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ASSOCIATION OF NEWLY FORMED VIRAL PROTEIN WITH SPECIFIC POLYRIBOSOMES

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The polyribosome or polysome, a cluster of ribosomes presumably bound together by a strand of messenger RNA, has been shown to be a functional unit for protein synthesis in rabbit reticulocytes,[1, 2] rat liver cells,[3, 4] and cultured HeLa cells.[5, 6] Moreover, infection of the latter cells with poliovirus, one of the small, RNA-containing viruses, leads to dissolution of the polyribosomes involved in the synthesis of cell protein and the formation of characteristic, larger polyribosomes.[5] The nature of the newly synthesized protein associated with the polyribosomes of poliovirus-infected cells has been investigated in the present experiments, and an appreciable portion of it has been identified immunologically as virus protein. The events following infection with vaccinia virus, a large DNA-containing virus which, like poliovirus, multiplies in the cytoplasm, have also been studied. In this case as well, the formation of characteristic polyribosomes active in the synthesis of viral protein has been demonstrated.

Materials and Methods.—Cells and virus: Strain S3 HeLa cells were grown in suspension by the "spinner" technique in modified Eagle's medium supplemented with 5% horse serum.[7] Cells in the phase of logarithmic growth were collected by low-speed centrifugation and infected as follows. For poliovirus infection, the cells were washed and resuspended at 8×10^6 cells/ml in warm medium containing the usual concentration of glutamine, $1/50$ the concentration of the other amino acids, and no serum. This medium, which does not support cell multiplication but does

permit optimal yields of virus,[8] was utilized to conserve isotopically labeled precursors of viral protein. The cells were infected with 100 plaque-forming units (PFU)/cell of Mahoney strain Type 1 poliovirus, and incubated at 37° with gentle stirring (magnetic bar). After a 20-min period of adsorption, the cell suspension was diluted with an equal volume of warm medium, and the incubation continued. The course of maturation was followed by measurements of infectivity by a plaque assay on HeLa cell monolayers.[9] Under these conditions, maturation of virus begins after a latent period of about 2.5 hr, and is complete at 5–6 hr.

A generally similar procedure was followed in the case of vaccinia virus, except that the cells were suspended at 3×10^6 cells/ml in growth medium with 5% horse serum and infected with an input multiplicity of about 25 PFU/cell of strain WR vaccinia virus. After incubation for 1 hr, the cells were washed three times with warm medium containing 20% dialyzed human serum and no amino acids except glutamine, and finally resuspended at 3.5×10^5 cells/ml in medium with the usual concentration of glutamine, $^1/_{10}$ the concentration of the other amino acids, and 5% dialyzed horse serum. The course of viral maturation was followed by a plaque assay on monolayers of chick embryo fibroblasts.[10] Formation of new virus under these conditions begins about 4 hr after infection, and is complete at 14 hr.

Antigens and antisera: Antisera to the complete poliovirus particle (D antigen) and to the hollow virus capsid (C antigen) were obtained as previously described.[11] Treatment of purified virus with guanidine, urea, or sodium dodecyl sulfate yields a soluble antigen (S antigen) distinct from D or C antigen, but identical to at least a portion of the soluble antigen(s) in the infected cell.[12] Antiserum to S antigen was prepared as follows. Purified virus[7] was treated with 6.35 *M* guanidine at pH 8.3 for 5 hr at 37°, followed by 24 hr at 25°. 0.1 ml of reaction mixture, corresponding to 300 μg of virus, was diluted with 3 volumes of 0.14 *M* NaCl and emulsified in complete Freund's adjuvant.[13] The emulsion was injected into the footpads of an albino rabbit, and the animal was bled 3 weeks later. The serum, which was characterized by agar diffusion with purified antigens according to techniques which will be given in detail,[12] contained antibodies directed against C antigen and S antigen.

Vaccinia antiserum was obtained as previously described[14] from rabbits infected with partially purified virus, and antiserum to bacteriophage λ was produced in rabbits by intravenous injection of phage purified by sedimentation in CsCl gradients.

Antiserum against rabbit γ globulin was obtained by immunizing sheep intramuscularly with a suspension of 5–10 mg of diphtheria toxoid–rabbit antitoxin immune precipitate in 0.5 ml of 0.14 *M* NaCl, emulsified in an equal volume of complete Freund's adjuvant. The resulting antiserum is directed primarily against 7S and 19S rabbit γ globulin.

Globulins were prepared from the rabbit antiviral sera by the addition of $^1/_2$ volume of saturated ammonium sulfate, and the point of equivalence with sheep antirabbit γ globulin was determined for each preparation.[15]

Quantitative measurement of viral antigens: The procedure, which has been briefly described previously,[11] is based on the fact that isotopically labeled amino acids in the medium of an infected cell culture are efficiently incorporated into viral protein.[16] The soluble complex of labeled antigen and its specific rabbit antibody is precipitated by the addition of sheep antiserum to rabbit γ globulin, and the amount of isotope in the precipitate is a measure of the amount of viral antigen. A sample of labeled purified virus can be nearly quantitatively precipitated with the appropriate antibody.[14, 17]

In practice, γ globulin prepared from poliovirus antiserum was added to one aliquot from an infected culture, and the mixture was incubated at 37° for 1.5–2 hr. A parallel incubation of another aliquot with γ globulin prepared from a rabbit immunized with bacteriophage λ provided an immunologically unrelated control. The amounts of antiviral and control γ globulin were adjusted so as to yield precipitates which carried down the same amount of nonspecific radioactivity in control incubations set up as described below with fractions from uninfected, labeled cells. An amount of sheep antiserum equivalent to the amount of rabbit γ globulin in the system was added, and the incubation continued for 2 additional hr. The resulting precipitates were collected and washed three times with cold 0.14 *M* NaCl, dissolved in 0.25 *N* acetic acid, and plated for radioactivity determinations. A similar procedure was followed for vaccinia virus except that unfractionated rabbit antiserum was used, and the control was pre-immune rabbit serum.

Isolation and characterization of polyribosomes of infected cells: The technique of Penman

et al.[5] was followed with minor modifications. After a 1-min exposure to C^{14} amino acids in the form of hydrolysate of algal protein, a suspension of 7–8 $\times$ 10^7 infected cells was poured into an equal volume of a frozen slurry of amino-acid–free medium. The cells were immediately sedimented at 1,500 rpm for 3 min, washed once with 8 ml of the same chilled medium, and resuspended in 2 ml of RSB (10^{-2} tris·HCl pH 7.4, 10^{-2} *M* KCl, and 1.5 $\times$ 10^{-3} *M* $MgCl_2$). After 5 min at 0° in this hypotonic environment, the cells were ruptured with three strokes of a tight-fitting, 7-ml-capacity Dounce homogenizer, and sucrose to a concentration of 0.15 *M* was added. Unbroken cells and nuclei were removed by centrifugation, the supernatant was made 0.25% with respect to sodium desoxycholate, layered over a linear gradient of 10–20% W/V sucrose in RSB, and centrifuged for 45–65 min at 25,000 rpm in a Spinco SW 25 rotor. The bottom of the tube was punctured, and the absorbancy of the effluent at 260 mμ was monitored with a Carey recording spectrophotometer equipped with a flow cell. Approximate sedimentation constants were calculated according to Martin and Ames,[18] assuming a value of 70S for the peak of single ribosomes. Fractions of 1.2 ml were collected. A portion of each fraction received 500 μg bovine serum albumin and an equal volume of 10% trichloroacetic acid (TCA) to determine the total precipitable radioactivity. The precipitates were washed with 5% TCA and dissolved in 0.5 ml of 0.25 *N* NH_4OH for plating. The remaining portion of each fraction was dialyzed overnight against three changes of RSB at 0°C. The volume of each dialyzed fraction was adjusted to 2 ml, of which 0.5 ml portions were utilized for specific and nonspecific immunological precipitation as described above. Another portion was precipitated with trichloroacetic acid. To correct for the 20–30% loss of viral proteins during the experimental manipulations, the net specific radioactivity in each dialyzed fraction was multiplied by the ratio of total acid-precipitable counts before and after dialysis. Several fractions of a gradient prepared from uninfected cells were always carried through as controls; in such fractions, the immune precipitates obtained with viral antibody and phage antibody each brought down about 10% of the total acid-precipitable radioactivity. In fractions from the polyribosome area of infected cells, the antiviral γ globulin typically brought down about 40% of the total counts, and the unrelated control γ globulin about 15%. The possibility of antigen excess, and consequent incomplete precipitation of viral antigen, was ruled out by parallel incubations with one-half portions from the peak fractions.

Labeled amino acids were obtained from the New England Nuclear Corp. The 7-ml Dounce homogenizer was manufactured by the Kontes Glass Co.

Results.—Association of nascent poliovirus antigen with specific polyribosomes: A characteristic series of events in HeLa cells pretreated with actinomycin and then infected with poliovirus was observed by Penman *et al.*[5] Analysis of cytoplasmic extracts of infected cells fractionated by sucrose-gradient centrifugation demonstrated an initial inhibition of protein synthesis and disappearance of polyribosomes, followed by resumption of protein synthesis associated with newly formed, larger polyribosomes.

Similar phenomena are demonstrable in cells infected without actinomycin. Four hours after infection, a culture was exposed to C^{14} amino acids for a 1-min period, and a cytoplasmic extract was prepared immediately afterward. The analysis of a sucrose gradient of such an extract is shown in Figure 1; a similar preparation from growing, uninfected cells is shown in Figure 3*A*. In accord with the finding of Penman *et al.*,[5] labeled protein was associated with the polyribosomes of both the uninfected and infected cells under these conditions. The peak of labeled protein in the uninfected cells was about 250S, in the infected cells about 400S. Uninfected cells maintained for 4 hours under the conditions used for infection yielded sucrose gradients similar to those from growing cells, except for some diminution in the peak of optical density around 250S, and a corresponding increase in the peak of single ribosomes.

A considerable portion of the nascent protein associated with the polyribosomes of the infected cells was specifically precipitated with antibody directed against

subunits of the viral protein. Before the immunological procedure, it was necessary to dialyze the fractions under conditions which dissociated polyribosomes, so that the actual immune precipitation did not involve nascent protein still attached to aggregates of ribosomes. The fraction of specifically precipitable protein in nine different experiments was between 15 and 40 per cent, constituting on the average 25 per cent of the total. With specific antibody directed against either structurally complete virions (D antigen) or empty capsids (C antigen), no more radioactivity was precipitated than with the immunologically unrelated control antibody.

Both the total and specifically precipitable radioactivity associated with polyribosomes were maximal about 4 hr after infection; qualitatively similar results were obtained before and after this time, with respect both to the fraction precipitable with specific antibody and to other characteristics of the nascent protein. Although the process of dissolution of cellular polyribosomes, and presumably the inhibition of cell protein synthesis, is accelerated in cells pretreated for 3 hr and infected in the presence of 5 μg/ml of actinomycin D,[5] a similar fraction of the total nascent, polyribosome-associated protein was specifically precipitable at 4 hr after infection in extracts of cells so treated.

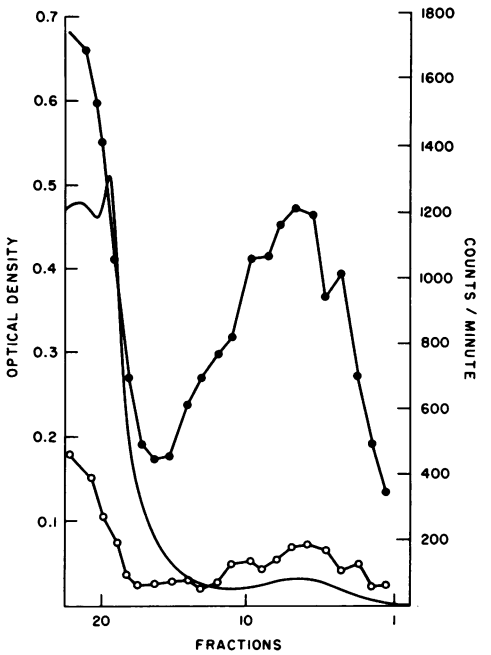


FIG. 1.—Relationship of optical density at 260 mμ (continuous line); newly synthesized poliovirus S antigen, O; and total nascent protein, ●, in fractions of an extract of infected HeLa cells after sedimentation through a sucrose gradient. 3.75 hr after infection, 8×10^7 cells were exposed to 50 μc of C^{14} protein hydrolysate (1 mC/0.6 mg) for 1 min. A cytoplasmic extract was prepared and layered over a 10–20% W/V sucrose gradient as described in the text. Fractions were collected and analyzed after centrifugation at 25,000 rpm for 55 min. The bottom of the gradient is to the right, and the peak in the optical density curve at fraction 19 represents single (~70S) ribosomes. The values for poliovirus antigen represent the amount of radioactive material precipitable with antiserum to degraded virus, less that precipitable with antiserum to bacteriophage λ.

Despite the brief period of exposure to C^{14} amino acids followed by disruption of the cells, a considerable amount of radioactivity was always found near the top of the sucrose gradient in the foregoing experiments, of which a portion was specifically precipitable (cf. Fig. 1). The conditions of centrifugation were designed to differentiate classes of polyribosomes, and provided limited data on the sedimentation characteristics of this smaller material. Accordingly, material corresponding to fractions 18–22 in Figure 1 was diluted with an equal volume of RSB, layered over a 10–20% sucrose gradient, and this time centrifuged for 2.5 hr at 25,000 rpm. Less than 20 per cent of the labeled protein was associated with the band of single ribosomes which sediments to the middle of the gradient under these conditions, the bulk of it remaining near the top of the tube. This result implies that most of this labeled material represents complete or incomplete polypeptide chains free or at-

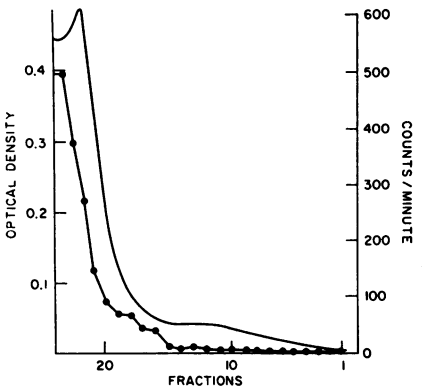


FIG. 2.—Failure of labeled, nascent protein to adsorb to polyribosomes. Material corresponding to fractions 20–22 in Figure 1 was mixed with an equal volume of an unlabeled extract of infected cells, layered over a second sucrose gradient, and centrifuged at 25,000 rpm for 45 min. Fractions were collected and analyzed as described for Fig. 1.

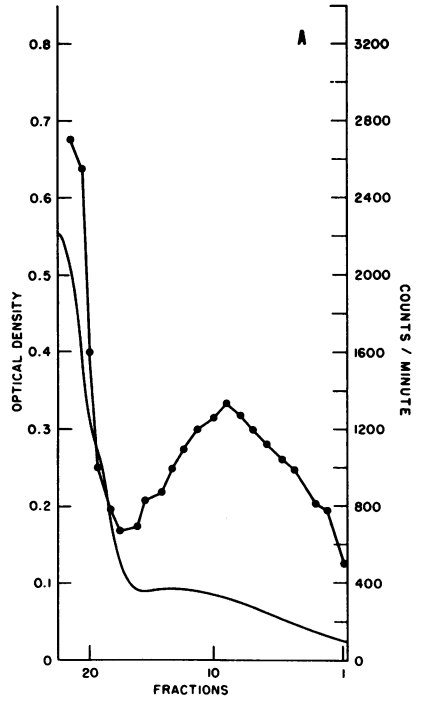
tached to soluble RNA. The small fraction which is associated with single ribosomes may arise, at least in part, from the degradation of polyribosomes during manipulations of the cell extracts.[5]

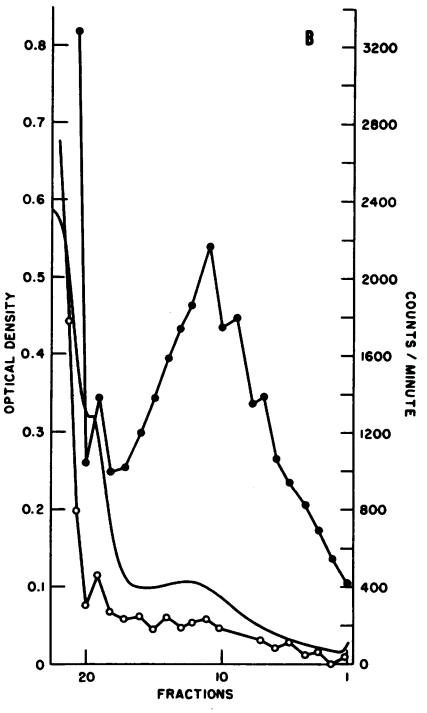
The results of the foregoing experiment render it unlikely that nonspecific adsorption of nascent polypeptide chains to polyribosomes is a significant factor under conditions of the present experiments. Further evidence on this point is given by the experiment illustrated in Figure 2. Material from the top of a gradient similar to that shown in Figure 1 was mixed with a whole cytoplasmic extract of infected, unlabeled cells. The mixture was sedimented through a sucrose gradient for 45 min. About 90 per cent of the radioactivity was recovered in the fractions corresponding to the original position of the labeled material in the gradient; a negligible amount was associated with polyribosomes.

Function of polyribosomes in the synthesis of vaccinia virus protein: In distinction to the procedure followed with poliovirus, cells were infected with vaccinia virus under conditions which support cell multiplication in the absence of infection. The analysis of a sucrose gradient prepared from cells growing under these conditions is shown in Figure 3*A*. There was a broad peak of functional polyribosomes centered at about 250S, and the single ribosomes were represented only by a slight shoulder on the optical density profile. Figure 3*B* indicates the situation 2.5 hr after infection, when the synthesis of virus protein was proceeding at a rapid rate, but before new virus is first detectable. There was an increase both in the amount of 250S polyribosomes and in the amount of nascent protein associated with them. Of the total polyribosome-associated nascent protein, about 15 per cent was specifically precipitable with antiserum. At 5 hr after infection (Fig. 3*C*), during the period of rapid maturation of virus, the amount of ribonucleoprotein in the polyribosome area and the amount of nascent protein were reduced, but a much larger fraction of the latter—about 30 per cent—was precipitable with viral antiserum. The distribution of nascent protein was broad and irregular, suggesting the existence of separate classes of functional polyribosomes of different sizes. A generally similar picture was observed later in the maturation cycle, at 8 hr after infection.

Another manifestation of the infectious process, noted also in the case of poliovirus, was the progressive increase with time in the amount of single ribosomes, presumably reflecting the dissolution of polyribosomes responsible for the synthesis of cell protein. Moreover, in accord with the results of Warner *et al.*,[2] treatment of extracts of cells (infected with either poliovirus or vaccinia virus) with 1 μg of ribonuclease per ml for 10 min at 0° converted the polyribosomes to single ribosomes, to which the nascent protein remained attached.

Discussion.—Specific immunological reactivity has previously been used to





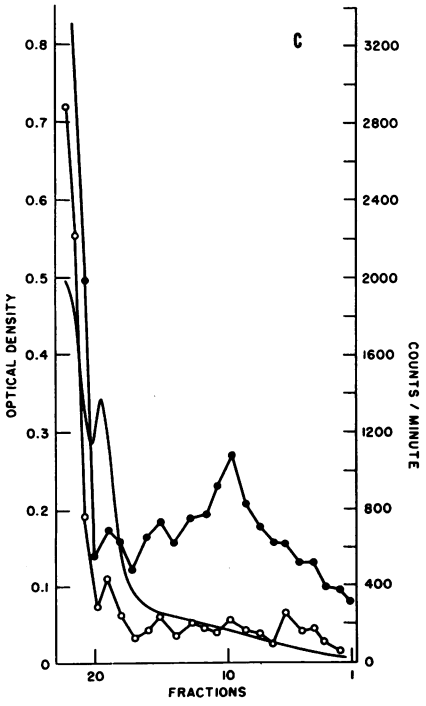


FIG. 3.—Relationship of optical density at 260 mμ (continuous line); newly synthesized vaccinia virus protein, O; and total nascent protein, ●, in fractions of HeLa cell extracts after sedimentation through a sucrose density gradient. Cells were infected, washed, and replicate cultures of 185 ml containing 3.5×10^5 cells/ml were established as described in the text. Uninfected cells (*A*) were similarly treated but without the addition of virus. At $2^1/_2$ hr (*B*) and 5 hr (*C*) after infection, a culture was centrifuged at 37°, and all but 15–20 ml of the medium removed. The cells were gently resuspended by stirring and, after 15 min at 37°, 15 μc of C^{14} protein hydrolysate was added for 1 min. A cytoplasmic extract was prepared and layered over a 10–20% sucrose density gradient. Fractions were collected and analyzed after centrifugation at 25,000 rpm for 65 min. The values for vaccinia virus protein represent the amount of radioactivity precipitated with antivaccinia serum less that precipitated with pre-immune serum.

demonstrate the association of several newly synthesized proteins,[19-22] including poliovirus protein,[23] with ribosomes. In the present experiments with poliovirus, care has been taken to preserve the polyribosomes of the infected cell during isolation and fractionation, and the data indicate that it is with these particles, rather than single ribosomes, that the bulk of immunologically identifiable nascent virus protein is associated. The results thus confirm the conclusion of Penman *et al.*[5] based on the characteristic larger size of the polyribosomes of the infected cell and their time of appearance and functional activity, that they are the site of synthesis of poliovirus protein.

Of the total protein labeled with a 1-min pulse of C^{14} amino acids and attached to the isolated polyribosomes, 15–40 per cent reacted specifically with antiserum directed against the soluble product of virus degraded with guanidine.[24] These values are corrected by subtraction of the amount of radioactivity brought down by an unrelated immune precipitate, and accordingly are low to the extent that immunologically reactive virus protein is occluded. Moreover, the immunologically identifiable fraction represents only those polypeptide chains sufficiently complete as to be antigenic, but not yet released from the site of synthesis. Thus, although the fraction cannot be precisely determined, it is apparent that a considerable portion of the total nascent protein has the antigenic character of subunits of the viral capsid. It is noteworthy that the fraction of specifically precipitable counts was virtually the same in all fractions of the broad peak of polyribosomes, implying that the synthesis of capsid protein is a function of all, rather than a special class, of the polyribosomes of the infected cell.

A new RNA polymerase has been identified in the cytoplasm of poliovirus-infected cells,[25] but otherwise nothing is known concerning enzymes or other proteins which may be specified by the viral genome and which are necessary for viral replication. One might reasonably assume that such proteins are immunologically distinct from capsid protein, and at least a portion of the immunologically unreactive nascent protein might be accounted for on this basis.

The evidence currently available indicates that the capsid is built up of one, or possibly two, polypeptide chains with a molecular weight of 25,000.[26] The single molecule of RNA which constitutes the genetic apparatus of the virus is comprised of about 6,000 nucleotides, and thus apparently contains sufficient information to specify about 10 polypeptides of this size. Moreover, the large size of the specific polyribosome suggests that the entire molecule is functioning as messenger RNA. If the foregoing assumptions are valid, it is necessary to postulate some mechanism for the selective synthesis of capsid protein to account for the large fraction of immunologically reactive nascent protein. Conceivably, the RNA could contain repeated sequences specifying the same capsid protein(s), or a particular sequence might be read more frequently than other sequences. Alternatively, capsid protein(s) may be released from polyribosomes more slowly than other nascent protein.

The results with vaccinia virus suggest that the formation of specific polyribosomes for the synthesis of virus protein may be a general phenomenon, at least with respect to viruses which multiply in the cytoplasm. In this case, specific polyribosomes of varied size appear in the infected cell early in the infectious cycle, and the first observed change in the optical density of the sucrose gradient profile is an increase in the amount of material in the 250S region. These results do not indicate

whether dissolution of cellular polyribosomes precedes the appearance of virus-specific polyribosomes, but suggest in any event that early in the infectious cycle both cellular and viral proteins are being synthesized. As early as 2.5 hr after infection, the latter accounts for an appreciable fraction of the total.

The vaccinia antiserum was produced by infecting rabbits with the virus and may contain antibody to nonstructural as well as to the various structural proteins. Nevertheless, as in the case of poliovirus, the maximum immunologically identifiable fraction of the polysome-associated protein labeled for 1 min (at 5 hr following infection) was about 30 per cent. With a 1-hr period of labeling at the same time in the infectious cycle, more than 50 per cent of the total labeled protein was immunologically reactive.[27] Even the latter figure is probably a low estimate of the amount of viral protein synthesis relative to total protein synthesis in the infected cell, since this value for viral protein is also corrected by subtraction of the amount of radioactivity precipitated with pre-immune serum. Experiments with actinomycin,[28] which completely inhibits the synthesis of viral protein, indicate that non-viral protein synthesis is progressively inhibited in the vaccinia-infected cell, and no net increase in cell proteins occurs after the fourth to sixth hr.

As the cycle of vaccinia infection proceeds, the data suggest the formation of polyribosomes which differ in size and which may be concerned with the synthesis of different viral proteins. The distribution of nascent specific protein does not form a discrete peak as in the case of poliovirus, but is distributed throughout the polyribosome region. This finding is in keeping with the complexity of the virus particle and the relatively large molecular weight (1.6×10^8) of its DNA.[29, 30] Clearly, unlike the case of poliovirus, there is no restriction to a single species of messenger RNA.

Summary.—Polyribosomes have been isolated from the cytoplasm of HeLa cells infected with poliovirus, a small, RNA-containing virus, and vaccinia virus, a large DNA-containing virus. In both cases, the polyribosomes have been shown to be the site of synthesis of immunologically identifiable virus protein.

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UV-INDUCED ELECTRON SPIN RESONANCES IN DNA AND THYMINE

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We have observed the electron spin resonance absorption spectra of UV-irradiated DNA and thymine. The principal free radical formed in both cases, as determined by analysis of the resonance data, consists of an unpaired electron on the 5-carbon of thymine. This radical is the same as that recently observed in both DNA[1] and thymine[2] after irradiation with γ rays.

The spectra were measured at 77°K in a Varian ESR spectrometer operating at 9,112 mc/sec with 100 kc/sec external field modulation and phase-sensitive detection. Samples were first irradiated at 77°K in a quartz dewar and then inserted into the microwave cavity dewar. In this way UV-induced paramagnetic centers in the quartz cavity dewar were avoided. The UV source consisted of an Osram 500-watt high-pressure mercury arc lamp followed by a filter which transmitted light between 2,400 and 4,800 A.

The first derivatives of the absorptions observed in lyophilized DNA (calf thymus DNA from Worthington Biochemical Corp.) and thymine after UV irradiation are shown in Figure 1*a* and *b*, respectively. The satellite structures in these spectra are identical to each other and to those previously observed in thymine[2] and in DNA[1] after γ irradiation. The radical proposed to explain these spectra was $-CH_2-\dot{C}(-CH_3)-$ from the 5- and 6-carbons of thymine. The pattern expected for this radical, on the basis of experimentally determined hf coupling constants of 20.8 oe with three methyl protons and of 37.7 oe with two methylene protons, is