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COMPOSITION OF FOWLPOX VIRUS AND INCLUSION MATRIX

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ABSTRACT

RANDALL, CHARLES C. (University of Mississippi School of Medicine, Jackson), LANELLE G. GAFFORD, ROBERT W. DARLINGTON, AND JAMES M. HYDE. Composition of fowlpox virus and inclusion matrix. J. Bacteriol. **87**:939-944. 1964.—Inclusion bodies of fowlpox virus infection are especially favorable starting material for the isolation of virus and inclusion matrix. Electron micrographs of viral particles and matrix indicated a high degree of purification. Density-gradient centrifugation of virus in cesium chloride and potassium tartrate was unsatisfactory because of inactivation, and clumping or disintegration. Chemical analyses of virus and matrix revealed significant amounts of lipid, protein, and deoxyribonucleic acid, but no ribonucleic acid or carbohydrate. Approximately 47% of the weight of the virus and 83% of the matrix were extractable in chloroform-methanol. The lipid partitions of the petroleum ether extracts were similar, except that the phospholipid content of the matrix was 2.2 times that of the virus. Viral particles were sensitive to diethyl ether and chloroform.

In this laboratory it was previously shown that fowlpox virus (FP) may be isolated from inclusion bodies and subsequently purified by differential centrifugation (Randall, Gafford, and Darlington, 1962). The virus is of the deoxyribonucleic acid (DNA) type, containing 35% guanine plus cytosine by either chemical (Randall et al., 1962) or physicochemical methods (Szybalski et al., 1963). Purification of FP from inclusions has great advantage over the use of whole tissue. At the onset, cell debris is virtually eliminated, and one has to contend only with inclusion matrix. The method of purification yields a highly infectious viral preparation without significant contamination, as recognized by electron microscopy. Other methods of purification were tried, as will be shown, but were abandoned since they did not yield virus of good quality. In this report, we present a chemical analysis of FP virus compared, where appropriate, with inclusion matrix.

MATERIALS AND METHODS

Virus and isolation of inclusions. The strain of FP, method of inoculation, preparation of infected tissue, and isolation of viral inclusions were described (Randall et al., 1962). The method of rupture by sonic treatment of the inclusions was also described in detail (Randall et al., 1962). The virus particles were separated from the inclusion matrix material by centrifugation at 12,100 × *g* for 20 min in a Servall RC-2 refrigerated centrifuge. The virus was suspended in 5 to 8 ml of distilled water, sonically treated for 1 to 2 min to effect complete dispersion, and centrifuged at 8,700 × *g* for 20 min. This cycle was repeated three to five times until the supernatant fluid was clear. The virus was resuspended in a measured amount of distilled water, and a sample was removed for particle counting and electron microscopy. The virus was pelleted, the supernatant was discarded, and the sedimented virus particles were vacuum-dried. The matrix material (supernatant) from the first high-speed centrifugation was further purified by centrifugation at 12,100 × *g* for 30 min. The supernatant fluid was removed, a sample was taken for electron microscopy, and the remainder of the sample was frozen overnight at −20 C. Upon thawing, the particulate matter could be sedimented by centrifugation at 27,000 × *g* for 30 min. The essentially clear supernatant fluid was removed, evaporated to a volume of 1 to 2 ml with a Rinco flash evaporator, and combined with the pellet. The entire sample of matrix material then was dried and stored under vacuum for subsequent analysis.

Density-gradient centrifugation. Centrifugation in potassium tartrate and in cesium chloride was carried out essentially as described by Darlington and Randall (1963).

Electron microscopy and particle counting. The

techniques of electron microscopy and counting viral particles were described (Darlington and Randall, 1963). Virus was stained with phosphotungstic acid (PTA) and uranyl acetate (UA; Smith and Melnick, 1962).

Estimation of particle weight. Samples of purified virus were dried under a stream of nitrogen and then under vacuum to constant weight as described previously (Darlington and Randall, 1963).

Chemical methods. Viral samples, dried to constant weight, were extracted twice with cold 0.4 N perchloric acid (PCA) at 4 C for 10 min. Lipid was removed by five extractions with chloroform-methanol (CM; 2:1) at room temperature. The extracts were evaporated to dryness under N_2, and the lipids were separated from the residue by repeated extractions with petroleum ether (boiling point, 30 to 60 C). The extracts were adjusted to a known volume for subsequent determination of weight and chemical analysis. The methods for various lipid and phosphorus analyses were described (Randall and Gafford, 1962). The residue after lipid extraction was further extracted with 0.5 N PCA for ribonucleic acid (RNA) and DNA analysis (Darlington and Randall, 1963), and the residue was digested in 1 N NaOH at 37 C overnight. If particulate matter remained, fresh NaOH was added, and the sample was heated in a boiling-water bath until solution; the digests were pooled. Proteins in the digests were determined by the Lowry method (Layne, 1957). The carbohydrate content was determined by the anthrone reaction (Hassid and Abraham, 1957).

RESULTS

Electron microscopy. Viral preparations were either shadow-cast or stained with PTA or UA to assess the purity. Stained preparations were easy to examine for nonviral debris and, in addition, gave some detail of the viral structure. Figure 1 presents a typical field of a PTA-stained grid in which no demonstrable debris may be identified. In scanning numerous grids, occasional minute debris was seen but was not judged to be significant. Representative fields were chosen for determination of particle size. From shadowed grids, the average size was 258 by 334 mμ (average of 20 measurements). The average size as

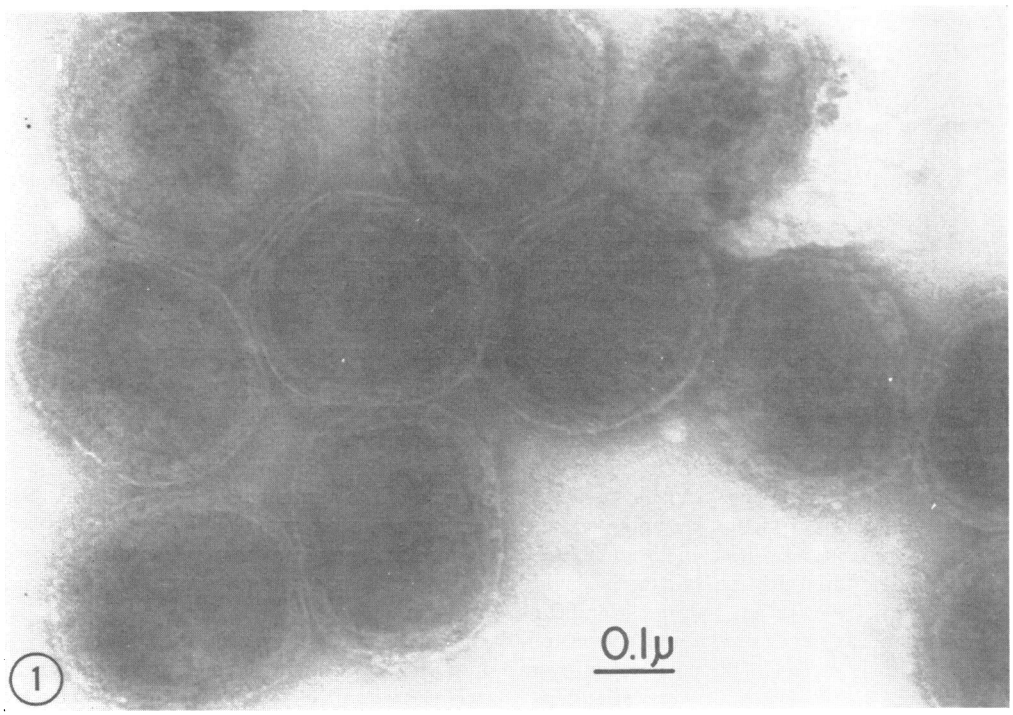

FIG. 1. *Electron micrograph of fowlpox virus stained with phosphotungstic acid.*

determined by PTA stains was 265 by 354 mμ (10 measurements).

Virus stained with UA contained a dense nuclear portion consistent with the appearance of the nucleoid (Fig. 2). The bits of irregular debris are artifacts of the staining method. Hollow particles were very infrequent. Electron micrographs of matrix material showed amorphous debris, and no viral particles were recognized.

Density gradient and electron microscopy. Samples of FP previously examined by electron microscopy were studied in density gradients. Samples of FP in 0.5 ml were carefully layered over preformed potassium tartrate gradients, and the tubes were centrifuged at 30,000 rev/min (73,449 × *g*) with an SW 39 rotor in a Spinco model L centrifuge. Two bands were formed, both rather broad and diffuse. Electron micrographs of virus from both bands were of similar appearance. A typical electron micrograph of a band (in this case, a lower band) stained with 0.5% UA is shown in Fig. 3. The preparations were overstained, thus emphasizing the interconnecting strands. At this point, the debris was abundant and was judged to be derived from disintegrating viral particles. The composition of these strands has not as yet been studied by enzymatic and histochemical methods. The infectivity of pooled virus from both bands was less than 1% of the original. Purification was not achieved by further centrifugation; for example, aspiration of the lower band and recentrifugation under identical conditions gave two bands as before, indicating further breakage of the particles.

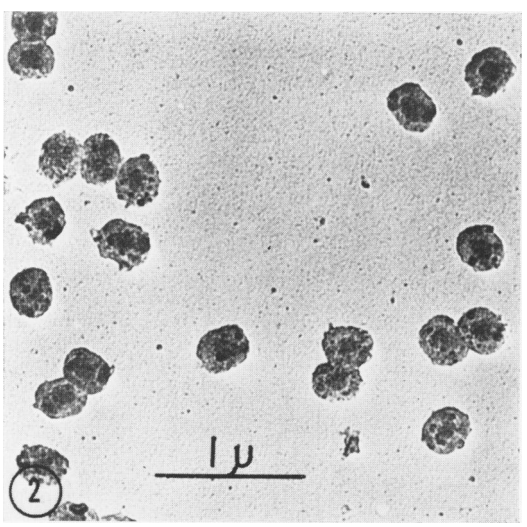

FIG. 2. *Electron micrograph of fowlpox virus stained with uranyl acetate.*

Virus centrifuged in CsCl also was unsatisfactory for chemical analysis, because after centrifugation for 20 to 24 hr it was totally inactivated. The virus tended to aggregate in a very dense band which could only be dispersed with difficulty by sonic treatment for 10 to 15 min. There was considerable destruction of the virus; under these conditions, particle counts and other analyses would be meaningless. This also would apply to virus from tartrate gradients. Other workers reported that Shope papilloma virus (Breedis, Berwick, and Anderson, 1962) becomes fragile in CsCl with some disintegration.

Chemical analyses of FP and matrix. Quantitative determination of the protein, lipid, and DNA content of FP virus is illustrated in Table 1. The particle weight of 2.04×10^{-14} g may be contrasted with the weight (5.34×10^{-15} g) of vaccinia virus (Smadel, Rivers, and Pickels, 1939), another member of the pox group. The weight of FP may be too high because of bound water which was not removed by the methods of drying employed. In the case of FP, the lipid and protein content equals or exceeds the dry weight of the vaccinia particles. No RNA was detected by the orcinol reaction, and neither the virus nor the matrix contained a significant amount of reducing sugar (determined with the anthrone reagent).

About 47% of the weight of the virus and 83% of the matrix was extractable by CM. After evaporation of this solvent system and re-extraction of the residue by petroleum ether, about 20% (average) of the total weight was not solubilized. The nature of the bulk of this material (other than protein) remains to be determined. The residue in question contains a significant but quite variable amount of protein: from 20 to 40% in four cases, but in the fifth the total residue was protein. The total protein is the sum obtained from NaOH digests of extracted virus and matrix, and their respective CM and hot PCA extracts, the last two containing about 18% of the total protein. The protein values are probably only relative, since the amino acid content may be significantly different from that of the standard (bovine serum albumin).

Lipid composition and phosphorus content of

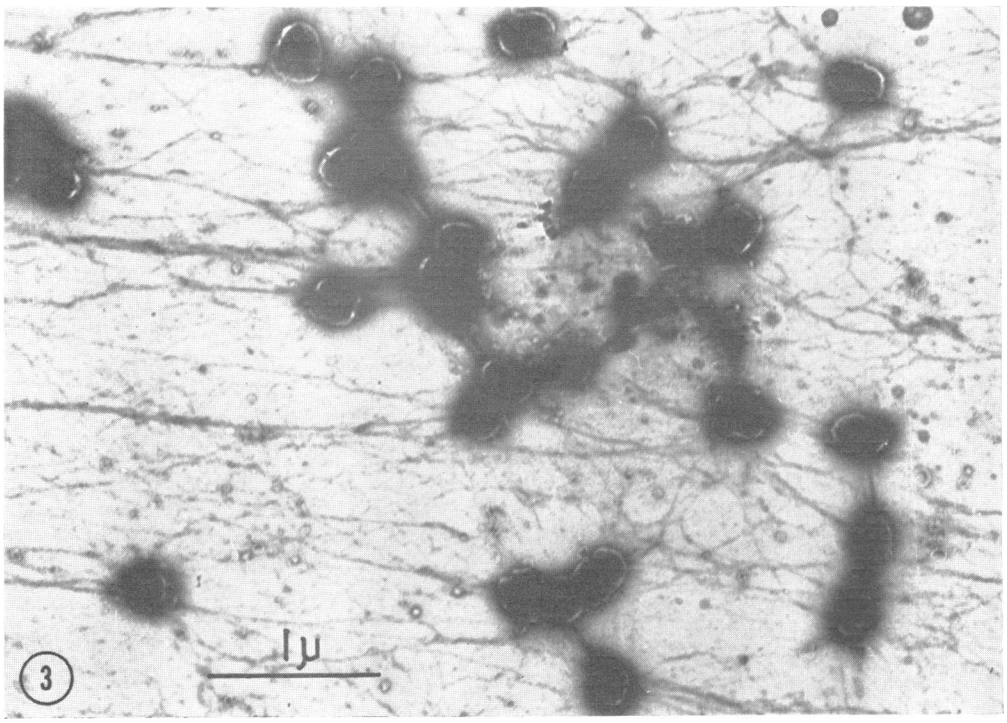

FIG. 3. *Electron micrograph of lower band obtained by centrifuging fowlpox virus in a potassium tartrat density gradient.*

TABLE 1. *Lipid, protein, and DNA content of fowlpox virus*

Passage no.	Particle wt. (g × 10^{-14})	Lipid per particle (g × 10^{-15})*	Protein per particle (g × 10^{-15})	DNA per particle (g × 10^{-16})
M3-11C	1.32	4.76	4.92	2.17
M3-13C	3.11	8.60	10.3	5.02
M3-15B	1.82	4.55	7.34	4.73
M3-15C	1.72	4.82	7.47	4.29
M3-15D	2.24	4.95	—	3.95
Average	2.04	5.54	7.51	4.03

* Weight of petroleum ether-soluble lipid.

virus and matrix. Analyses of purified virus and matrix are shown in Tables 2, 3, and 4. The data are the averages of duplicate or triplicate analyses of five different preparations. Significant differences are evident in the percentage composition of the petroleum ether-soluble components (Table 2) and in the distribution of phosphorus (Table 4), which was extracted in several different ways. The sum of the components does not equal 100% (Table 3). This discrepancy may have the same explanation as was found for fowlpox-infected and normal chick scalp, in which the difference was due to the contribution of total fatty acids (Todd, Randall, and Coniglio, 1958). It is of interest that the lipid composition (Table 3) of virus and matrix is very similar except that the phospholipid content of the matrix is 2.2 times that of the virus. The data in Table 1, in terms of content per particle, are relatively absolute values and are preferred to percentage figures.

TABLE 2. *Chemical composition of separated inclusion components**

Component	Virus	Matrix
Lipid	27.4	62.8
DNA	1.98	0.36
Protein	36.8	15.3
Phosphorus	0.52	0.48

* All values are expressed as percentages of dry weight. Lipid values after chloroform-methanol extraction were 47% of the virus and 83% of the matrix. Values shown are after re-extraction by petroleum ether.

DISCUSSION

The chemical and electron microscopic evidence indicates that differential centrifugation of ruptured inclusions is an adequate procedure for the purification of virus from inclusion matrix. Further attempts to study FP in potassium tartrate and CsCl gradients were unsatisfactory because of inactivation of the virus and the obvious disruption of the virus in the former; in addition, aggregation rendered particle counts highly inaccurate. For these reasons, the DNA content was not determined on virus preparations exposed to these solvents, although on purely morphological grounds there is no appreciable loss of DNA because the nucleoid stained well with UA. Other gradients, such as sucrose, might be resorted to with more success. In any case, the DNA content of FP reported in this paper is consistent with data reported for a number of DNA viruses of bacterial, insect, and animal origin (Allison and Burke, 1962).

It is not unreasonable to expect that the virus particles would have a high lipid content, since in the inclusion they are imbedded in this material. Because the viral and matrix lipid have a similar composition, it is plausible that viral and matrix lipid have the same biochemical origin. In a recent electron microscopic study (Arhelger and Randall, 1964) of the development of FP in the chorioallantoic membrane, evidence was presented to suggest that the outer layer of the virus particles is derived from the inclusion body. It is of interest that FP is sensitive to diethyl ether (Randall and Gafford, *unpublished data*), confirming the observation of Bang, Levy, and Gey (1951*a*, *b*). A 10^6 titer was reduced 2 to 3 log units by the method of Andrewes and Horstmann (1949). After 10 min in $CHCl_3$, according to the techniques of Feldman and Wang (1961), the same material was totally noninfectious.

It appears likely that removal of the lipid affects the viability of the virus adversely, although the effect of the solvents employed on virus protein remains to be determined.

TABLE 3. *Lipid composition of separated inclusion components**

Component	Virus	Matrix
Phospholipid	3.8	8.4
Total cholesterol	22.0	20.5
Free cholesterol	1.5	1.5
Esterified cholesterol	20.4	19.0
Glycerol	3.9	3.9
Free fatty acid	31.5	25.5

* All values are expressed as percentages of total petroleum ether-soluble lipid.

TABLE 4. *Phosphorus distribution in separated inclusion components**

Fraction	Virus	Matrix
Cold PCA extract	3.8	11.9
Hot PCA extract	39.3	17.6
Chloroform-methanol extract	29.6	64.8
NaOH digest	27.3	5.7

* Expressed as percentage of total phosphorus.

ACKNOWLEDGMENTS

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