs, coincides with the period of maximal viral mRNA synthesis (13), and therefore probably represents the period of maximal virus-specified protein synthesis.

The time course of synthesis of protein which reacts with antiserum to vaccinia virus [which can be detected on polyribosomes (165)] has been determined by Shatkin (169). Such protein is first detectable at 2 hr, but the maximal rate of synthesis is not reached until much later: almost 50% of it becomes detectable only between 6 and 10 hr. This is much later than the estimate obtained by measuring the overall rate of protein synthesis. The two techniques probably measure different processes. There are many reasons why it is unlikely that protein synthesis proceeds at a rapid rate at a time as late as 6 to 10 hr after infection: very little mRNA is then made (13), there are very few polyribosomes in the cells (13), the mRNA at that time has a short half-life and therefore cannot continue to function for long (170), and the presence of FPA from 6 hr on does not affect virus growth (112). It therefore seems likely that the process measured by the immunological technique is not polypeptide synthesis, but the conversion of polypeptides to such precursors of virus as are precipitable by antiserum.

Immunodiffusion analysis has also been used to follow the synthesis of virus-specified proteins (9). Again, material capable of forming precipitin lines is first detectable at about 2 hr after infection, 4 to 6 lines are detectable by 6 hr, 8 to 10 by 8 hr, and up to 20 at the end of the multiplication cycle (5). Again, there is the phenomenon that most of the lines only become detectable *after* the time of maximal protein synthesis.

Salzman et al. (161) found that protein synthesis is necessary until 30 min before maturation. Loh and Payne pointed out that this observation is at variance with their observation that FPA has no effect on virus multiplication from 6 hr on. The most likely explanation of this discrepancy is that formation of a certain protein is indeed necessary up to at least 30 min before the onset of maturation (see below), but that the rate of synthesis of this protein exceeds the rate of maturation so that it accumulates and maturation, once it has started, becomes less and less dependent on the continued synthesis of protein.

Apart from the early enzymes already discussed, certain other virus-coded proteins can be identified by specific techniques in infected cells, and the time course of their synthesis has been measured. Those are: the antigen(s) combining with neutralizing antibody, the synthesis of which begins 2 hr before the onset of maturation and is 50% complete at 6 hr, when maturation commences (6); the "LS" and "NP" antigens, neither of which is probably a single molecular species, the synthesis of which starts at 4 hr and 5 to 6 hr, respectively (111); and the viral hemagglutinin, the nature of which has been discussed

above and the synthesis of which commences about 2 hr after the onset of maturation (111).

Immunodiffusion analysis has also been used to gauge the effect of various inhibitors on viral protein synthesis. Thus, the general metabolic inhibitor azide allows the synthesis of only a few of the 20 or so viral antigens normally detectable, the most prominent being one formed early (4). In the presence of IBT (see below) also, only some of the early viral antigens are formed (4, 9). FPA is a poor inhibitor of the formation of viral antigens, owing no doubt to the fact that it is extensively incorporated into protein which may or may not be functional (21); at a concentration of 0.002 M, virtually the normal complement of viral antigens is formed (although no mature virions), and even at 0.01 M six are detectable (8). Finally, in cells infected with ultraviolet-inactivated virus also some viral antigens were detectable, the pattern being similar to that observed in cells infected in the presence of azide (4); but the conditions of ultraviolet-inactivation and subsequent infection were not detailed precisely enough to allow comparison with the studies describing uncoating of ultraviolet-inactivated virus, or induction of the formation of thymidine kinase.

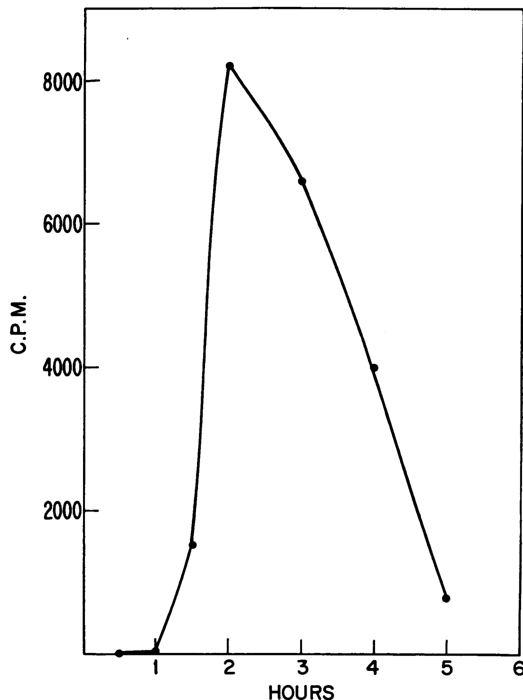

FIG. 5. *Time course of synthesis of vaccinia virus strain WR DNA in HeLa S3 cells in suspension culture (90).*

Replication of Poxvirus DNA

Salzman first defined the time course of vaccinia virus DNA synthesis by adding 5-fluorodeoxyuridine at various times after infection and measuring the virus yield at 24 hr (160). Viral DNA synthesis commenced at about 2 hr after infection, and by 5 hr enough DNA had been synthesized to allow the formation of maximal virus yields. Essentially similar results have been obtained by other workers (5, 113). Increased incorporation of thymidine-C^{14} into cells infected with vaccinia virus was first demonstrated by Magee et al. (114), but, in the absence of adequate techniques for specifically measuring incorporation of label into viral DNA rather than into total DNA, the information gained from such experiments was limited. On the other hand, the importance of the quantitative autoradiographic analysis of Kato et al. (100) has already been mentioned. Shatkin and Salzman (168) showed that at limiting concentrations thymidine is used as a precursor for viral DNA rather than for host cell DNA.

An adequate technique for studying viral DNA alone rather than a mixture of cellular and viral DNA was provided by the technique of homogenizing cells in Dounce homogenizers (141). With this technique, it is possible to prepare cytoplasmic material contaminated by less than 1% of nuclear material, and, since poxvirus DNA replicates in the cytoplasm, it alone can be examined. Figure 5 shows the rate of incorporation of thymidine-C^{14} into cytoplasmic DNA by use of 5-min labeling pulses (90). The fact that the material into which label is incorporated is viral DNA is readily shown by density gradient analysis as well as by chase experiments; the labeled material appears in due course in mature viral progeny. Viral DNA synthesis starts at about 1.5 hr after infection, reaches a maximal rate at 2.5 hr, and is 90% complete by 4 hr. There is evidently very tight control over viral DNA replication. The rate of viral DNA synthesis at 2.5 hr exceeds that of cellular DNA synthesis in uninfected cells by three- to fivefold; this is due to the fact that the total amount of viral DNA synthesized in an infected HeLa cell is equivalent to about 50% of the host cell's DNA complement and that this amount of DNA is synthesized in a period of about 2.5 hr rather than in 24 hr, the generation time of nonsynchronized host cells.

Viral DNA replicates in very large aggregates, the stability of which is dependent on the presence of magnesium (90); these aggregates appear to correspond to the factories which can be observed by microscopic or autoradiographic

techniques. As expected, the parental viral DNA is also present in these aggregates (90). However, they contain no detectable amounts of viral mRNA; although it is presumably transcribed there, it is evidently transported from there rapidly. Studies on cytoplasmic fractions enriched for these aggregates have indicated that they catalyze some DNA synthesis in vitro (94). At the time when viral DNA is synthesized at the maximal rate, *all* viral DNA is present in the form of aggregates. Subsequently, the aggregates break up; by 7 hr after infection, only about 50% of the viral DNA is in the form of aggregates (90). This biochemical analysis correlates well with the cytological pattern described above.

Maturation

There are numerous electron microscopic studies on the maturation of poxviruses. These will not be reviewed here; the reader should consult Dales and Siminovitch (22). Developmental forms and immature particles have been described and schemes concerning the sequence in which they arise have been proposed. However, it is very difficult to decide which of these developmental forms contain DNA. It seems safe to assume that the maturation process is not a reversal of the uncoating process.

Another technique of studying the maturation process is to examine the fate of the newly formed progeny DNA molecules. Infected cells are exposed to a 5-min pulse of thymidine-C^{14} at, say, 2.5 hr after infection, when DNA is replicating rapidly, and at various times thereafter the physical state of the DNA molecules synthesized during that 5-min period is determined (90). At the time when the molecules are synthesized, they are susceptible to deoxyribonuclease, and they continue to be so until about 5 hr after infection. The labeled molecules then apparently become associated with protein, but they remain sensitive to deoxyribonuclease. Some 30 to 60 min later, they then become insensitive to deoxyribonuclease without yet however being in complete virions. While in these two stages, the DNA molecules are said to be in "subvirion" form, corresponding to immature viral developmental forms. Finally, at 6 to 6.5 hr after infection, the first progeny DNA molecules appear in completed mature virions. DNA molecules are withdrawn at random from the pool of replicated DNA to undergo the maturation process (90); DNA molecules formed at 4.5 hr after infection, at the very end of the period of DNA replication, have the same chance of appearing in a virion at 7 hr after infection as the molecules synthesized at 1.5 hr, at the beginning of the period of DNA replication.

This maturation continues over a period of about 10 hr, until at 16 to 20 hr, at the end of the multiplication cycle, one-third to one-half of the DNA molecules which were synthesized are incorporated into virions. Maturation is thus not a very efficient process, probably for topographical reasons. Under optimal conditions, of the order of 10,000 virions and 20,000 to 30,000 viral DNA molecules are synthesized per cell (90).

The sequence of synthesis of the various proteins necessary for maturation has been analyzed by adding puromycin at various times after pulse-labeling at 2 hr, and determining the state of the DNA at 7 hr after infection (90). Synthesis of the protein which converts naked DNA molecules to the deoxyribonuclease susceptible subvirion form commences at about 4 hr after infection; that of the protein which converts DNA molecules to the deoxyribonuclease-resistant subvirion form commences at 4.5 to 5 hr, and the final protein necessary for complete virion formation first appears at 5.5 to 6 hr, 30 to 60 min before the first mature virions are detectable (90). At no time is there a significant accumulation of immature viral forms. The time necessary to transform a naked DNA molecule into a mature virion is therefore relatively short, certainly not longer than 1 hr.

We thus have the following picture of the maturation process. Whereas there is very tight control over DNA replication, 90% of which occurs during the 2.5-hr period from 1.5 to 4 hr after infection, the maturation period is highly asynchronous, for the number of mature virions increases from 6 to 16 hr. However, the maturation period for each individual DNA molecule is short, no more than 1 hr. Thus, it appears that, after DNA replication has ceased, DNA molecules are withdrawn at random from the pool for a period of about 10 hr and put through a process which involves the stepwise addition of protein so that each molecule appears successively as naked, associated with protein but sensitive to deoxyribonuclease, resistant to deoxyribonuclease, and finally in a mature virion (90). There is a large and fruitful field here for characterizing these various immature viral forms with respect to morphology and chemical and antigenic composition. It should be possible to separate them into classes by means of density gradient centrifugation; they can of course be identified by their content of labeled viral DNA. A similar analysis is now being undertaken with labeled amino acids to determine whether there are any immature viral forms lacking the viral genome (Joklik, *unpublished data*).

Effect of Poxvirus Infection on Host Cell Functions

Although various effects of infection on the host cell have already been mentioned in passing, it may be useful to summarize them here. (i) Infection interrupts the cell division cycle (19, 100, 103). There is no evidence that an infected ceil can undergo cell division unless infection occurs immediately before metaphase. (ii) Infection causes CPE which vary with the poxvirus strain used. These CPE take the form of progressive rounding of cells in the period from 2 to 6 hr after infection, followed by cell fusion or giant-cell formation (4, 96). Inhibitors of virus multiplication like azide or IBT (see below) limit CPE to the cell-rounding stage. Virus irradiated with ultraviolet light produces only rounding of cells but not fusion when rabbitpox virus infects ERK cells (probably a strain of HeLa cells) (4), but is said to produce giant-cell formation (i.e., cell fusion) when vaccinia strain IHD infects L cells (97). Even heat-inactivated IHD virus is capable of killing cells at relatively low multiplicity (64). The simplest explanation of all these results is that, although the later cytopathic changes are probably caused by the metabolic lesions induced by the replicating virus, the initial changes are caused by the introduction into the cell of viral protein. (iii) Infection quickly inhibits cellular DNA replication (90, 100, 103). This is also caused by ultraviolet-inactivated virus, even in the presence of puromycin (90) and is thus not caused by a protein coded from the viral genome after infection. However, host cell DNA is not degraded in virus-infected cells (104). (iv) Infection does not cause any immediate inhibition in the *overall* rate of RNA synthesis, which reflects mainly the rate of ribosomal RNA synthesis. However, by 2 to 3 hr after infection the transport of cellular mRNA to the cytoplasm is greatly inhibited, since during that interval the only newly formed mRNA found on polyribosomes is viral mRNA (13, 162). After 3 hr, the rate of overall cellular RNA synthesis decreases, though its pattern remains essentially normal for up to 6 hr (162). (v) Protein synthesis is inhibited soon after infection (170). This process occurs in the presence of actinomycin D, and must therefore be caused by the viral protein which is introduced into the cell. The best way to assess the effect of infection on host cell protein synthesis is to follow the synthesis of specific proteins. The synthesis of uridine kinase is inhibited in cells infected with vaccinia virus (107), and TK$^-$ mutants of vaccinia strain IHD turn off the synthesis of cellular thymidine kinase (30).

SPECIALIZED ASPECTS

Genetic Recombination

A considerable amount of genetic work has been carried out with poxviruses. Excellent markers are available, as first pointed out by Fenner (38), who characterized a large number of poxvirus strains of the vaccinia subgroup. Among the markers are: characteristics of the pocks formed on the chorioallantoic membrane of the developing chick embryo (appearance, color, size, virus content); plaqueing characteristics on different host cells (among the ones tested are HeLa, L, KB, FL, pig kidney, rabbit kidney, and chick embryo cells); heat stability; production of hemagglutinin; and virulence for mouse and rabbit. Ceiling growth temperature (14, 31) could no doubt also be used.

The more closely related poxvirus strains are, the greater the efficiency with which they recombine. Thus, if cells are mixedly infected with two members of the vaccinia subgroup, such as vaccinia strain Lederle 7N and rabbitpox strain Utrecht (RP u$^+$), which differ in a large number of the above mentioned markers, then between one-third and one-half of the progeny are recombinants, with all combinations of markers represented (39). When vaccinia and ectromelia strains are the parents, recombinants are rare and selective procedures must be used to isolate them (185). Recombination between parents of the vaccinia and myxoma-fibroma subgroups is practically nonexistent. However, if both parents are members of the myxoma-fibroma subgroup, recombinants can be found (185).

A knowledge of the biological characteristics of the parents enables one to apply selective pressures for the isolation of virus strains with desired markers, i.e., enables one to construct poxvirus virus strains, just as it is possible to construct bacterial or phage strains. Similarly, such methods can be applied for obtaining virus yields consisting almost entirely of recombinants. An ingenious example of this is the following. A cross is carried out at such a temperature that one of the parents cannot multiply, though its genome will replicate. The other parent is heat-inactivated. Consequently, under conditions of the cross, neither parent separately can multiply. However, the "heat-tethered" virus enables the heat-inactivated virus to multiply (see below, *Poxvirus Reactivation*), and recombination between the two genomes occurs. The heat-tethered parent is not represented at all among the yield in the form of the parental genome, and the fact that the recombinants multiply reduces the yield of the pure genome of the heat-inactivated parent

by interference; as a result, about 90% of the yield are recombinants. This cross has been carried out between smallpox or alastrim viruses as the heat-tethered parents and rabbitpox or cowpox as the heat-inactivated parents (31).

The ease with which poxviruses recombine has led to speculations regarding the origin of poxvirus strains. In particular, the possibility exists that the "vaccinia" viruses arose as recombinants between smallpox (the highly pathogenic human virus) and cowpox virus (the highly pathogenic bovine virus).

The most interesting genetic work with poxviruses has been done with a series of mutants of rabbitpox virus. While wild-type rabbitpox virus (RP u^+) forms red pocks on the chorioallantoic membrane of the developing chick embryo, about 1% of the pocks are white; these are the u mutants. A number of these mutants have been isolated and characterized with regard to heat stability, hemagglutinin production, and plaque morphology on a variety of host cells (55). On mixed infection, many pairs of these mutants yield wild-type virus among the progeny. Early hopes of measuring recombination frequencies and map distances have not been realized, as no sufficiently quantitative system is yet available. However, by carrying out pairwise infection and scoring simply for absence or presence of wild-type virus among the yield, it proved possible to arrange a number of the u mutants in a linear order (54, 55). Pairs of mutants yielding no detectable wild-type recombinants were assumed to be identical; however, in at least one case a pair of mutants not doing so could be distinguished by plaque morphology (54).

More detailed genetic analysis is no doubt possible. However, the system presents formidable technical difficulties. It seems remarkable that no recombination yielding wild-type virus has been found to occur among the various u mutants of cowpox virus which otherwise appear to be analogous to those of rabbitpox (42). It may be anticipated that the main use made of genetic recombination among poxviruses will be the construction of virus strains to be used for biochemical studies of the processes involved in poxvirus multiplication.

Rescue Phenomena

The availability of easily scored markers has made possible studies on certain interactions between poxviruses which may be termed "rescue phenomena." There has been a tendency to interpret these phenomena in terms of mechanisms, in particular breakage and reunion between viral genomes, elucidated in the phage field. It would seem, however, that the evidence is as yet too scanty to permit such evaluation. Four specific phenomena will be discussed here.

Rescue of ultraviolet-irradiated virus. Abel (1) and Galasso and Sharp (50) observed changes in the slope of ultraviolet-inactivation curves which suggested multiplicity reactivation; in effect, they observed that at low survival levels the apparent number of survivors increased disproportionately with increasing inoculum size. It is doubtful, however, whether this effect is to be explained by interaction between inactivated viral genomes. Abel observed this phenomenon only in chick embryo fibroblast cells, not in KB cells, an effect attributed to the much larger size of the latter and a consequently smaller chance of the genomes being able to interact. However, the corresponding phenomenon described by Galasso and Sharp did occur in L cells, which are as large as KB cells. Furthermore, topography plainly plays no role in the interaction between the genomes of RP u6/2 and other RP u mutants (see below). An additional difficulty is the fact that ultraviolet-irradiated virus is uncoated poorly, if at all (89, 95); in fact, inactivation of poxviruses by ultraviolet-irradiation can be accounted for by damage to the uncoating mechanism rather than to the genome. It is therefore very unlikely that two or more uncoated genomes would have been able to interact under the conditions used. A more likely explanation of this rescue phenomenon is that the efficiency of uncoating of ultraviolet-irradiated virus particles increases with multiplicity by some additive if not cooperative effect, and that in the studies of Abel (1) and Galasso and Sharp (50) uncoating did occur in a very small proportion of cells receiving a high multiplicity. The effect of particle aggregation on the survival of ultraviolet-irradiated vaccinia virus has been investigated and discussed in terms of multiplicity reactivation (52*a*), but the effects observed are also explicable in terms of the hypothesis advanced here.

Marker rescue. Abel (1) compared the sensitivity to ultraviolet-irradiation of infectivity and of the u^+ marker (by scoring on cells infected with an RP u mutant). The sensitivity of the u^+ marker was much less, an effect attributed to marker rescue mediated by interaction of the genomes. However, it is also possible that uncoating of ultraviolet-irradiated RP u^+ was more efficient in cells infected with an RP u mutant than in uninfected cells.

Rescue of virus temporarily prevented from multiplying. Easterbrook (33) studied the early stages of vaccinia virus infection in the presence of azide. Under the conditions used neither un-

coating nor early mRNA formation was inhibited (since some viral antigens were formed), but viral DNA failed to replicate. Virus prevented from multiplying for 24 hr failed to multiply if the inhibitor was then removed, unless the cells were superinfected with another virus of the vaccinia subgroup; superinfection with myxoma virus failed to "rescue." The mechanism of this rescue phenomenon is not known. As pointed out by Easterbrook (33), rescue is very efficient, and it is possible that the entire genome of the temporarily blocked virus remains intact throughout and capable of replication once the rescuing virus has performed some essential function.

Rescue of conditional lethal mutants in nonpermissive hosts. McClain and Greenland (124) studied the interaction of RP u6/2 and RP u1 in pig kidney cells, which are nonpermissive for the former. The yields from mixedly infected cells contained a high proportion of RP u^+ (over 50%), demonstrating efficient genetic recombination, as well as a 1,000-fold larger number of virus particles with the U6/2 phenotype than was found in cells infected with RP u6/2 alone, demonstrating efficient rescue of the U6/2 marker. However, rescue was also achieved with rabbitpox strains which did not recombine with RP u6/2, indicating that rescue proceeded by a nongenetic mechanism. The rescue of vaccinia strain Dairen I by vaccinia strain IHD in L cells, which are nonpermissive for the former, may be another example of this phenomenon (139).

It seems clear that further investigation of all these four phenomena, along both biological and biochemical lines, would be profitable.

Poxvirus Reactivation

Although the basic mechanism of each of the four phenomena just discussed may involve nongenetic reactivation rather than direct interaction between genomes by means of breakage-reunion, it seems best to reserve the term "poxvirus reactivation" for the particular phenomenon to which it was first applied, namely, the ability of infectious poxvirus particles to carry out certain intracellular functions as a result of which heat-inactivated poxvirus particles can multiply (40, 41, 61, 62, 79, 80, 101). The only viruses capable of reactivating heat-inactivated poxviruses are poxviruses (41).

Poxvirus reactivation was observed first almost 30 years ago. In 1936 Berry and Dedrick (15) noted that rabbits inoculated with a mixture of heat-inactivated highly virulent myxoma virus and infectious benign fibroma virus died, and that active myxoma virus could be isolated from these animals. The explanation of this "myxoma-fibroma transformation" was thought to be the transformation of fibroma virus into myxoma virus by a heat-stable principle in the heated myxoma virus preparation, presumably myxoma virus DNA. The fact that, in the model system with two strains of the vaccinia subgroup which differed in six genetic characters, the yield contained virus indistinguishable from the inactivated parent made this transformation theory extremely unlikely, as it would have required the "transformation" of one DNA molecule into another in all these markers (40). A more likely explanation of the reactivation is that heating damages an essential protein which may be supplied by reactivating virus particles. The genome of the reactivating virus would thus play no role in the reactivation process; it would merely be necessary that the reactivating parent should contain in undenatured form the protein inactivated by heat in the other parent. This proposition was tested directly by using as the reactivating parents virus particles the genome of which was inactivated, but the protein moiety of which was intact (81). The first attempts utilized ultraviolet-inactivated virus, but most were unsuccessful. This was also found by Hanafusa (63). The explanation became evident later when it was found that ultraviolet irradiation abolishes the capacity to initiate synthesis of the uncoating protein (89). However, RP u^+ inactivated with nitrogen mustard, which alone was noninfectious was able to reactivate RP u1 or RP u8 inactivated by heat (81). The U^+ marker did not appear among the progeny; the ability to reactivate heated virus was far more resistant to nitrogen mustard than the U^+ marker, which itself was more resistant than infectivity. Reactivation thus involves no genetic interaction between the two genomes, but is due to the introduction into the cell of viral protein, the function of which is the induction of the synthesis of uncoating protein.

The properties of heat-inactivated reactivable virus have been examined in some detail (80). Inactivation of the heat-labile essential protein, the viral inducer protein, by a number of reagents has also been studied (89).

In addition to this type of reactivation, Kitamura (109) described reactivation between virus inactivated by Nagarse and virus inactivated by ultraviolet irradiation. This reactivation probably depends on aggregation outside the cell and on the uptake of large clumps; sonic treatment of the mixture of the two virus preparations, which had to be incubated at 37 C for 60 min, prevented reactivation. It cannot be explained in terms of the preceding, as Nagarse presumably inactivates

the essential viral inducer protein, as does ultraviolet irradiation (89).

Interference with Poxvirus Multiplication by Poxvirus Inactivated by Heat and Ultraviolet Irradiation

The only type of interference with poxvirus multiplication which will be discussed here is that caused by heat-inactivated and ultraviolet-inactivated poxvirus particles.

Heat-inactivated virus. At a multiplicity of 10 particles per cell, vaccinia virus strain WR inactivated by heating at 56 C for 45 min markedly inhibits cellular growth (49). At a somewhat higher multiplicity, vaccinia virus strain IHD markedly inhibits cellular DNA synthesis within 6 to 8 hr, and causes cell shrinkage and death within 24 hr (64). As few as two heat-inactivated particles of WR virus per cell cause detectable interference with the multiplication of homologous virus (51). The higher the ratio of the number of inactivated to active particles, and the longer the time interval by which infection with inactivated virus precedes infectious virus, the greater the degree of inhibition: 100 heat-inactivated particles per cell added 21 hr before challenge inhibit multiplication by 95% (46). Interference with the multiplication of homologous and closely related virus strains was also observed by Joklik et al. (79); however, Hanafusa found that heat-inactivated vaccinia strain IHD did not interfere with the multiplication of ectromelia virus added 2 hr later (66).

As would be anticipated, the inactivation process must be carefully controlled; heating at 60 C readily inactivates the ability to interfere. The ability to combine with antibody is more resistant. The velocity constant for inactivation of infectivity is 45 times larger at 56 C than that for inactivation of interfering capacity; at 37 C they are equal (52).

Ultraviolet-inactivated virus. At multiplicities of fewer than 10 particles per cell ultraviolet-inactivated vaccinia virus does not inhibit cellular growth (50); but at multiplicities of 100 or more it causes CPE similar to the early CPE exhibited by active virus (4), and completely inhibits cellular DNA synthesis within 3 hr (50, 66, 90). This inhibition occurs even in the presence of puromycin, and is thus due to the introduction into the cell of viral protein (90). Ultraviolet-inactivated virus also interferes with virus multiplication; again, this effect manifests itself only at multiplicities of 100 or more (50).

Nothing is known concerning the molecular basis of the interference caused by heat- or ultraviolet-inactivated virus. However, the elucidation of the mechanisms by which as few as 10 virus particles can strongly inhibit cellular growth and virus multiplication is likely to be of great importance to our understanding of the normal cell and the viral growth cycle.

Expression of Viral Functions Under Conditions Preventing Viral DNA Replication

In the normal viral multiplication cycle, there is an ordered progression of events culminating in assembly of mature progeny. Some of these events occur before viral DNA has replicated, such as the induction of the synthesis of the early enzymes, which occurs at least 30 min before the first parental genome replicates; others occur after DNA replication has ceased. In attempting to gain an insight into the control mechanisms which regulate this progression of events, one of the first questions one might ask is whether all viral functions can express themselves if replication of DNA is prevented. Therefore, it seems worthwhile to assemble here all the relevant data.

It is difficult to ensure complete inhibition of DNA replication. Both 5-fluorodeoxyuridine and aminopterin have been used for this purpose, and it will be assumed that complete suppression of DNA synthesis can be achieved although it is recognized that this may not be so. In fact, the results of Easterbrook (36), which conflict with what follows, may have been due to incomplete inhibition of DNA replication. Easterbrook found a sixfold increase in the amount of virus associated with cells between 11 and 24 hr after infection, although DNA replication was seemingly completely inhibited. He postulated that no replication of the viral genome occurred, but that all viral functions were expressed, that all viral antigens necessary for the assembly of mature virions were produced, and that efficient recoating of the parental DNA molecules occurred. A more likely explanation is that DNA synthesis was not completely arrested and that progeny genomes did in fact arise within the cell.

Synthesis of protein identifiable as viral by immunological means commences at 2 hr after infection (169). When viral DNA replication is inhibited with 5-fluorodeoxyuridine, viral protein synthesis begins normally and then continues, at first rapidly (from 4 to 6 hr), and then more slowly, until by 14 hr about one-third of the amount of viral protein normally found at that time has been synthesized in the presence of 5-fluorodeoxyuridine (169). When the inhibition is relieved, the protein which was formed in the presence of 5-fluorodeoxyuridine is not utilized for the formation of progeny virions; *de novo* synthesis of protein is required for this purpose

(161). Even in the presence of 5-fluorodeoxyuridine, the only protein synthesized from 4 hr on is viral protein (169).

It would be interesting to know what sort of proteins are coded by the parental genomes between 2 and 14 hr. The technique of immunodiffusion should be useful here, but has not yet been applied. Appleyard and Westwood (5) found that about the same amount of antigen is formed in the presence as in the absence of 5-bromodeoxyuridine, and concluded that only the parental strands code for protein. However, this seems very unlikely. Vaccinia virus DNA replicates in the presence of 5-bromodeoxyuridine, and there is no reason for believing that mRMA cannot be transcribed from DNA molecules containing 5-bromodeoxyuridine. No analysis was made of the nature of the antigens formed in the presence of 5-bromodeoxyuridine.

Finally, there is the switchoff phenomenon which has already been discussed above (see *Early enzymes*). In brief, this phenomenon involves the prevention of the synthesis of the early virus-induced enzymes, which are known to be coded by long-lived mRNA molecules transcribed from the parental genomes (122, 94), at about 4 hr after infection (121). The essential point here is that switchoff does *not* occur when viral DNA synthesis is prevented (121, 122, 94): enzyme synthesis is then "uncontrolled." Mechanisms to account for switchoff have been considered above; the most plausible theory, but by no means the only possible one, is that there is coded from progeny genomes a protein which cannot be coded from parental genomes, and the function of which is to prevent the mRNA coding for the early enzymes from functioning.

In summary, it seems important to determine whether there are proteins which cannot be transcribed from parental genomes. One possible approach is to determine the nature of the structural viral proteins coded from parental DNA; another is to investigate further the switchoff phenomenon.

Mechanism of Action of Certain Antiviral Compounds

Only two classes of compounds will be discussed here: halogen-substituted pyrimidines and IBT.

5-Bromodeoxyuridine (BUDR) and 5-iododeoxyuridine (IUDR). The progress of infection in the presence of 5-fluorodeoxyuridine has already been discussed in detail. Here we are concerned with the action of BUDR and IUDR. Both compounds are sometimes used in place of 5-fluorodeoxyuridine to determine whether a virus contains DNA or RNA; they are used both in the Salzman-type experiment (160) and in simple experiments comparing the yield in the presence and absence of the compound. It must be recognized, however, that the mechanisms of action of 5-fluorodeoxyuridine on the one hand and of BUDR and IUDR on the other are quite different. 5-Fluorodeoxyuridine in its phosphorylated form is a very powerful inhibitor of thymidylate synthetase; no thymidylate synthetase resistant to the inhibitor has yet been found. In its clear-cut mechanism of action, 5-fluorodeoxyuridine approaches the ideal of a perfect metabolic inhibitor. The position is quite different with BUDR and IUDR. Not only do both of these compounds in their phosphorylated form inhibit to varying degrees the enzymes forming thymidine triphosphate, but they are also themselves incorporated into DNA and do not inhibit DNA replication. There is no way of predicting whether the resultant DNA will be a good template or whether the information, distorted to a greater or lesser degree, will, on transcription and translation, yield functional protein. It should be noted, for instance, that T2 containing DNA completely substituted with BUDR has about 10% of normal specific infectivity. When one further takes into account the fact that whether BUDR and IUDR will act at all depends on the presence of appreciable amounts of the enzyme phosphorylating them, namely thymidine kinase, the action of which is influenced very greatly by allostery, it is clear that it is impossible to foresee what effect BUDR or IUDR will have, and very difficult to explain the effects that they do have. 5-Fluorodeoxyuridine also has to be phosphorylated, but 5-fluorodeoxyuridine monophosphate is so active that much less enzyme is needed to furnish sufficient amounts of inhibitor than when BUDR or IUDR is used.

Easterbrook and Davern (35) found that the presence of BUDR between 2 and 6 hr after infection resulted in an almost total abolition of the infectivity of vaccinia virus progeny. Viral DNA replication did proceed; under the conditions used, there was 13% replacement of thymine by 5-bromouracil as calculated from its density in CsCl. The yield per cell was about 1,000 particles, only one in 5,000 of which, however, was infectious; this corresponded to a 200-fold reduction of infectivity. BUDR-containing virus cannot be reactivated and does not reactivate; its morphology is atypical. It is uncoated prematurely, immediately on being taken up by the cell, and its DNA, which appears to be fragmented (35),

is broken down as soon as it is uncoated (89). Vaccinia strain V-MH appears to be more affected by the presence of BUDR during the infection cycle than RP or vaccinia strain IHD, since the same amount of BUDR allowed the yield of more infectious virus of the latter two than of the former (35). Elucidation of the reason for this would be instructive; possibly there is a difference in the DNA polymerases coded for by these strains.

The formation of viral antigens in the presence of BUDR (5) has already been commented on. In the light of the preceding, it can certainly not be concluded that in the presence of BUDR all antigens are coded from the parental strands.

Kit and his co-workers have utilized an ingenious technique for the isolation of poxvirus strains resistant to BUDR (30). This technique involves in the use of host cells devoid of thymidine kinase (TK^- cells) (106); the BUDR-resistant virus strains are those that are themselves unable to induce the formation of thymidine kinase, so that BUDR can therefore not be phosphorylated and is thus without effect on virus growth. Strains resistant to BUDR are also resistant to IUDR.

IUDR can also be incorporated into vaccinia DNA (154); under conditions when there was 18% replacement of thymine by 5-iodouracil, the virus yield was decreased by about 90%. The emergence of poxvirus strains resistant to IUDR by repeated passage in the presence of increasing concentrations of the compound has been reported (43). It is possible that the cells used (chick embryo fibroblasts) contain only a low level of thymidine kinase so that TK^- strains were selected. More work is necessary before it can be decided whether all IUDR-resistant strains really are TK^- strains or whether there is some other reason for their resistance. Virus strains resistant to IUDR are said to be less pathogenic for rabbits.

IBT. IBT inhibits the formation of mature vaccinia virus progeny (178). Derivatives of IBT are also active, and the nature of the chemical modification determines the antiviral spectrum, which for certain compounds includes not only poxviruses but even some enteroviruses (11, 140). One of the derivatives, *N*-methyl-IBT, is currently being used in clinical smallpox control trials in India (12).

On the basis of the observation that some viral DNA and structural protein are synthesized in the presence of IBT, the suggestion has been made that the compound interferes with the maturation of viral progeny (10, 34). There is no evidence that it acts as an amino acid or nucleic acid base analogue or that it is incorporated by covalent linkage into either protein or nucleic acid. Since the elucidation of the mode of antiviral action at the molecular level of a relatively small molecule could well have far-reaching implications for antiviral chemotherapy, a search has been initiated for the primary locus of action at which IBT inhibits the formation of mature progeny of vaccinia virus (187).

Both uncoating and viral DNA replication proceed normally in the presence of IBT. mRNA synthesis is not affected, and all the proteins known to be formed during the first 3 hr of the infection cycle, such as early enzymes (Jungwirth and Joklik, *unpublished data*; 115) and early viral antigens (9), are synthesized in the presence of IBT. However, no further viral antigens appear after this time (9), progeny viral DNA is not coated (187), and only immature viral forms are found within cells (34). However, there is no accumulation of immature viral forms, merely a marked reduction in the number of virions (187).

These results suggest interference with the synthesis of structural viral proteins. Protein synthesis is indeed inhibited abruptly at 3 hr after infection in the presence of IBT, and polyribosomes disappear at that time (187). Thus, although normal amounts of mRNA are still synthesized after the first 3 hr, these molecules do not form polyribosomes. Not only does this situation hold when IBT is present from the time of infection, but, if IBT is added to normally infected cells at 4 hr after infection, the polyribosomes then present are disrupted within less than 1 hr if sufficiently high concentrations of IBT are used. Protein synthesis is not necessary for IBT to exert this effect (Woodson and Joklik, *unpublished data*). On the other hand, high concentrations of IBT fail to disrupt polyribosomes in either uninfected cells or during the first 3 hr of the infection cycle.

Further analysis has shown that the nascent viral mRNA synthesized after 3 hr in the presence of IBT is normal in size and that it *is* incorporated into polyribosome structure, but that within 3 to 5 min ribosomes become detached from it and its sedimentation coefficient decreases from about 16*S* to about 8*S*. It is not yet known which of these two events is the primary one. However, the net result is that in the presence of IBT the effective half-life of vaccinia mRNA from 3 hr on is decreased from not less than 20 to 30 min to about 3 to 5 min (187).

The final elucidation of the manner in which IBT acts must account for the fact that it suddenly begins to act at 3 hr after infection. It could well be significant that this is the time when DNA replication proceeds at the maximal rate. On the one hand, one could suppose that IBT in some

way modifies the process of DNA replication so that the progeny DNA is a defective template and therefore codes for mRNA molecules which are inherently unstable. No molecular basis for such modifications of either DNA or mRNA structure exists. On the other hand, one could suppose that the mRNA synthesized is normal, but that IBT causes scission of the mRNA, which then results in dissociation of ribosomes, or that IBT promotes dissociation of ribosomes from the mRNA after which its breakdown is automatic; there is some evidence (Woodson and Joklik, *unpublished data*) that mRNA is broken more rapidly in the presence of puromycin, when it is less loaded with ribosomes, than in its absence.

It is interesting to speculate that the time at which IBT becomes effective is close to the time when switchoff of the synthesis of early enzymes occurs. If it is true, as discussed earlier, that the reason for switchoff is selective destruction of certain mRNA molecules, then IBT might abolish the selectivity of the mechanism involved and so cause all mRNA to be degraded. However that may be, the overall result of the presence of IBT is that any proteins programmed to be synthesized *after* the first 3 hr are not synthesized and since, as discussed above, many structural viral proteins are programmed to be synthesized after this time, no mature virions can be formed.

Conditional Lethal Mutants

A glance at the table compiled by Fenner (38) on the genetic markers of a number of virus strains of the vaccinia subgroup shows how diverse are the strains commonly classed as "vaccinia." It would be expected that these various virus strains would differ in the degree to which the various biochemical reactions which have been discussed above proceed. They might be expected to differ in the degree to which they inhibit host cell macromolecular synthesis, in the time course of DNA replication, in the extent to which synthesis of early enzyme is induced, etc. Investigation along these lines should throw much light on the infectious cycle. (It also follows from these considerations that the strain of vaccinia virus used in any investigation should be described as completely as possible; the practice of merely referring to "vaccinia virus" is to be deplored.)

By the same token, a biochemical investigation of various mutants of rabbitpox and cowpox could be expected to prove instructive. The u sets of mutants are already available (38), and sets of temperature-sensitive and host range mutants will most probably be isolated soon. Investigation of one of the u mutants of RP has already commenced (163). RP u6/2, a derivative of RP u6, is unable to grow on a number of cell lines, in particular HeLa, L, and pig kidney cells, although it grows well on chick embryo fibroblasts (CEF; 124). It is thus a conditional lethal mutant, and the problem is to decide why this virus mutant cannot carry out certain essential reactions in HeLa and L cells when these reactions evidently proceed perfectly well in CEF. RP u6/2 is uncoated equally well in CEF, HeLa, L, and pig kidney cells, acts as the reactivating virus for heat-inactivated viruses, arrests host cell DNA replication, and causes induction and switchoff of the synthesis of thymidine kinase in all four cell types (163). However, in pig kidney cells viral DNA does not replicate, though it does so in the other three; and, whereas 10 to 11 antigens as well as mature progeny are produced in CEF, only 5 to 6 antigens and no progeny appear in HeLa cells, only 3 to 4 antigens and no progeny in L cells, and neither antigens nor progeny in pig kidney cells (163). Further elucidation of the pattern of macromolecular biosynthesis directed by RP u6/2 in these four cells, as well as by other conditional lethal mutants, would no doubt be most instructive.

In Vitro Uncoating and Infectious Poxvirus DNA

Abel (2) recently reported that certain viruses of the vaccinia subgroup can be uncoated in vitro to yield DNA that can replicate and cause the formation of mature viral progeny in both CEF and transformable *B. subtilis* cells. In essence, her experiments are as follows. When an extract of cells infected for from 2 to 8 hr with a poxvirus, e.g., vaccinia Lederle 7N, is added to a preparation of RP u^+ which has been completely inactivated by heat, then there occurs a time-dependent reactivation reaction, as a result of which infectivity is partially restored. The infective units are sensitive to deoxyribonuclease and band at 1.72 in CsCl. The efficiency of reactivation is of the order of 10^{-6}. Abel found that not only heat-inactivated but also intact virions are uncoated.

These results have been interpreted as signifying that the extract of infected cells contains factors, among them presumably the uncoating enzyme discussed earlier, which are capable of uncoating the heat-inactivated virus particles through *both* stages of uncoating and liberating viral DNA molecules which are infectious, a remarkable property for molecules over 80 μ long. The DNA molecules are presumably *free* from any adherent protein since their density is 1.72; this value, which was only crudely determined, has led to the most unlikely suggestion that the infectious DNA is single-stranded.

Such in vitro uncoating cannot be demonstrated by chemical means; DNA of highly purified labeled virus does not become accessible to deoxyribonuclease under these conditions (Joklik, *unpublished data*). Obviously, both the first stage of uncoating, which in vivo occurs within phagocytic vesicles, as well as the second stage, would have to be accomplished. The efficiency with which the reactivation occurs in Abel's experiments is not particularly low when it is considered that the specific infectivity of infectious DNA molecules may be quite low as is that of the much smaller infectious RNA molecules. Still, the fact that in vitro uncoating cannot be detected by biochemical means suggests that it may not be virions which are uncoated by Abel's enzyme preparation, but immature subvirion forms present in her unpurified viral preparations. Such subvirion forms would not have to undergo the first stage of uncoating; indeed, it is hard to see how this first stage could occur in vitro. The suggestion that such immature forms only are uncoated in Abel's experiments would also account for the fact that attempts to repeat her work when using highly purified virus preparations as starting material have failed.

Abel has reported that the DNA which is infectious for CEF may be able to replicate and cause the formation of vaccinia virions in transformable cells of *B. subtilis*. The evidence is that the infectious units which are added to bacterial cells are susceptible to deoxyribonuclease, whereas those emerging are resistant to this enzyme but susceptible to antiviral serum. However, insufficient work has been carried out to establish beyond doubt that the vaccinia virus DNA replicates in *B. subtilis* and that synthesis of viral proteins proceeds.

CONCLUSION

The unique advantage of poxviruses is that they replicate in the cytoplasm. It is therefore possible to observe the replication and functioning of a relatively small piece of genetic material. The aim of this review has been to assess and summarize the groundwork that has been laid and to indicate promising avenues of future research.

LITERATURE CITED

1. ABEL, P. 1962. Multiplicity reactivation and marker rescue with vaccinia virus. Virology 17:511–519.
2. ABEL, P. M. 1963. Reactivation of heated vaccinia virus *in vitro*. Z. Vererbungslehre 94:249–252.
3. ALLISON, A. C., AND D. C. BURKE. 1962. The nucleic acid contents of viruses. J. Gen. Microbiol. 27:181–194.
4. APPLEYARD, G., J. C. N. WESTWOOD, AND H. T. ZWARTOUW. 1962. The toxic effect of rabbitpox virus in tissue culture. Virology 18:159–169.
5. APPLEYARD, G., AND J. C. N. WESTWOOD. 1964. The growth of rabbitpox virus in tissue culture. J. Gen. Microbiol. 37:391–401.
6. APPLEYARD, G., H. T. ZWARTOUW, AND J. C. N. WESTWOOD. 1964. A protective antigen from the poxviruses. I. Reaction with neutralizing antibody. Brit. J. Exptl. Pathol. 45:150–161.
7. APPLEYARD, G., AND J. C. N. WESTWOOD. 1964. A protective antigen from the poxviruses. II. Immunization of animals. Brit. J. Exptl. Pathol. 45:162–173.
8. APPLEYARD, G., AND H. T. ZWARTOUW. 1965. The effect of p-fluorophenylalanine on the replication of rabbitpox virus and its nucleic acids. J. Gen. Microbiol. 38:429–436.
9. APPLEYARD, G., V. B. M. HUME, AND J. C. N. WESTWOOD. 1965. The effect of thiosemicarbazones on the growth of rabbitpox virus in tissue culture. Ann. N.Y. Acad. Sci. 130: 92–104.
10. BACH, M. K., AND W. E. MAGEE. 1962. Biochemical effects of isatin-β-thiosemicarbazones on development of vaccinia virus. Proc. Soc. Exptl. Biol. Med. 110:565–567.
11. BAUER, D. J. 1963. The chemotherapy of ectromelia infection with isatin-β-dialkyl thiosemicarbazones. Brit. J. Exptl. Pathol. 44:233–242.
12. BAUER, D. J. 1965. Clinical experience with the antiviral drug marboran® (1-methylisatin 3-thiosemicarbazone). Ann. N.Y. Acad. Sci. 130:110–117.
13. BECKER, Y., AND W. K. JOKLIK. 1964. Messenger RNA in cells infected with vaccinia virus. Proc. Nat. Acad. Sci. U.S. 51:577–585.
14. BEDSON, H. S., AND K. R. DUMBELL. 1961. The effect of temperature on the growth of poxviruses in the chick embryo. J. Hyg. 59:457–469.
15. BERRY, G. T., AND H. M. DEDRICK. 1936. A method for changing the virus of rabbit fibroma (Shope) into that of infectious myxomatosis (Sanarelli). J. Bacteriol. 31:50–51.
16. BLOOM, S., B. GOLDBERG, AND H. GREEN. 1965. The lifetime of messenger RNA for collagen and cell protein synthesis in an established mammalian cell line. Biochem. Biophys. Res. Commun. 19:317–321.
17. BRESNICK, E., Y. B. THOMPSON, H. P. MORRIS, AND A. G. LIEBELT. 1964. Inhibition of thymidine kinase activity in liver and hepatomas by TTP and d-CTP. Biochem. Biophys. Res. Commun. 16:278–284.
18. BÜTTNER, D., H. GIESE, G. MÜLLER, AND D. PETERS. 1964. Die Feinstruktur reifer Elementarkörper des Ecthyma contagiosum und der Stomatitis papulose. Arch. Ges. Virusforsch. 14:657–673.
19. CAIRNS, H. J. F. 1960. The initiation of vaccinia infection. Virology 11:603–623.
20. CHAPPLE, P. J., AND J. C. N. WESTWOOD. 1963.

Electron microscopy of myxoma virus. Nature **199**:199–200.

20a. COGNIAUX-LE CLERK, J. 1965. Inhibitory effect of 8-azaguanine on the synthesis of vaccinia virus deoxyribonucleic acid. Biochim. Biophys. Acta **108**:5–10.

21. COHEN, G. N., AND R. MUNIER. 1959. Effets des analogues structuraux d'amino acides sur la croissance, la synthèse de protéines et la synthèse d'enzymes chez *Escherichia coli*. Biochim. Biophys. Acta **31**:347–356.

22. DALES, S., AND L. SIMINOVITCH. 1961. The development of vaccinia virus in Earle's L strain cells as examined by electron microscopy. J. Biophys. Biochem. Cytol. **10**:475–502.

23. DALES, S. 1962. An electron microscopic study of the early association between two mammalian viruses and their hosts. J. Cell Biol. **13**:303–322.

24. DALES, S. 1962. Attachment and uptake of animal viruses as studied by electron microscopy. Cold Spring Harbor Symp. Quant. Biol. **27**:132–136.

25. DALES, S. 1963. The uptake and development of vaccinia virus in strain L cells followed with labeled viral deoxyribonucleic acid. J. Cell Biol. **18**:51–72.

26. DALES, S., AND R. KAJIOKA. 1964. The cycle of multiplication of vaccinia virus in Earle's strain L cells. I. Uptake and penetration. Virology **24**:278–294.

27. DALES, S. 1965. Penetration of animal viruses into cells. Progr. Med. Virol. **7**:1–43.

28. DAVIDSON, E. H., V. G. ALLFREY, AND A. E. MIRSKY. 1963. Gene expression in differentiated cells. Proc. Natl. Acad. Sci. U.S. **49**: 53–60.

29. DAVISON, P. F. 1959. The effect of hydrodynamic shear on the deoxyribonucleic acid from T2 and T4 bacteriophages. Proc. Natl. Acad. Sci. U.S. **45**:1560–1568.

30. DUBBS, D. R., AND S. KIT. 1964. Isolation and properties of vaccinia mutants deficient in thymidine kinase inducing activity. Virology **22**:214–225.

31. DUMBELL, K. R., AND H. S. BEDSON. 1964. The use of ceiling temperature and reactivation with isolation of pox virus hybrids. J. Hyg. **62**:133–140.

32. EASTERBROOK, K. 1961. The multiplication of vaccinia virus in suspended KB cells. Virology **15**:404–416.

33. EASTERBROOK, K. B. 1961. Analysis of the early stages of vaccinia virus infection in KB cells using sodium azide. Virology **15**:417–427.

34. EASTERBROOK, K. B. 1962. Interference with the maturation of vaccinia virus by isatin-β-thiosemicarbazone. Virology **17**:245–251.

35. EASTERBROOK, K. B., AND C. I. DAVERN. 1963. The effect of 5-bromodeoxyuridine on the multiplication of vaccinia virus. Virology **19**:509–520.

36. EASTERBROOK, K. B. 1963. Conservation of vaccinial DNA during an abortive cycle of multiplication. Virology **21**:508–510.

36a. EASTERBROOK, K. B. 1965. Controlled degradation of vaccinia virions in vitro: an electron microscopy study. J. Ultrastruct. Res., *in press*.

37. FENNER, F., AND F. M. BURNET. 1957. A short description of the poxvirus group (vaccinia and related viruses). Virology **4**:305–314.

38. FENNER, F. 1958. The biological characteristics of several strains of vaccinia, cowpox and rabbitpox viruses. Virology **5**:502–529.

39. FENNER, F. 1959. Genetic studies with mammalian poxviruses. II. Recombination between two strains of vaccinia virus in single HeLa cells. Virology **8**:499–507.

40. FENNER, F., I. H. HOLMES, W. K. JOKLIK, AND G. M. WOODROOFE. 1959. Reactivation of heat-inactivated poxvirus: a general phenomenon which includes the fibroma-myxoma virus transformation of Berry and Dedrick. Nature **183**:1340–1341.

41. FENNER, F., AND G. M. WOODROOFE. 1960. The reactivation of poxviruses. II. The range of reactivating viruses. Virology **11**:185–201.

42. FENNER, F., AND J. F. SAMBROOK. 1964. The genetics of animal viruses. Ann. Rev. Microbiol. **18**:48–94.

43. FERRARI, W., G. L. GESSA, B. LODDO, AND M. L. SCHIVO. 1965. Decreased pathogenicity for rabbit skin of IDU-resistant vaccinia virus. Virology **26**:154–155.

44. FRIEDMAN-KIEN, A. E., W. P. ROWE, AND W. G. BANFIELD. 1963. Milker's nodules: isolation of a poxvirus from a human case. Science **140**:1335–1336.

45. FUJIO, Y. 1963. Effects of actinomycin on growth and hemagglutinin production of vaccinia virus. Biken J. **6**:197–203.

46. GALASSO, G. J., AND D. G. SHARP. 1961. Inhibitory effect of heated vaccinia virus on growth of vaccinia virus in Earle's L cells. Proc. Soc. Exptl. Biol. Med. **107**:957–960.

47. GALASSO, G. J., AND D. G. SHARP. 1963. Quality and yield of vaccinia virus from L cell cultures. Proc. Soc. Exptl. Biol. Med. **113**:43–47.

48. GALASSO, G. J., AND D. G. SHARP. 1963. The effect of horse serum on the quality of vaccinia virus grown in L cell cultures. J. Immunol. **90**:647–653.

49. GALASSO, G. J., AND D. G. SHARP. 1963. Homologous inhibition with heated and ultraviolet-treated vaccinia virus in cultures of L cells. Virology **20**:1–13.

50. GALASSO, G. J., AND D. G. SHARP. 1963. Homologous inhibition, toxicity, and multiplicity reactivation with ultraviolet-irradiated vaccinia virus. J. Bacteriol. **85**:1309–1314.

51. GALASSO, G. J., AND D. G. SHARP. 1964. Relative plaque-forming, cell-infecting, and interfering qualities of vaccinia virus. J. Bacteriol. **88**:433–439.

52. GALASSO, G. J., AND D. G. SHARP. 1965. Effects of heat on the infecting, antibody-absorbing, and interfering powers of vaccinia virus. J. Bacteriol. **89**:611–616.

52a. GALASSO, G. J., AND D. G. SHARP. 1965. Effect of particle aggregation on the survival of

irradiated vaccinia virus. J. Bacteriol. **90**: 1138–1142.

53. GAUSH, C. R., AND J. S. YOUNGNER. 1963. Studies on the lipids of virus-infected cells. I. Lipid analysis of a soluble hemagglutinin from chorioallantoic membranes infected with vaccinia virus. Virology **19**:573–579.
54. GEMMELL, A., AND H. J. F. CAIRNS. 1959. Linkage in the genome of an animal virus. Virology **8**:381–383.
55. GEMMELL, A., AND F. FENNER. 1959. Genetic studies with mammalian poxviruses. III. White (u) mutants of rabbitpox virus. Virology **11**: 219–235.
56. GEORGIEV, G. P., O. P. SAMARINA, M. I. LERMAN, M. H. SMIRNOV, AND A. H. SEVERTZOV. 1963. Biosynthesis of messenger and ribosomal ribonucleic acids in the nucleolo chromosomal apparatus of animal cells. Nature **200**:1291–1294.

56*a*. GERSTL, F. 1964. Ein Beitrag zur Diagnostik und Morphologie des Erregers des ansteckenden Lippengrindes (Ecthyma contagiosum) bei Gemsen. Zentr. Bakteriol. Parasitenk. Abt. I Orig. **195**:182–189.

57. GIRARD, M., H. LATHAM, S. PENMAN, AND J. E. DARNELL. 1965. Entrance of newly formed messenger RNA and ribosomes into HeLa cell cytoplasm. J. Mol. Biol. **11**:187–201.

57*a*. GRAUSGRUBER, W. 1964. Lippengrind (Ecthyma contagiosum) bei Gemsen. Zentr. Bakteriol. Parasitenk. Abt. I Orig. **195**:175–181.

58. GREEN, M. 1962. Studies on the biosynthesis of viral DNA. Cold Spring Harbor Symp. Quant. Biol. **27**:219–233.
59. GREEN, M., M. PINA, AND V. CHAGOYA. 1964. Biochemical studies on adenovirus multiplication. V. Enzymes of deoxyribonucleic acid synthesis in cells infected by adenovirus and vaccinia virus. J. Biol. Chem. **239**:1188–1197.
60. HALL, B. D., AND P. DOTY. 1959. The preparation and physical chemical properties of ribonucleic acid from microsomal particles. J. Mol. Biol. **1**:111–126.
61. HANAFUSA, T., H. HANAFUSA, AND J. KAMAHORA. 1959. Transformation phenomena in the pox group of viruses. II. Transformation between several members of pox group. Biken's J. **2**:85–91.
62. HANAFUSA, T., H. HANAFUSA, AND J. KAMAHORA. 1959. Transformation of ectromelia into vaccinia virus in tissue culture. Virology **8**:525–527.
63. HANAFUSA, H. 1960. Reactivation phenomena in the pox group of viruses. III. Some properties of heat-inactivated vaccinia virus. Biken's J. **3**:41–56.
64. HANAFUSA, H. 1960. Killing of L-cells by heat- and UV-inactivated vaccinia virus. Biken's J. **3**:191–199.
65. HANAFUSA, T. 1961. Enzymatic synthesis and breakdown of deoxyribonucleic acid by extracts of L cells infected with vaccinia virus. Biken's J. **4**:97–110.
66. HANAFUSA, H. 1962. Factors involved in the initiation of multiplication of vaccinia virus. Cold Spring Harbor Symp. Quant. Biol. **27**:209–216.
67. HAREL, L., J. HAREL, A. BOER, J. IMBENOTTE, AND N. CARPENI. 1964 Persistence d'une synthèse de D-RNA dans le foie de rat traité par l'actinomycine. Biochim. Biophys. Acta **87**:212–218.
68. HARRIS, W. J., AND J. C. N. WESTWOOD. 1964. Phosphotungstate staining of vaccinia virus. J. Gen. Microbiol. **34**:491–495.
69. HERSHEY, A. D., AND E. BURGI. 1960. Molecular homogeneity of the deoxyribonucleic acid of phage T2. J. Mol. Biol. **2**:143–152.
70. HINZE, H. C., AND D. L. WALKER. 1964. Response of cultured rabbit cells to infection with the Shope fibroma virus. I. Proliferation and morphological alteration of infected cells. J. Bacteriol. **88**:1185–1194.
71. HOAGLAND, C. L., J. E. SMADEL, AND T. M. RIVERS. 1940. Constituents of elementary bodies of vaccinia. I. Certain basic analyses and observations on lipid components of the virus. J. Exptl. Med. **71**:737–750.
72. HOAGLAND, C. L., G. I. LAVIN, J. E. SMADEL, AND T. M. RIVERS. 1940. Constituents of elementary bodies of vaccinia. II. Properties of nucleic acid obtained from vaccinia virus. J. Exptl. Med. **72**:139–147.
73. HOAGLAND, C. L., S. M. WARD, J. E. SMADEL, AND T. M. RIVERS. 1941. Constituents of elementary bodies of vaccinia. IV. Demonstration of copper in the purified virus. J. Exptl. Med. **74**:69–80.
74. HOAGLAND, C. L., J. E. SMADEL, S. M. WARD, AND T. M. RIVERS. 1941. Constituents of elementary bodies of vaccinia. V. A flavin associated with the purified virus. J. Exptl. Med. **74**:133–144.
75. HOAGLAND, C. L., S. M. WARD, J. E. SMADEL, AND T. M. RIVERS. 1942. Constituents of the elementary bodies of vaccinia. VI. Studies on the nature of enzymes associated with the purified virus. J. Exptl. Med. **76**:163–173.
76. HYDE, J. M., L. G. GAFFORD, AND C. C. RANDALL. 1965. Fine structure of the coat and nucleoid material of fowlpox virus. J. Bacteriol. **89**:1557–1569.
77. ISRAELI, E., AND L. SACHS. 1964. Cell-virus interaction with the Shope fibroma virus on cultures of rabbit and rat cells. Virology **23**: 473–485.
78. JOKLIK, W. K., AND J. MCN. RODRICK. 1959. Biochemical studies on vaccinia in cultured cells. I. Incorporation of adenine-8-C^{14} into normal and infected cells. Virology **9**:396–416.
79. JOKLIK, W. K., G. M. WOODROOFE, I. H. HOLMES, AND F. FENNER. 1960. The reactivation of poxviruses. I. The demonstration of the phe-

nomenon and technique of assay. Virology 11:168–184.
80. JOKLIK, W. K., I. H. HOLMES, AND M. J. BRIGGS. 1960. The reactivation of poxviruses. III. Properties of reactivable particles. Virology 11:202–218.
81. JOKLIK, W. K., P. ABEL, AND I. H. HOLMES. 1960. Reactivation of poxviruses by a nongenetic mechanism. Nature 186:992–993.
82. JOKLIK, W. K. 1962. The purification of four strains of poxvirus. Virology 18:9–18.
83. JOKLIK, W. K. 1962. The preparation and characteristics of highly purified radioactively labeled poxvirus. Biochim. Biophys. Acta 61:290–301.
84. JOKLIK, W. K. 1962. The multiplication of poxvirus DNA. Cold Spring Harbor Symp. Quant. Biol. 27:199–208.
85. JOKLIK, W. K. 1962. Some properties of poxvirus DNA. J. Mol. Biol. 5:265–274.
86. JOKLIK, W. K. 1962. Reactivation of poxviruses: fate of reactivable virus within the cell. Nature 196:556–558.
87. JOKLIK, W. K. 1964. The intracellular uncoating of poxvirus DNA. I. The fate of radioactively labeled rabbitpox virus. J. Mol. Biol. 8:263–276.
88. JOKLIK, W. K. 1964. The intracellular uncoating of poxvirus DNA. II. The molecular basis of the uncoating process. J. Mol. Biol. 8:277–288.
89. JOKLIK, W. K. 1964. The intracellular fate of rabbitpox virus rendered noninfectious by various reagents. Virology 22:620–633.
90. JOKLIK, W. K., AND Y. BECKER. 1964. The replication and coating of vaccinia DNA. J. Mol. Biol. 10:452–474.
91. JOKLIK, W. K. 1965. The molecular basis of the viral eclipse phase. Progr. Med. Virol. 7:44–96.
92. JOKLIK, W. K., AND Y. BECKER. 1965. Studies on the genesis of polyribosomes. I. The origin and significance of the subribosomal particles. J. Mol. Biol. 13:496–510.
93. JOKLIK, W. K., AND Y. BECKER. 1965. Studies on the genesis of polyribosomes. II. The association of nascent messenger RNA with the 40S subribosomal particles. J. Mol. Biol. 13:511–520.
94. JUNGWIRTH, C., AND W. K. JOKLIK. 1965. Studies on "early" enzymes in HeLa cells infected with vaccinia virus. Virology 27:80–93.
95. KAJIOKA, R., L. SIMINOVITCH, AND S. DALES. 1964. The cycle of multiplication of vaccinia virus in Earle's strain L cells. II. Initiation of DNA synthesis and morphogenesis. Virology 24:295–309.
96. KAKU, H., AND J. KAMAHORA. 1964. Giant cell formation in L cells infected with active vaccinia virus. Biken J. 6:299–315.
97. KAKU, H., AND J. KAMAHORA. 1964. A study on giant cell formation of L cells infected with the UV inactivated vaccinia virus. Biken J. 7:37–40.
98. KATES, M., A. C. ALLISON, D. A. J. TYRELL, AND A. T. JAMES. 1962. Origin of lipids in influenza virus. Cold Spring Harbor Symp. Quant. Biol. 27:293–301.
99. KATO, S., AND J. KAMAHORA. 1962. Significance of the inclusion formation of poxvirus group and herpes simplex virus. Symp. Cell Chem. 12:47–90.
100. KATO, S., M. OGAWA, AND H. MIYAMOTO. 1964. Nucleocytoplasmic interaction in poxvirus-infected cells. I. Relationship between inclusion formation and DNA metabolism of the cells. Biken J. 7:45–56.
101. KILHAM, L. 1960. The fibroma-myxoma transformation. Advan. Virus Res. 7:103–129.
102. KIT, S., D. R. DUBBS, AND L. J. PIEKARSKI. 1962. Enhanced thymidine phosphorylating activity of mouse fibroblasts (strain LM) following vaccinia infection. Biochem. Biophys. Res. Commun. 8:72–75.
103. KIT, S., AND D. R. DUBBS. 1962. Biochemistry of vaccinia-infected mouse fibroblasts (strain L-M). I. Effects on nucleic acid and protein synthesis. Virology 18:274–285.
104. KIT, S., AND D. R. DUBBS. 1962. Biochemistry of vaccinia-infected mouse fibroblasts (strain LM). II. Properties of the chromosomal DNA of infected cells. Virology 18:286–293.
105. KIT, S., D. R. DUBBS, AND L. J. PIEKARSKI. 1963. Inhibitory effects of puromycin and fluorophenylalanine on induction of thymidine kinase by vaccinia infected L-cells. Biochem. Biophys. Res. Commun. 11:176–181.
106. KIT, S., D. R. DUBBS, L. J. PIEKARSKI, AND T. C. HSU. 1963. Deletion of thymidine kinase activity from L cells resistant to bromodeoxyuridine. Exptl. Cell Res. 31:297–312.
107. KIT, S., Y. VALLADORES, AND D. R. DUBBS. 1964. Effects of age of culture and vaccinia infection on uridine kinase activity of L-cells. Exptl. Cell Res. 34:257–265.
108. KIT, S., AND D. R. DUBBS. 1965. Properties of deoxythymidine kinase partially purified from noninfected and virus-infected mouse fibroblast cells. Virology 26:16–27.
109. KITAMURA, T. 1963. Reactivation of protease-inactivated vaccinia virus. Virology 21:286–289.
110. LEVINTHAL, C., A. KEYNAN, AND A. HIGA. 1962. Messenger RNA turnover and protein synthesis in *B. Subtilis* inhibited by actinomycin D. Proc. Nat. Acad. Sci. U.S. 48:1631–1638.
111. LOH, P. C., AND J. L. RIGGS. 1961. Demonstration of the sequential development of vaccinial antigens and virus in infected cells: observations with cytochemical and differential fluorescent procedures. J. Exptl. Med. 114:149–160.
112. LOH, P. C., AND F. E. PAYNE. 1965. Effect of p-fluorophenylalanine on the synthesis of vaccinia virus. Virology 25:560–574.
113. LOH, P. C., AND F. E. PAYNE. 1965. Effect of

5-fluoro-2′-deoxyuridine on the synthesis of vaccinia virus. Virology **25**:575–584.
114. MAGEE, W. E., M. R. SHEEK, AND M. J. BURROUS. 1960. The synthesis of vaccinial deoxyribonucleic acid. Virology **11**:296–299.
115. MAGEE, W. E., AND M. K. BACH. 1965. Biochemical studies on the antiviral activities of the isatin-β-thiosemicarbazones. Ann. N.Y. Acad. Sci. **130**:80–91.
116. MAITLAND, H. B., AND R. POSTLETHWAITE. 1959. Vaccinia virus in HeLa cells. Symp. Soc. Gen. Microbiol. **9**:185–199.
117. MANDELL, J. D., AND A. D. HERSHEY. 1960. A fractionating column for analysis of nucleic acids. Anal. Biochem. **1**:66–77.
118. MARQUARDT, J., S. E. HOLM, AND E. LYCKE. 1964. Preparation of a purified vaccinia virus freed from soluble antigens. Proc. Soc. Exptl. Biol. Med. **116**:112–116.
119. MARQUARDT, J., S. E. HOLM, AND E. LYCKE. 1965. Immunoprecipitating factors of vaccinia virus. Virology **27**:170–178.
120. MCAUSLAN, B. R., AND W. K. JOKLIK. 1962. Stimulation of the thymidine phosphorylating system in HeLa cells on infection with poxvirus. Biochem. Biophys. Res. Commun. **8**:486–491.
121. MCAUSLAN, B. R. 1963. Control of induced thymidine kinase activity in the poxvirus-infected cell. Virology **20**:162–168.
122. MCAUSLAN, B. R. 1963. The induction and repression of thymidine kinase in the poxvirus-infected HeLa cell. Virology **21**:383–389.
123. MCAUSLAN, B. R. 1965. Deoxyribonuclease activity of normal and poxvirus-infected HeLa cells. Biochem. Biophys. Res. Commun. **19**:15–20.
123*a*. MCAUSLAN, B. R., P. HERDE, D. PETT, AND J. ROSS. 1965. Nucleases of virus-infected animal cells. Biochem. Biophys. Res. Commun. **20**:586–591.
124. MCCLAIN, M. E., AND R. M. GREENLAND. 1965. Recombination between rabbitpox virus mutants in permissive and nonpermissive cells. Virology **25**:516–522.
125. MONOD, J., J. WYMAN, AND J. P. CHANGEUX. 1965. On the nature of allosteric transitions: a plausible model. J. Mol. Biol. **12**:88–118.
126. MOSCOVICI, C., E. P. COHEN, J. SANDERS, AND S. S. DELONG. 1963. Isolation of a viral agent from pseudo cowpox disease. Science **141**:915–916.
127. MÜLLER, D., AND D. PETERS. 1963. Substrukturen des Vaccinevirus, dargestellt durch Negativkontrastierung. Arch. Ges. Virusforsch. **13**:435–451.
128. MUNYON, W., AND S. KIT. 1965. Inhibition of thymidine kinase formation in LM(TK⁻) cells simultaneously infected with vaccinia and a thymidine kinaseless vaccinia mutant. Virology **26**:374–377.
129. NAGINGTON, J., AND R. W. HORNE. 1962. Morphological studies of Orf and vaccinia viruses. Virology **16**:248–260.
130. NAGINGTON, J., W. PLOWRIGHT, AND R. W. HORNE. 1962. The morphology of bovine papular stomatitis virus. Virology **17**:361–364.
131. NAGINGTON, J., A. A. NEWTON, AND R. W. HORNE. 1964. The structure of Orf virus. Virology **23**:461–472.
131*a*. NAGINGTON, J., G. H. TEE, AND J. S. SMITH. 1965. Milker's nodule virus infections in Dorset and their similarity to Orf. Nature **208**:505–507.
132. NEFF, B. J., W. W. ACKERMANN, AND R. E. PRESTON. 1965. Studies of vaccinia hemagglutinin obtained from various vaccinia infected tissues. Density gradient centrifugation and electron microscopy. Proc. Soc. Exptl. Biol. Med. **118**:664–671.
133. NISHMI, M., AND R. KELLER. 1962. Observations on the mode of spread of vaccinia virus in HeLa cell monolayer cultures. Virology **16**: 91–92.
134. NISHMI, M., AND R. KELLER. 1962. The microepidemiology of vaccinial infection as studied in HeLa cell stationary cultures. Virology **18**:109–117.
135. NOYES, W. F. 1962. Further studies on the structure of vaccinia virus. Virology **18**:511–516.
136. NOYES, W. F. 1965. Observations on two poxtumor viruses. Virology **25**:666–669.
137. ODA, M. 1963. Vaccinia-virus HeLa interaction. II. Effect of mitomycin C on the production of infective virus, complement-fixing antigen, and hemagglutinin. Virology **21**:533–539.
138. ODA, M. 1964. Plaque-hemadsorption technique for the detection of non-hemagglutinating vaccinia virus. Virology **23**:432–434.
139. ODA, M. 1965. Rescue of dermovaccinia abortive infection by neurovaccinia virus in L cells. Virology **25**:664–666.
140. O'SULLIVAN, D. G., AND P. W. SADLER. 1961. Agents with high activity against Type 2 poliovirus. Nature **192**:341–343.
141. PENMAN, S., K. SCHERRER, Y. BECKER, AND J. E. DARNELL. 1963. Polyribosomes in normal and poliovirus-infected HeLa cells and their relationship to messenger RNA. Proc. Natl. Acad. Sci. U.S. **49**:654–662.
142. PERRY, R. P. 1963. Selective effects of actinomycin D on the intracellular distribution of RNA synthesis in tissue culture cells. Exptl. Cell Res. **29**:400–406.
143. PETERS, D. 1957. Untersuchungen am Vaccine-Virus. Z. Naturforsch. **12b**:697–715.
144. PETERS, D. 1960. Struktur und Entwicklung der Pockenviren. Intern. Kongr. Elektronmikroskopie, 4th, Berlin, 1958, p. 552–573.
145. PETERS, D. 1962. Substrukturen des Vaccine-Virus. Intern. Congr. Electronmicroscopy, 5th, p. 77–78.
146. PETERS, D., AND D. BÜTTNER. 1964. Das Triplett des Vaccinevirus nach Anwendung verschiedener Fixierungsmittel. European Regional Conf. Electronmicroscopy, 3rd, Prague, vol. B, p. 377.
147. PETERS, D., G. MÜLLER, AND D. BÜTTNER. 1964.

The fine structure of paravaccinia viruses. Virology 23:609–611.
148. PFAU, C. J., AND J. F. MCCREA. 1962. Release of deoxyribonucleic acid from vaccinia virus by mercapto-ethanol and pronase. Nature 194: 894–895.
149. PFAU, C. J., AND J. F. MCCREA. 1963. Studies on the deoxyribonucleic acid of vaccinia virus. III. Characterization of DNA isolated by different methods and its relation to virus structure. Virology 21: 425–435.
150. PICKELS, E. G., AND J. E. SMADEL. 1938. Ultracentrifugal studies on the elementary bodies of vaccinia virus. J. Exptl. Med. 68:583–606.
151. PLANTEROSE, D. N., C. NISHIMURA, AND N. P. SALZMAN. 1962. The purification of vaccinia virus from cell cultures. Virology 18:294–301.
152. PLOWRIGHT, W., AND R. D. FERRIS. 1959. Ether sensitivity of some mammalian poxviruses. Virology 7:357–358.
153. PRESCOTT, D. N., AND M. A. BENDER. 1962. Synthesis of RNA and protein during mitosis in mammalian tissue culture cells. Exptl. Cell Res. 26:260–268.
154. PRUSOFF, W. H., Y. S. BAKHLE, AND J. F. MCCREA. 1963. Incorporation of 5-iodo-2′-deoxyuridine into the deoxyribonucleic acid of vaccinia virus. Nature 199:1310–1311.
155. RANDALL, C. C., L. G. GAFFORD, AND R. B. ARHELGER. 1961. Electron microscopic examination of isolated fowlpox inclusions. Virology 14:381–382.
156. RANDALL, C. C., L. G. GAFFORD, R. W. DARLINGTON, AND J. HYDE. 1964. Composition of fowlpox virus and inclusion matrix. J. Bacteriol. 87:939–944.
157. REISNER, A. H., W. R. SOBEY, AND D. CONOLLY. 1963. Differences among the soluble antigens of myxoma viruses originating in Brazil and California. Virology 20:539–541.
158. REVEL, M., AND H. H. HIATT. 1964. The stability of liver messenger RNA. Proc. Natl. Acad. Sci. U.S. 51:810–818.
159. RONDLE, C. J. M., AND K. R. DUMBELL. 1962. Antigens of cowpox virus. J. Hyg. 60:41–49.
160. SALZMAN, N. P. 1960. The rate of formation of vaccinia deoxyribonucleic acid and vaccinia virus. Virology 10:150–152.
161. SALZMAN, N. P., A. J. SHATKIN, AND E. D. SEBRING. 1963. Viral protein and DNA synthesis in vaccinia virus-infected HeLa cell cultures. Virology 19:542–550.
162. SALZMAN, N. P., A. J. SHATKIN, AND E. D. SEBRING. 1964. The synthesis of a DNA-like RNA in the cytoplasm of HeLa cells infected with vaccinia virus. J. Mol. Biol. 8:405–416.
163. SAMBROOK, J. F., M. E. MCCLAIN, K. B. EASTERBROOK, AND B. R. MCAUSLAN. A mutant of rabbitpox virus defective at different stages of its multiplication in three cell types. Virology 26:738–745.
164. SCHAECHTER, M., E. P. PREVIC, AND M. E. GILLESPIE. 1965. Messenger RNA and polyribosomes in *B. megaterium*. J. Mol. Biol. 12:119–129.
165. SCHARFF, M. D., A. J. SHATKIN, AND L. LEVINTOW. 1963. Association of newly formed viral protein with specific polyribosomes. Proc. Natl. Acad. Sci. U.S. 50:686–694.
166. SCHERRER, K., H. LATHAM, AND J. E. DARNELL. 1963. Demonstration of an unstable RNA and of a precursor to ribosomal RNA in HeLa cells. Proc. Natl. Acad. Sci. U.S. 49:240–248.
167. SHARP, D. G., P. SADHUKHAN, AND G. J. GALASSO. 1964. Quality changes in vaccinia virus during adaptation to growth in cultures of Earle's L cells. J. Bacteriol. 88:309–312.
168. SHATKIN, A. J., AND N. P. SALZMAN. 1963. Deoxyribonucleic acid synthesis in vaccinia virus-infected HeLa cells. Virology 19:551–560.
169. SHATKIN, A. J. 1963. The formation of vaccinia virus protein in the presence of 5-fluorodeoxyuridine. Virology 20:292–301.
170. SHATKIN, A. J. 1963. Actinomycin D and vaccinia virus infection in HeLa cells. Nature 199:357–358.
171. SHATKIN, A. J. 1965. Vaccinia virus directed RNA: its fate in the presence of actinomycin. Science 148:87–90.
172. SMADEL, J. E., AND M. J. WALL. 1937. Elementary bodies of vaccinia from infected chorioallantoic membranes of developing chick embryos. J. Exptl. Med. 66:325–335.
173. SMADEL, J. E., T. M. RIVERS, AND E. G. PICKELS. 1939. Estimation of the purity of preparations of elementary bodies. J. Exptl. Med. 70: 379–385.
174. SMADEL, J. E., G. I. LAVIN, AND R. J. DUBOS. 1940. Some constituents of elementary bodies of vaccinia virus. J. Exptl. Med. 71:373–389.
175. SOBEY, W. R., D. CONOLLY, A. H. REISNER, E. BURNETT, AND K. H. ADAMS. 1965. Purification and morphological studies of myxoma virus. Virology (*in press*).
176. STAEHELIN, T., F. O. WETTSTEIN, AND H. NOLL. 1963. Breakdown of rat-liver ergosomes *in vivo* after actinomycin inhibition of messenger RNA synthesis. Science 140:180–183.
177. SZYBALSKI, W., R. L. ERIKSON, G. A. GENTRY, L. G. GAFFORD, AND C. C. RANDALL. 1963. Unusual properties of fowlpox virus DNA. Virology 19:586–589.
178. THOMPSON, R. L., J. DAVIS, P. B. RUSSELL, AND G. H. HITCHINGS. 1953. Effect of aliphatic oxime and isatin thiosemicarbazones on vaccinia infection in the mouse and in the rabbit. Proc. Soc. Exptl. Biol. Med. 84:496–499.
179. VERNA, J. J. 1965. Cell-culture response to fibroma virus. J. Bacteriol. 89:524–528.
180. VILLA-TREVINO, S., E. FARBER, T. STAEHELIN, F. O. WETTSTEIN, AND H. NOLL 1964. Breakdown and reassembly of rat liver ergosomes after administration of ethionine or puromycin. J. Biol. Chem. 239:3826–3833.
181. WAGNER, R. R. 1964. Inhibition of interferon

biosynthesis by actinomycin D. Nature **204:** 49–51.

182. WATSON, J. D., AND J. W. LITTLEFIELD. 1960. Some properties of DNA from papilloma virus. J. Mol. Biol. **2:**161–165.

183. WESTWOOD, J. C. N., W. J. HARRIS, H. T. ZWARTOUW, D. H. J. TITMUSS, AND G. APPLEYARD. 1964. Studies on the structure of vaccinia virus. J. Gen. Microbiol. **34:**67–78.

184. WESTWOOD, J. C. N., H. T. ZWARTOUW, G. APPLEYARD, AND D. H. J. TITMUSS. 1965. Comparison of the soluble antigens and virus particle antigens of vaccinia virus. J. Gen. Microbiol. **38:**47–53.

185. WOODROOFE, G. M., AND F. FENNER. 1960. Genetic studies with mammalian poxviruses. IV. Hybridization between several different poxviruses. Virology **12:**272–282.

186. WOODROOFE, G. M., AND F. FENNER, 1962. Serological relationships within the poxvirus group: an antigen common to all members of the group. Virology **16:**334–341.

186*a*. WOODROOFE, G. M., AND F. FENNER. 1965. Viruses of the myxoma-fibroma subgroup of the poxviruses. I. Plaque production in cultured cells, plaque-reduction tests, and cross-protection tests in rabbits. Australian J. Exptl. Biol. Med. Sci. **43:** 123–142.

187. WOODSON, B., AND W. K. JOKLIK. 1965. The inhibition of vaccinia virus multiplication by istain-β-thiosemicarbazone. Proc. Natl. Acad. Sci. U.S. **54:**946–953.

188. ZWARTOUW, H. T., J. C. N. WESTWOOD, AND G. APPLEYARD. 1962. Purification of poxviruses by density gradient centrifugation. J. Gen. Microbiol. **29:**523–529.

189. ZWARTOUW, H. T. 1964. The chemical composition of vaccinia virus. J. Gen. Microbiol. **34:**115–123.

190. ZWARTOUW, H. T., J. C. N. WESTWOOD, AND W. J. HARRIS. 1965. Antigens from vaccinia virus particles. J. Gen. Microbiol. **38:**39–45.