THE INTRACELLULAR DISTRIBUTION OF LANSING POLIOMYELITIS VIRUS IN THE CENTRAL NERVOUS SYSTEM OF INFECTED COTTON RATS*

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Enzyme chemists have developed methods for the isolation of various cellular particulate components from tissue homogenates and have learned something of the metabolic activities of these particles (1). It would be interesting to determine the intracellular location of poliomyelitis virus by similar technics. Such information can serve as a beginning for metabolic studies of normal and virus-infected neurons which may yield clues to the site and manner of virus reproduction in the infected cell.

In the present study an attempt was made to determine the distribution of virus infectivity among the intracellular particles isolated by centrifugation from the brains and cords of cotton rats infected with Lansing virus. The reliability of estimates of such virus distributions depends in great measure upon the reproducibility of the bioassay method. An analysis was made, therefore, of the variability of the titrations done to determine relative virus concentrations, and the significance of the experimental data obtained was considered in the light of this variation.

Materials and Methods

Virus.—The Lansing strain of poliomyelitis virus used for this study was obtained from Armstrong (2) September 27, 1950, in the form of an infected mouse brain and cord representing the 379th mouse passage. It was passed twice through cotton rats in this laboratory. The second passage material was homogenized to a 20 per cent suspension in distilled water with the aid of a Waring blendor and served as a stock pool.

Assay of Virus Infectivity.—Estimates of virus infectivity of homogenates and fractions were made by the inoculation of serial threefold dilutions into Swiss mice. Each dilution was inoculated intracerebrally into eight mice. The volume of the inoculum was 0.02 to 0.03 ml. Fifty per cent infectivity end-points (ID_{50}) were calculated by the Reed and Muench method (3). They represent dilutions of original tissue which infect half the animals within the period of 3 weeks. Specific infectivity end-points similarly calculated refer to the concentrations of *protein* in grams per milliliter which infect half the mice within the same time period. The latter method of expressing infectivity titer serves as an index of the relative concentration of virus in the fractions compared.

Statistical Estimate of the Reproducibility of Titration End-Points.—Of the 68 assays of virus infectivity carried out in this study, 11 were randomly chosen and a standard error

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(S.E.) calculated for each by the method of Pizzi (4, 5). When the ID_{50} were expressed as the negative log of the dilution of tissue infecting half the rats inoculated, the average S.E. for the 11 titrations was found to be 0.17 log unit and the range 0.15 to 0.19.

In order to test the significance of a difference between any two titration end-points, the S.E. of the difference between end-points must be calculated by the formula

$$\text{S.E. difference} = \sqrt{\text{S.E.}_1^2 + \text{S.E.}_2^2} \quad (1)$$

in which S.E._1 and S.E._2 are the standard errors of the ID_{50} of the two titrations under consideration. Since the same number of animals, dilution interval, and precision of technic were used for all titrations, the average value of 0.17 may be used for the S.E. of any two titration end-points in equation (1) which would then reduce to

$$\text{S.E. difference} = 0.17\sqrt{2}$$

or 0.24. Any difference between 50 per cent infective doses should be at least twice this value to be considered outside the chance range. Threefold or greater differences between the relative infectivity of any two fractions assayed, therefore, were considered significant.

Fractionations.—Homogenates of freshly harvested tissue from infected cotton rat central nervous system (CNS) were prepared in sterile 0.44 M sucrose solution adjusted to pH 7.4 with NH_4OH. Animals that showed definite paralysis 48 hours after intracerebral inoculation with 1 per cent virus stock pool were anesthetized with ether and perfused with cold 0.44 M sucrose solution. The brains and cords were excised quickly and aseptically, weighed, and immediately placed in an ice-chilled, sterile glass homogenizer (6) containing sucrose solution. The tissue was homogenized for approximately 2 minutes at 4° C. and diluted with 0.44 M sucrose solution to yield a 20 per cent suspension of tissue. The homogenate was filtered through sterile linen and immediately adjusted from approximately pH 6.7 to pH 7.4 with 0.1 N NH_4OH. All subsequent manipulations of the virus-infected homogenates were carried out at 0 to 4° C. Each homogenate represented CNS tissue from 4 to 8 cotton rats.

In general, homogenates were separated into four principal fractions in hypertonic sucrose solutions by a modification of the centrifugation method developed for liver tissue suspensions by Hogeboom, Schneider, and Palade (7). Preliminary experiments have shown that early removal of inorganic salts and low density lipid material from CNS homogenates facilitated subsequent fractionation of particulate components. The soluble fraction, *S*, was removed first, therefore, by the highest centrifugal field applied in the fractionation procedure, in contrast to the method used on liver homogenates. A flow diagram outlining the procedure is presented in Fig. 1.

It was found that the most satisfactory separation of fractions in the centrifuge resulted when a suspension of particles of grossly different sizes was layered over pure sucrose solution of slightly higher density. This permitted the sedimentation of the largest particles to form a pellet virtually free of slower sedimenting components. Consequently, pellets were always resuspended in 0.40 M sucrose and the suspension sedimented through an equal volume of 0.44 M sucrose solution if further fractionation was desired.

Fifteen to 30 ml. of 20 per cent homogenate was layered over an equal volume of 0.44 M sucrose solution in lusteroid tubes and centrifuged for 90 minutes at 0° C. in an average centrifugal field of 102,000 *g*. High speed centrifugations were all done in the preparative Spinco centrifuge, model L. The supernatant liquid or soluble fraction, *S*, was aspirated and saved. The sediment was resuspended to the volume of the original 20 per cent homogenate in 0.40 M sucrose and homogenized for 1 minute. It was then layered over an equal volume of 0.44 M sucrose and centrifuged for 10 minutes at 600 *g* in a clinical centrifuge. Cell debris and nuclei sedimented to form a gelatinous pellet from which the supernatant liquid was care-

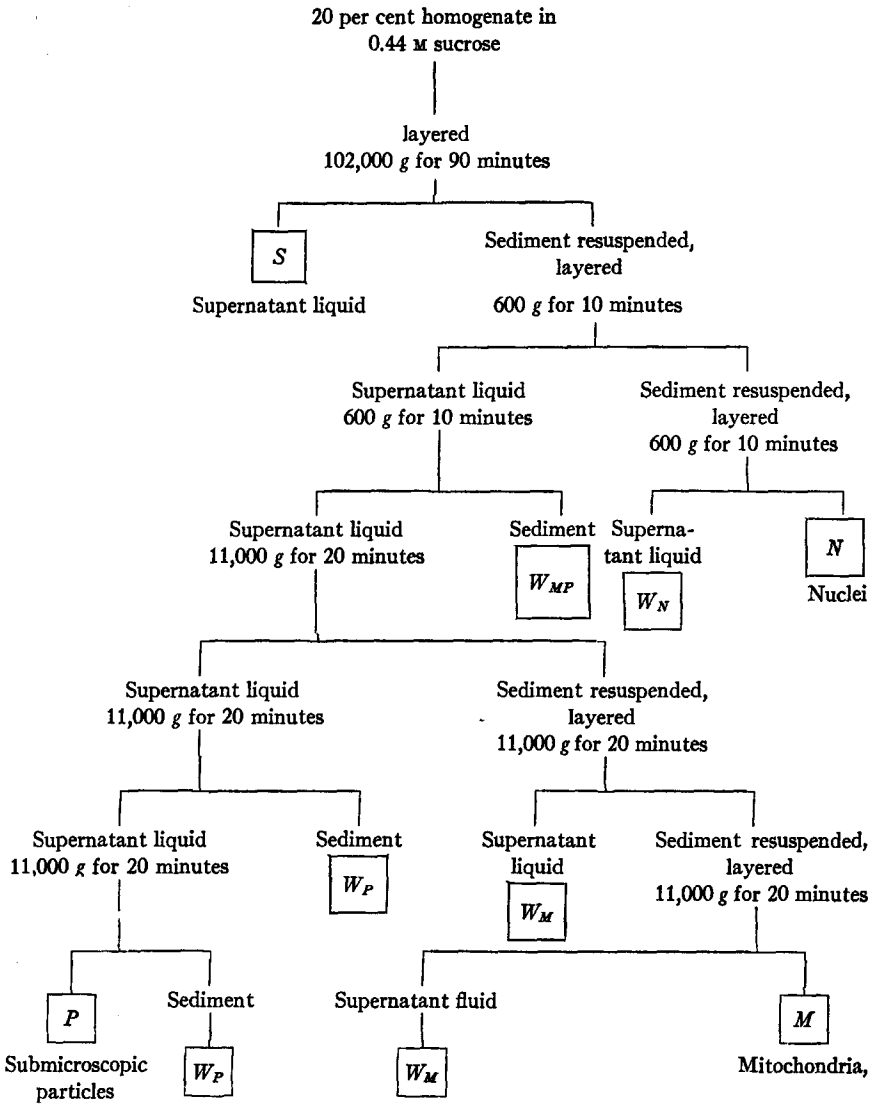

FIG. 1. Flow diagram of fractionation procedure.

fully removed and saved. The pellet was homogenized, layered, and centrifuged again at 600 *g*. The supernatant liquid which is the nuclear wash, W_N, was removed and the pellet resuspended to original volume. This suspension which consisted principally of nuclei and cellular debris was referred to as fraction *N*.

The supernatant liquid from the first centrifugation at 600 *g* was centrifuged at 600 *g* to remove any residual nuclei (W_{MP}) and then fractionated further into mitochondria and sub-

microscopic particles by centrifugation at 11,000 *g* for 20 minutes. The resulting pellet, which consisted of a brown core covered by a fluffy white layer, was subsequently found to contain virtually all the mitochondria. It was usually washed twice by resuspension in 0.40 M sucrose, layering over 0.44 M sucrose, and centrifugation at 11,000 *g* for 20 minutes. The washed mitochondria, referred to as fraction *M*, were resuspended to the volume of the original homogenate in 0.44 M sucrose.

The supernatant liquid containing the submicroscopic particles was washed free of mitochondria by one or two centrifugations at 11,000 *g* for 20 minutes but without layering and was referred to as fraction *P*. Any variation of the above fractionation procedure is stated in the text of the experimental section.

The effectiveness of the fractionation of the cellular particulate components was followed microscopically and biochemically. The distribution of nuclei between the *N* and *M* fractions and their washings was estimated using the aceto-orcein-fast-green stain of Kurnick and Ris (8). By means of this technic nuclear material is stained red-brown while cytoplasmic constituents appear green. Mitochondria were identified cytologically by their size and ability to stain vitally with Janus green B (9). For various centrifugal fractions a direct correlation was noted between the concentration of such particles and the succinic dehydrogenase content. From these observations it was concluded that the intact mitochondria contain all the succinic dehydrogenase present in the homogenate as had been found for liver tissue (7).

The centrifugal separation of *P* and *S* fractions is an arbitrary one based upon the relative particle sizes and densities of their constituents. This fractionation cannot be followed microscopically and does not lend itself readily to a biochemical identification of the fractions since both undoubtedly contain particles of numerous kinds. One biochemical characteristic which helped to distinguish *P* from other fractions was its high pentose nucleic acid phosphorus/nitrogen ratio which was determined for all fractions in several experiments.

Biochemical Assays.—Early in the study protein analyses were done by the biuret method (10) but all subsequent determinations were made by Kjeldahl digestion followed by the Koch and McMeekin modification of the Nessler reaction (11). Since the biuret values were consistently higher, they have been corrected by an appropriate factor arrived at by a comparison of the two methods applied to analogous fractions. The turbidity of the protein solutions appeared to be responsible for the falsely high protein values determined colorimetrically by the biuret test. Nitrogen determinations by the Nessler reaction were always made on cold 5 per cent trichloracetic acid (TCA) precipitates of the fractions to be analyzed.

Trichloracetic acid precipitates washed twice with cold 5 per cent TCA and twice with 95 per cent alcohol at room temperature were analyzed for pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA) in two ways. In one procedure the nucleic acids were extracted from the precipitate with hot TCA and aliquots analyzed directly for PNA and DNA by the orcinol and diphenylamine reactions, respectively, according to Schneider's method (12). The second method was that of Schmidt and Thannhauser as used by Schneider (13) in which the PNA and DNA are separated quantitatively on the basis of their relative resistances to hydrolysis in warm alkali. The unhydrolyzed DNA was separated from the hydrolyzed PNA by precipitation with TCA and assayed by the diphenylamine and orcinol color reactions, respectively. The latter method was preferred for CNS homogenates since a violet color not due to DNA was obtained with the diphenylamine reaction when the extraction with warm alkali was not used.

The succinic dehydrogenase activity of homogenates and various fractions was assayed by the manometric method described by Schneider and Potter (14). Results were expressed as microliters of oxygen taken up per 60 minutes by the material obtained from 1 ml. of 10 per cent homogenate.

EXPERIMENTAL RESULTS

1. The Distribution of Infectivity among Particulate Components of the CNS Cells of Infected Cotton Rats.—Experiments on the centrifugal fractionation of infected CNS homogenates in 0.44 M sucrose solution into nuclei, mitochondria, submicroscopic particles, and soluble material revealed a consistent trend with regard to the location of the infectivity. The bulk of the virus activity appeared in the submicroscopic particle fraction, *P*, which also possessed the highest specific infectivity, indicating that the ratio of virus to non-viral substances was greatest in this fraction.

The conditions for the initial separation of fraction *S* from the remaining fractions were decided upon after a series of preliminary experiments in which samples of layered homogenate were centrifuged at 102,000 *g* for 90 minutes in sucrose solutions of different concentrations. Decreasing amounts of infectivity were found in the *S* fraction with decreasing concentrations of sucrose from 1.32 M to 0.44 M. In the most dilute sucrose solution less than 2 per cent infectivity was consistently found in fraction *S*. This concentration of sucrose was chosen, therefore, for all fractionation experiments since it served not only to separate infectivity sharply between fractions *S* and *P* but also maintained the integrity of the mitochondria by virtue of its hypertonicity (7).

Succinic dehydrogenase activity measurements of the *M* and *P* fractions indicated 11,000 *g* as the minimum average field applicable which still resulted in essentially complete sedimentation of succinic dehydrogenase in the *M* fraction.

The distribution of infectivity, protein, and succinic dehydrogenase activity of six fractionation experiments is summarized and averaged in Table I. These results indicate that the *P* fraction contains on the average four-fifths of the isolable infectivity and has a specific infectivity three times that of the original homogenate. Fourteen per cent of the total homogenate protein is present in fraction *P* but only 2 per cent of the succinic dehydrogenase activity.

A comparison of the relative infectivities of analogous fractions of these six experiments shows considerable irregularity. This is a reflection largely of the variability of the animal assay for infectivity since a statistical analysis has shown that differences among relative infectivities must be three fold or greater to be considered significant. The trend in the distribution of per cent recoveries of infectivity among the fractions of any one experiment, nevertheless, is consistent. It should be emphasized at this point that a precise knowledge of the amount of virus present in each fraction is not implied by the figures recorded in Table I for the per cent recovery of infectivity. They merely serve as guides for a rough approximation of the distribution of the virus.

2. Attempts to Dissociate Virus Infectivity from Cellular Particles.—Small amounts of infectivity were regularly found associated with nuclear and mito-

chondrial fractions. It was of interest to determine whether infectivity was firmly bound to or loosely held by these particles.

(*a*) *Effects of Varying pH.*—An attempt was made to determine the effect of pH upon the distribution of infectivity among the particulate components. Aliquots of a 30 per cent homogenate of infective tissue in 0.44 M sucrose were adjusted to pH 5.8, 7.7, and 9.0 with 0.15 M phosphate or veronal buffers in 0.44 M sucrose. The final concentrations were 20 per cent homogenized CNS in 0.44 M sucrose and 0.05 M buffer. When these homogenates were subjected

TABLE I

Distribution of Infectivity, Protein, and Succinic Dehydrogenase Activity among the Particulate Cellular Components of Homogenates of Lansing-Infected Cotton Rat CNS

Experiment No.	Relative infectivity* of Fractions§					Per cent‡ protein in Fractions						Per cent recovery‡ of infectivity in Fractions						Per cent succinic‡ dehydrogenase activity in Fractions					
	N	*M*	*P*	*S*	*W*	*N*	*M*	*P*	*S*	*W*	Fraction total	*N*	*M*	*P*	*S*	*W*	Fraction total	*N*	*M*	*P*	*S*	*W*	Fraction total
1‖	4	25	500	10	—	6	25	7	15	23	76	0.3	6	36	1.5	—	44	—	—	—	—	—	—
2	30	25	100	—	—	8	35	7	—	17	—	2.6	9	37	—	—	49	4	68	1	—	—	>73
3	30	20	60	1	—	7	28	13	24	34	106	2	5	8	0.2	—	15	3	77	4	1	22	107
4	25	10	400	—	—	4	20	18	25	28	95	1	2	72	—	—	75	2	58	2	—	—	>62
5	130	30	600	<2	—	5	30	20	17	30	102	6	10	127	0.3	—	143	2	74	2	—	4	82
6	50	7	50	1	—	12	29	21	23	19	104	6	3	16	0.2	—	25	12	54	2	0	8	76
Average .	45	20	285	4	—	7	28	14	21	25	97	3	6	49	0.6	—	59	5	66	2	0	11	>80

* A comparison of the specific infectivities of the fractions with those of the original homogenate. The latter is arbitrarily assigned the value 100.

‡ Whole homogenate equals 100 per cent.

§ Fractions: *N* = nuclei, *M* = mitochondria, *P* = submicroscopic particles, *S* = soluble fraction, *W* = combined washings.

‖ Experiment 1 was assayed for infectivity by inoculating serial decimal dilutions instead of the usual threefold dilutions.

to the usual centrifugal fractionation procedure, 50 to 60 per cent of the total protein was found in fraction *N* instead of the usual 7 per cent obtained from unbuffered homogenate. Regardless of the pH, aggregation of mitochondria and submicroscopic particles occurred at this salt concentration making a fractionation of the particulate components impossible. Alteration of pH did affect the sedimentation characteristics of the infective particles, however, since approximately 90 per cent of the infectivity was found in the atypically voluminous *N* fraction at pH 5.8, 45 per cent at pH 7.7, and 20 per cent at pH 9.0.

If the pH of an infected CNS homogenate was adjusted to 9 with NH_4OH, the ionic strength of the homogenate was sufficiently low to permit a fractionation typical with respect to protein, succinic dehydrogenase, and infectivity

distribution. The results of the fractionation of two aliquots of an infected homogenate, one adjusted to pH 9 with NH_4OH and the other with veronal buffer, are compared in Table II.

It is difficult to assess the importance of H ion concentration with regard to the distribution of infectivity among the particulate cell components of CNS homogenates since an increase in electrolyte concentration by the buffers employed to adjust pH caused aggregation of the liberated particulate components of the ruptured cells. In the one case in which the pH of a homogenate was raised to 9 with NH_4OH of low concentration, no difference was found in the distribution of infectivity from that observed in the usual fractionation at pH 7.4.

TABLE II

Infectivity, Protein, and Succinic Dehydrogenase Activity Distributions among Centrifugal Fractions of Homogenates Adjusted to pH 9 with NH_4OH and Veronal Buffer

Fractions*	Buffer	Relative‡ infectivity	Per cent§ recovery of infectivity	Per cent§ protein	Per cent§ succinic dehydrogenase activity
N	0.01 *N* NH_4OH	30	3	8	4
M		25	9	35	68
P		100	37	37	1
N	0.05 M veronal	20	9	42	32
M		10	2	18	29
P		1000	71	7	0

* See Table I.
‡ See Table I.
§ See Table I.

(*b*) *Effect of Repeated Washing of Mitochondria.*—If the small amount of virus infectivity associated with the mitochondria is an integral part of such particles or of particles similar in sedimentation characteristics to mitochondria, repeated washings in the centrifuge should not result in a loss of virus from the mitochondrial fraction or a lowering of its specific infectivity. On the other hand, if virus infectivity is weakly adsorbed to the mitochondrial surfaces, repeated centrifugal washings should result in an elution of virus infectivity from fraction *M*.

To test this point, a 20 per cent homogenate of infected cotton rat CNS was prepared in 0.44 M sucrose and adjusted to pH 7.4 with NH_4OH in the usual way. It was layered over an equal volume of 0.44 M sucrose and centrifuged at 11,000 *g* for 20 minutes to sediment nuclei and mitochondria. The supernatant liquid containing fractions *P* and *S* was discarded while the sediment was rehomogenized and diluted in 0.44 M sucrose to a volume equal to that of the original homogenate. This suspension in turn was centrifuged at 600 *g* for 10 minutes to

sediment nuclei. The supernatant liquid, M, containing the mitochondria was sampled for protein analysis, succinic dehydrogenase activity, and virus infectivity assays. It was then layered over an equal volume of 0.44 M sucrose and centrifuged at 11,000 g for 20 minutes, the supernatant liquid, A, removed, and the sedimented mitochondria, M_1, resuspended to the original homogenate volume. After removal of a small sample for analysis and assays, the M_1 suspension was again layered over sucrose, centrifuged at 11,000 g for 20 minutes and the supernatant liquid B and sediment M_2 separated. The mitochondria were then washed twice again for a total of four times. The results of protein analyses and enzyme and virus activity assays of the mitochondria after each washing by centrifugation and also those of their corresponding supernatants are summarized in Table III.

TABLE III

The Effect of Repeated Washings upon the Succinic Dehydrogenase Activity and Virus Infectivity of the Mitochondrial Fraction

Fractions*	Per cent of original homogenate protein	μl. O_2/60 min.‡ / ml.	μl. O_2/60 min. / mg. protein	ID_{50}§	Specific‖ ID_{50}	Per cent recovery of infectivity
H	100	564	41	4.5	5.4	100
M	47	468	72	3.7	4.9	16
M_1	33	432	96	3.5	4.8	10
M_2	26	345	96	3.2	4.6	5
M_3	25	336	96	3.1	4.6	4
M_4	26	318	88	2.8	4.2	2
A	17	3	1	—	—	—
B	4	—	—	—	—	—
C	1	4	20	—	—	—
D	1	0	0	—	—	—

* See text.

‡ Results are expressed as microliters of oxygen taken up per 60 minutes by the material obtained from 1 ml. of 10 per cent homogenate.

§ Negative log of dilution of tissue which infects half the intracerebrally inoculated mice within 3 weeks.

‖ Negative log of the concentration of protein in gm./ml. which infects half the intracerebrally inoculated mice within 3 weeks

These data show that the composition of the mitochondrial fraction remained fairly constant with respect to protein and succinic dehydrogenase content throughout all four centrifugal washings. The total and specific virus infectivity, on the other hand, appeared to diminish gradually. The differences in titers between successive washings are certainly within the experimental error range of the bioassay method. The 0.7 of a log unit difference between the ID_{50} of the one time– and four times–washed mitochondria, however, was found to lie outside the chance range (0.48 log unit). The small amount of infectivity associated with the mitochondrial fraction apparently can be eluted with some difficulty from the mitochondrial particles or from particles of similar size and density which cannot be separated from mitochondria by centrifugal methods.

3. Further Centrifugal Fractionation of the Submicroscopic Particle Fraction. —A rough estimate of the particle sizes isolated in the submicroscopic particle fraction indicated they might range from 20 mμ to 400 mμ. An attempt was made, therefore, to effect a greater concentration of the infective particle by further centrifugal fractionation of the *P* fraction.

The submicroscopic particle fraction was obtained in 0.44 M sucrose in the manner described under Materials and Methods. It was then subjected to a centrifugal field of 23,000 *g* for 20 minutes. The resulting pellet, P_1, was readily separated from the supernatant fluid. The latter was centrifuged twice again: first at 48,000 *g* for 20 minutes to yield pellet P_2 and subsequently at 81,000 *g* for 30 minutes to give a final pellet, P_3, and the supernatant fluid P_4. All three pellets, which were firm and amber colored, were easily resuspended in a volume of 0.44 M sucrose equal to that of the original *P* fraction. These four subfractions of *P* were then assayed for infectivity and analyzed for protein and in one experiment for pentose nucleic acid and desoxypentose nucleic acid. The results of three experiments are listed in Table IV.

There is no marked concentration of virus infectivity in any of the four subfractions of *P* although it appears to increase to a slight maximum in subfraction P_3. The rise in PNA phosphorus/protein ratio from P_1 through P_4 shows that the physical separation of the particles in fraction *P* on the basis of sedimentation characteristics, though arbitrary, did yield fractions of differing chemical composition. These results indicate that an original submicroscopic particle fraction contains particles which vary with respect to nucleic acid content as well as physical properties.

The widespread distribution of total infectivity among the four subfractions of *P* suggests either that the virus particles have suffered various degrees of aggregation or have adsorbed to particles of various sizes or that the centrifugal fractionation procedure employed was inefficient in resolving particles of one size from an array of particles embracing the size range found in *P*. The following experiments using bacterial chromatophores and bushy stunt virus particles were performed in an attempt to elucidate this point.

In the first experiment a submicroscopic particle fraction obtained from a homogenate of normal cotton rat CNS in 0.44 M sucrose was mixed with 0.1 volume of concentrated chromatophores isolated from *Rhodospirillum rubrum*. These particles which have a high extinction coefficient at $\lambda = 880$ mμ are spherical with an anhydrous diameter of approximately 60 mμ and are obtained in a fairly homogeneous state by high speed centrifugation from *R. rubrum* ruptured by sonic irradiation (15). The chromatophore—*P* fraction mixture was centrifugally fractionated as above into subfractions P_1, P_2, P_3, and P_4 and each subfraction assayed for chromatophore concentration by determining its optical density in a Beckman spectrophotometer at wave length 880 mμ. Accuracy of measurement was within 10 per cent. Optical density readings corrected for turbidity and non-chromatophore absorption were 0.17, 0.21, 0.31, and 0.25 for fractions P_1 through P_4, respectively.

In the second experiment a suspension of purified bushy stunt virus (BSV)[1] was subjected to a centrifugal fractionation similar to that applied to the bacterial chromatophores. In this

[1] We are indebted to Dr. C. A. Knight for this preparation of purified BSV.

case, however, the BSV was suspended in 0.44 M sucrose alone rather than in a sucrose solution of submicroscopic particles from the CNS homogenate of a normal cotton rat. These virus particles are spherical and have an anhydrous diameter of approximately 26 mμ (16). The concentration of BSV in the four subfractions analogous to P_1 through P_4 was determined with an accuracy of 10 per cent by spectrophotometry at a wave length of 260 mμ.

The distribution of BSV particles in the four centrifugal subfractions is compared in Table V with that of bacterial chromatophores and poliomyelitis virus infectivity in the P_1 through P_4 subfractions. The poliomyelitis virus infectivity

TABLE IV

The Distribution of Infectivity, Protein, and Nucleic Acids among the Centrifugal Subfractions of the Submicroscopic Particle Fraction, P

Fractions	Relative infectivity* in Experiments				Per cent protein‡ in Experiments				Per cent recovery‡ of infectivity in Experiments				γPNA-*P* / mg. protein	γDNA-*P* / mg. protein
	5	6	7	Av.	5	6	7	Av.	5	6	7	Av.	Experiment 7	Experiment 7
H§													1.15	0.85
N	130	50	—	90	5	12‖	—	9	6	6	—	6	—	—
M	30	7	—	20	30	29	—	30	10	3	—	7	—	—
P_1	300	20	250	190	10	8	7	8	32	2	18	18	1.3	0.7
P_2	1000	100	400	500	3	3	4	3	30	3	17	17	2.0	0
P_3	1250	160	1000	800	3	2	3	3	38	4	34	25	3.2	0.5
P_4	800	100	160	350	4	8	8	7	27	7	8	14	3.7	0
S	<3	1	—	<2	20	23	—	22	<0.3	0.2	—	0.2	—	—
Washings	—	—	—	—	33	19	—	26	—	—	—	—	—	—
Fraction total....					108	104	—	108	143	25	—	87		

* See Table I.

‡ See Table I.

§ Whole homogenate.

‖ This nuclear fraction was not washed.

and the chromatophore particles revealed a similarity in distribution among analogous subfractions, with each attaining a maximum concentration in subfraction P_3. BSV, in the absence of any *P* fraction proteins, also showed a widespread distribution among the four subfractions but with a peak concentration in fraction P_4. Since the suspension of BSV is one of homogeneous particles, it was concluded that the distribution of chromatophores or of poliomyelitis virus in a suspension of CNS microsomes is due to the inefficiency of centrifugal fractionation rather than to adsorption to submicroscopic particles of different sizes. The fact that the maximum concentration of chromatophores was found in P_3 while that of BSV was found in P_4 can be explained by the difference in particle sizes.

The exact distribution of poliomyelitis virus infectivity among the four subfractions of P and an estimate of the mass of the infectious particle may be questioned owing to the inaccurate assay method for virus activity. The patterns of distribution of infectivity are similar in the three experiments recorded in Table IV, however, and correspond roughly to those found for chromatophores and BSV. It is postulated, therefore, that the virus particles in fraction P are free in solution and not adsorbed to submicroscopic particles of various sizes.

4. Fractionation of Homogenates of Normal Cotton Rat CNS Contaminated with Active Virus.—In two experiments the distribution of virus infectivity was determined among the principal particulate fractions obtained from a normal cotton rat CNS homogenate to which partially purified Lansing virus had

TABLE V

Comparison of the Distributions of Poliomyelitis Virus Infectivity, Chromatophore Concentration, and BSV Concentration in 0.44 M Sucrose upon Centrifugal Fractionation

Subfractions	Per cent virus infectivity or particle concentration in the four subfractions of:		
	P fraction of poliomyelitis-infected CNS homogenate*	*P* fraction of normal CNS homogenate + chromatophores	Purified BSV in 0.44 M sucrose
P_1 — sediment at 23,000 *g* for 20 min.	24	18	6
P_2 — sediment at 48,000 *g* for 20 min.	23	22	17
P_3 — sediment at 81,000 *g* for 30 min.	34	33	33
P_4 — supernate at 81,000 *g* for 30 min.	19	27	44

* This column represents the average of the three experiments listed in Table IV.

been added. The experiments were carried out for the purpose of distinguishing between adsorption and poor centrifugal separation.

(a) Normal Homogenate Contaminated with Partially Purified Virus Concentrate.—

A 20 per cent homogenate of CNS from a normal cotton rat was prepared in 0.44 M sucrose to which had been added a Lansing poliomyelitis virus concentrate prepared by the Loring and Schwerdt method (17). Electron micrographs of such preparations had shown the particle size range of 15 to 34 mμ to predominate (18). The Lansing-contaminated normal homogenate was fractionated in the centrifuge in the usual way to yield the four principal fractions N, M, P, and S. The mitochondria which are obtained by sedimentation at 11,000 *g* for 20 minutes were resuspended and fractionated further by centrifugation at 2,000 *g* for 10 minutes. The sedimented fraction was referred to as M_1 while the supernatant liquid was called M_2. Further fractionation of the submicroscopic particle fraction as described in the preceding section yielded subfractions P_1, P_2, P_3, and P_4. Virus and succinic dehydrogenase assays as well as protein and pentose and desoxypentose nucleic acid analyses were made of all fractions. The results are listed in Table VI.

The distribution of protein and succinic dehydrogenase activity was similar to that found in fractions of homogenized infected tissues. The pentose nucleic acid/protein ratios increased from fraction P_2 through P_4 as had also been found previously with infectious tissue fractions, while the desoxypentose nucleic acid/protein ratios were compatible with what would be expected if the nuclei were isolated intact. Virus infectivity, however, was distributed largely among the last three subfractions P_2, P_3, and P_4 with little or none found associated with P_1, nuclear, and mitochondrial fractions.

TABLE VI

Distribution of Virus Infectivity, Succinic Dehydrogenase Activity, Protein, and Pentose and Desoxypentose Nucleic Acids among Particulate Components of Homogenate of Normal Cotton Rat CNS Contaminated with a Purified Virus Concentrate

Fractions	Relative* infectivity	Per cent protein	Per cent recovery of virus	Per cent succinic dehydrogenase activity in	$\frac{\gamma\text{PNA-}P}{\text{mg. protein}}$	$\frac{\gamma\text{DNA-}P}{\text{mg. protein}}$
H	(100)	(100)	(100)	(100)	1.6	0.9
N	<40	4	<1	4	1.6	8.7
M_1	<10	13	<2	25	0.7	1.0
M_2	<10	15	<2	31	0.7	0.1
P_1	80	4	3	2	1.1	0
P_2	500	6	29	1	0.9	0
P_3	1000	5	45	<1	1.9	0
P_4	400	8	32	<1	3.9	0.3
S	<4	21	<1	<1	1.0	0

* See Table I.

(b) Homogenate of Normal Tissue Prepared in an Infectious P_4 Fraction.—

Subfraction P_4 was prepared from a 0.44 M sucrose homogenate of the CNS from an infectious cotton rat and served as solvent for the homogenization of CNS tissue from a normal cotton rat. The normal homogenate, contaminated with virus infectivity and submicroscopic particles of the smallest size, was then fractionated by centrifugation as described in the preceding experiment. Table VII summarizes these data.

The results are similar to those found in the preceding experiment, in which a normal homogenate was contaminated with a partially purified virus concentrate, with the exception that the P_1 subfraction contained an amount of infectivity equivalent to that found in the *lighter* subfractions of P and the nuclear fraction contained a small yet measurable amount of infectivity. It would appear, therefore, that virus infectivity introduced as a P_4 fraction into a normal CNS homogenate was distributed among the subfractions P_1 through P_4 and possibly adsorbed to nuclei but not to mitochondria.

The fractionation of normal CNS homogenates contaminated with partially

purified virus resulted, for the most part, in the localization of the infectivity in the subfractions of *P* with little or none found in nuclear and mitochondrial fractions. This evidence suggests that the association of infectivity with the nuclei and mitochondria of infected CNS homogenates may be due not to poor centrifugal separation but possibly to adsorption or to the fact that the virus s an integral part of these large particles.

5. Succinic Dehydrogenase Activity.—In the course of this work five determinations of succinic dehydrogenase activity of normal CNS and ten determinations on infected CNS homogenates were performed. In view of the observation by Howe and Flexner (19) that the anterior horns of spinal cord en-

TABLE VII

Distribution of Virus Infectivity, Succinic Dehydrogenase Activity, Protein, and Pentose and Desoxypentose Nucleic Acids among Cellular Particulate Components of Normal Cotton Rat CNS Tissue Homogenized in Infectious P_4 Fraction

Fractions	Relative* infectivity	Per cent protein	Per cent recovery of virus	Per cent succinic dehydrogenase activity in	$\frac{\gamma PNA\text{-}P}{mg.\ protein}$	$\frac{\gamma DNA\text{-}P}{mg.\ protein}$
H	(100)	(100)	(100)	(100)	1.3	0.9
N	100	6	6	3	1.1	12.2
M_1	<10	14	<1	29	0.6	0.6
M_2	<10	13	<1	24	0.7	0.5
P_1	400	8	34	2	1.7	0.6
P_2	800	4	35	0	2.4	2.0
P_3	1300	2	29	0	4.0	0
P_4	250	5	13	0	4.2	0.5
S	—	18	—	—	1.1	0.2
Washings	—	15	—	13	1.2	1.4

* See Table I.

largements of infected monkeys contained less succinic dehydrogenase activity than normal tissue, it seemed of interest to compare the activities. The normal tissue had a $QO_2(N) = 408$ with a standard deviation of 50 (range 350 to 470) while the infected tissue had a $QO_2(N) = 347$ with a standard deviation of 49 (range 270 to 420). Since the difference of 61 between these means is 2.2 times the standard deviation of the means, 27, the probability that the results could differ by chance is less than 0.05 and, hence, we conclude that the succinic dehydrogenase activity of infected CNS is significantly less than that of normal tissue. The true difference between the enzyme activity of virus-infected and normal cells is in all probability much greater than our data would indicate since the mass of neurons affected by the virus of poliomyelitis represents only a small fraction of the total CNS (20).

DISCUSSION

By the application of a relatively mild method of homogenization and fractionation, cellular particulate components of poliomyelitis virus-infected CNS have been separated. A large part of the total activity and high specific infectivity has been found to reside predominantly in the submicroscopic particle fraction. Experiments on further centrifugal fractionation showed a wide distribution of infectivity among the subfractions of the submicroscopic particle material. Evidence is presented, however, for the existence of most of the virus in a free state. Small yet measurable amounts of infectivity were found in nuclear and mitochondrial fractions isolated from infectious homogenates. Infectivity could be washed from such mitochondria only with difficulty. When homogenates of normal CNS were contaminated with partially purified poliomyelitis virus, almost all the added infectivity was isolated with the submicroscopic particle fraction, none was found associated with the mitochondria, and a small amount was found in the nuclear fraction of only one of two such fractionation experiments. Virtually no infectivity was found in the *S* fraction which consisted principally of low molecular weight proteins and soluble substances of a non-protein nature.

The above evidence suggests that in the intact neuron the greatest portion of active poliomyelitis virus is free or associated with the microsome fraction. The fact, however, that a small amount of infectivity is always associated with the nuclei and mitochondria of infected homogenates but is not isolated with the latter particles when a normal CNS homogenate is contaminated with partially purified virus suggests that the association between virus particles and mitochondria in the infected cell might be more than simple adsorption. The data are insufficient, however, to attach too great significance to this finding.

The inference that most of the virus is free or associated with the submicroscopic particle portion of the intact neuron is always subject to the criticism that the distribution of virus found among the isolated fractions of the tissue homogenate is an artifact and does not represent the distribution in the whole infected cell. Hence the question may properly be asked: Is most of the virus infectivity found in the microsome fraction because homogenization results in the release of the infectious particles from mechanically ruptured nuclei or mitochondria? This seems unlikely. DNA phosphorus assays of all fractions showed that most of the DNA was present in fraction *N* which contains intact nuclei as shown by the aceto-orcein staining method. Succinic dehydrogenase activity is almost solely recovered in the *M* fraction which is evidence for unbroken mitochondria. The homogenization of soft tissues in isotonic or slightly hypertonic sucrose solution by means of a Potter-Elvehjem glass homogenizer has generally been found to be a mild method of breaking cells which leaves the nuclei and large organized particles of the cytoplasm whole (1).

It must be borne in mind, however, that the virus-infected neurons represent

but a small fraction of the total CNS and it is conceivable that the nuclei or mitochondria of this small sample of cells are easily ruptured by this method of homogenization and do release virus in large quantities. Hence caution must be exercised in postulating the intracellular location of the virus infectivity.

An estimation of the intracellular distribution of poliomyelitis virus is greatly complicated by the variability of the bioassay for infectivity. Titers of any one fraction are subject to errors as great as 300 per cent. Confidence in the significance of a difference in virus content between any two fractions is dependent, therefore, upon the reproducibility of distribution patterns in repeated fractionation experiments. In our opinion the results of the six fractionation experiments on infectious homogenates recorded in this study show that the great bulk of the virus infectivity was isolated with the microsome fraction.

SUMMARY

A procedure has been described for the centrifugal fractionation of the cellular particulate components of CNS tissue infected with poliomyelitis virus. A study of the distribution of infectivity among these components revealed that approximately four-fifths of the virus is found free in the submicroscopic particle fraction. The validity of the conclusion that the virus is located in this fraction of the intact neuron is discussed.

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