THE RELATION OF HERPES VIRUS TO HOST CELL MITOCHONDRIA*

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Francis and Kurtz have applied centrifugation techniques for the separation of structural units of cells to the problem of the intracellular distribution of virus (1). Although the inclusions produced by herpes virus are nuclear in location, no evidence was found to indicate that virus is selectively bound to nuclei or to nuclear nucleoprotein. On the contrary, their results suggested that the virus was more richly associated with cytoplasmic material and that the inclusions are not herpes virus. This view is also supported by the observations of Bang (2).

A relation has been established between certain phases of mitochondrial metabolism and the propagation of herpes and influenza viruses (3–5). The concept that these cellular organelles function in the development of virus is supported further by the present report which describes an intimate association of the virus with mitochondria. The nature and significance of that association are discussed.

Methods and Materials

Virus.—The Armstrong strain of herpes virus used had undergone 48 passages in mouse brain and was adapted to the embryonate egg by 40 passages to the chorio-allantoic membrane (1). A 10 per cent suspension of such infected membranes was prepared in saline containing 10 per cent horse serum. After centrifugation, this pool of virus was used to initiate infection in the experimental tissue studied.

Tissues.—12-day-old eggs were infected *via* the chorio-allantoic membrane, and after 3 days gross lesions had occurred in the livers and hearts of the embryos. The infected livers were harvested at that time, washed in cold 0.25 M sucrose, and used immediately for cell fractionation. In some experiments normal livers were removed in an identical manner from uninfected eggs of the same age.

Fractionation.—A 10 per cent homogenate of liver was prepared in 0.25 M sucrose using the Potter-Elvehjem glass homogenizer (6). The resulting preparation was fractionated essentially by the procedure of Hogeboom and Schneider (7). All operations were performed at temperatures between 4 and 0°C.

To sediment nuclei the homogenate was diluted to 2 per cent with sucrose and subjected to a centrifugal force 300 times gravity for 10 minutes. The supernate obtained by sedimenting a 10 per cent homogenate at 300 × *G* for 10 minutes was employed for the isolation of mito-

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chondria. These particulates were sedimented with a centrifugal force of 2400 × *G* applied for 20 minutes. The supernate resulting from this last operation contained microsomes, fat particles, and soluble proteins.

All fractions were examined by phase microscopy. After two washings, the fraction labelled nuclei was found to contain some blood cells, some mitochondria, and very few intact liver cells. The mitochondrial preparation was free of nuclei and fat particles. In the final supernate, it was possible to see considerable numbers of fat droplets and some visible particles which sedimented with difficulty and were considerably smaller than the mitochondria.

Virus Titrations.—The amount of virus was estimated by determining the infectious titer for eggs. Tenfold serial dilutions of virus were prepared in 0.25 M sucrose and were inoculated onto the chorio-allantoic membrane of embryonate eggs. After 3 days the eggs were opened and examined for the formation of pocks. The 50 per cent infectious titer was calculated using the method of Reed and Muench (8).

Nuclei Counts.—To determine the number of nuclei per unit weight of liver, suitable dilutions of a 10 per cent homogenate of tissue were made in 1 per cent citric acid (9). The nuclei hardened by citric acid were then counted in a hemocytometer.

Nitrogen.—The nitrogen content of the mitochondrial fractions was determined by the Koch-McMeeken modification of Nessler's method (10).

RESULTS

Intracellular Distribution of Herpes Virus.—Cellular fractions corresponding to nuclei, mitochondria, and supernatant fluid were prepared by differential centrifugation. The titers of virus in the embryonic liver used for this purpose ranged from $10^{-6.3}$ to $10^{-7.0}$ with an average for six experiments of $10^{-6.6}$. The nuclear and mitochondrial particulates were washed twice in 0.25 M sucrose and each component was diluted to give a final volume equal to that of the original 10 per cent homogenate. These fractions were titered in embryonate eggs, and the final titers expressed correspond to the number of infectious doses per 0.1 gm. of moist liver in the case of the whole homogenate or to the number of infectious doses per cellular fraction obtained from 0.1 gm. of liver tissue.

The results of these titrations are tabulated in Table I. The nuclei, mitochondria, and supernatant fluid contained respectively 1.3, 16, and 80 per cent of the virus found in the whole homogenate.

Effect of Repeated Sedimentation on the Viral Content of the Supernatant Fluid.—Preliminary to the study of the relation of virus to the cellular components, it was desirable to know something of the sedimentation characteristics of the virus under these conditions. A centrifugal force of 2400 × *G* applied for 20 minutes will sediment both the nuclei and mitochondria from an homogenate in 0.25 M sucrose. The supernatant fluid was centrifuged repeatedly under these conditions. After each operation, the lower twentieth of the fluid was discarded, and the remainder was thoroughly mixed and titered. The original titer of the 10 per cent homogenate was $10^{-5.5}$; and after 5 sedimentations, the titer was $10^{-5.4}$ (Fig. 1). Thus, repeated centrifugation at this speed did not remove the virus from the supernatant fluid. Further, during such an operation, the virus did not undergo detectable deterioration.

In contrast if one repeats the operation employing a centrifugal force of 24,000 × *G*, the virus is readily sedimented. Each centrifugation decreases the titer by approximately $10^{-0.8}$ and sediments about 85 per cent of the virus which is present (Fig. 1).

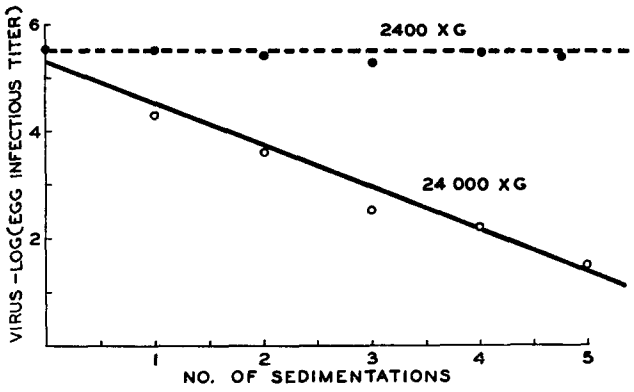

FIG. 1. Effect of repeated sedimentations on the viral content of the supernatant fluid.

TABLE I

Intracellular Distribution of Herpes Virus

Experiment	Virus titers* of cellular fractions			
	Whole homogenate	Nuclei	Mitochondria	Supernatant fluid and microsomes
I	7.0	4.8	—	—
II	6.5	4.5	—	—
III	6.5	—	6.0	—
IV	6.3	—	5.6	—
V	6.8	—	—	6.5
VI	6.4	—	—	6.4
Average	6.6	4.7	5.8	6.5
Percentage	100	1.3	16	80

* The 50 per cent infectious dose for eggs is expressed as the reciprocal of the log of the dilution of the cell fraction obtained from 100 mg. of liver.

Effect of Repeated Sedimentation on the Viral Content of Mitochondria.—Since the mitochondria had a considerable titer, $10^{-5.8}$, and were associated with 16 per cent of the total virus of the cell (Table I), an attempt was made to determine whether the viral activity was a superficial contamination of this particulate fraction.

Mitochondria obtained from infected livers were washed repeatedly in cold isotonic sucrose. After each washing, the residue was sedimented under conditions of time and centrifugal force (2400 × *G* for 20 minutes) which did not sediment the virus (*cf.* Fig. 1). However, titrations of the virus in the mitochondria after each of 5 successive washings revealed a constant titer by the 2nd washing. This demonstrated that the virus was being repeatedly sedimented with the mitochondria (Fig. 2). Thus, a considerable amount of the virus was associated with the mitochondria in what was apparently an intimate attachment.

In contrast mitochondria prepared from normal liver were suspended in a supernatant fluid obtained by homogenizing and centrifuging infected tissue

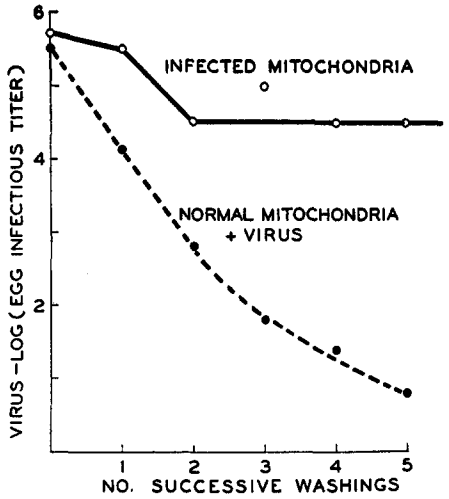

FIG. 2. Effect of repeated washings on the viral content of mitochondria.

in the cold with sucrose. Using the procedure described above, the mitochondria were readily separated from the virus (Fig. 2). Thus, under the same conditions employed to isolate infected mitochondria, there was no significant union of virus and normal mitochondria. In addition attempts to achieve union of normal mitochondria with virus of infected supernatant fluid in the presence of ionic medium (horse serum in saline) or at elevated temperatures (37°) were without success.

Influence of Immune Serum on Virus Associated with Infected Mitochondria.— Although it was not possible to obtain a union of virus and mitochondria *in vitro* nor to wash infected mitochondria free of virus, the associated virus could be inactivated by immune serum. The immune serum was prepared by immunizing rabbits with a preparation of mouse brain containing herpes virus. Tenfold dilutions of the mitochondria were made in normal and immune serum.

After incubation for 1 hour at 4°, the mixtures were inoculated on chorio-allantoic membranes. A $10^{-5.4}$ dilution made in a 1:160 dilution of normal serum produced pocks in 50 per cent of the eggs. In a similar concentration of immune

TABLE II

Accessibility to Immune Sera of Virus Associated with Infected Mitochondria

Dilution of serum	Viral titers*	
	Mitochondria‡	Supernatant fluid‡
Normal 1:8	5.16	—
Immune 1:8	3.50	—
Normal 1:160	5.38	—
Immune 1:160	3.75	—
Normal 1:160	—	6.00
Immune 1:160	—	4.83

* Viral titer is expressed as the reciprocal of the log of dilution.

‡ Amount of cell fraction corresponds to that found in 100 mg. of moist liver.

TABLE III

Effect of Herpes Infection on the Amount of Mitochondria per Cell

Experiment*	Tissue	Nuclei/gm. liver × 10^{-7}	Mitochondrial nitrogen	
			mg./gm. liver	μg./nuclei × 10^{6}
I	Normal	50.9	5.76	11.3
	Infected	54.3	4.68	8.6
II	Normal	72.2	6.88	9.5
	Infected	80.0	4.36	5.5
III	Normal	73.0	5.15	7.1
	Infected	84.0	3.45	4.1
Average	Normal	65.4	5.92	9.1
	Infected	72.8	4.19	5.8

* In each experiment a pool of livers obtained from 18 eggs was used.

serum, a $10^{-3.8}$ dilution of mitochondria was necessary to produce a 50 per cent egg infectious dose (Table II). When supernatant fluid containing virus was employed, like results were seen (Table II). Thus, the virus is held in association with the mitochondria in such a manner that it is still accessible to the action of immune serum.

Effect of Herpes Infection on the Concentration of Mitochondria per Cell.—In

view of the intimate attachment, as well as biochemical relation of the virus to the mitochondria, the possibility was considered that these organelles may be the intracellular site of viral synthesis. Later deterioration of the mitochondria on maturation of virus would account for the accumulation of large amounts of free virus in the cytoplasm. To detect any selective destruction of mitochondria, an estimation was made of the amount of mitochondrial material in the cells of normal and infected livers.

A 10 per cent homogenate was prepared in 0.25 M sucrose. Suitable dilutions were made of one aliquot in 1 per cent citric acid, and the nuclei were counted in a hemocytometer and the number of nuclei per gram of liver was estimated. From another portion of the homogenate, the mitochondria were isolated by differential centrifugation, and the amount of mitochondrial nitrogen per gram of liver was determined. The amount of mitochondrial nitrogen per nucleus was then calculated.

In each pool of infected livers there was, in comparison with normal liver, a marked decrease (30 per cent) in the mitochondrial nitrogen per gram of liver, as well as a decrease (36 per cent) in the mitochondrial nitrogen per nucleus (Table III). These data indicate that in livers infected with herpes virus there is a selective destruction or deterioration of mitochondria.

DISCUSSION

Cellular fractionation has been employed most extensively in the study of the intracellular localization of enzymatic proteins. The limitations of the technique in this relation have been reviewed elsewhere (7). Most of the same limiting considerations are applicable here and they preclude a rigid interpretation of the results in terms of the original distribution of the virus in the cell. However, it appears that a considerable portion of the virus is firmly bound to the mitochondria in such a manner that it is unlikely that any appreciable quantity was eluted during the fractionation procedure (Fig. 2). Also, it is unlikely that virus present in the supernatant fluid was adsorbed to the mitochondria during the isolation, since normal mitochondria can be separated readily from the virus of supernatant fluid (Fig. 2).

The low viral activity of the nuclear fraction may be accounted for in terms of the impurity of the preparation (Table I). The presence of some mitochondria and whole cells in this fraction, as demonstrated by phase microscopy, undoubtedly contributed in part to the viral activity found in the nuclear sediment. It cannot be proved on the basis of present information that virus is not released from the nuclei when the cells are disrupted by the procedures of purification employed, but the high count of nuclei in preparations of infected liver indicates that if virus is released from these bodies, there is not sufficient concurrent destruction of them to account for the low concentration of virus in that cellular fraction (Table III). But in this type of experiment, needless to say, the

finding of a component in a cell particulate may be more significant than the observation that it is not present.

It has been demonstrated previously that certain of the oxidative reactions of the Krebs cycle which are localized in the mitochondria are essential to the propagation of influenza virus (4, 5). Furthermore, changes in these enzymatic activities have been observed in tissues infected with herpes virus (3). The data reported here show the existence not only of a biochemical but also of a physical relationship of this virus to the mitochondria. The interpretation is tentatively advanced that these organelles are a site of viral synthesis in the cell. The virus found in the cytoplasm may result from a deterioration of the mitochondria at the time of viral maturation. These inferences are supported in part by visual data previously obtained through electron microscopy (2). Information most pertinent to this concept is the ratio of viral activity of the mitochondria to that of the cytoplasmic residue expressed as a function of time. Experiments designed to provide such data are now in progress.

SUMMARY

The intracellular distribution of herpes virus in embryonic liver was investigated. 80 per cent of the virus was found in the cytoplasm in what appears to be a form uncombined with mitochondria or nuclei. A significant amount of the virus (16 per cent) was found bound to the mitochondria by an intimate attachment. Furthermore evidence was obtained that the mitochondria from liver tissue undergo a selective deterioration when infected with herpes virus. The concept is advanced that these organelles function in the development of virus.

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