Centrifugation Studies on the Infectivities of Cellular Fractions Derived from Mouse Brain Infected with Scrapie ('Suffolk strain')

BY D. L. MOULD, W. SMITH AND A. McL. DAWSON

Animal Diseases Research Association, Moredun Institute, Gilmerton, Edinburgh

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SUMMARY

Cellular components in homogenates of brain tissue from scrapie-affected mice have been separated by centrifugation in sucrose and caesium chloride density gradients with the objective of location of the scrapie agent and concentration of scrapie activity. Improvements in the relative activity of the fractions removed from sucrose gradients were small but recovery was high in the material sedimented through 0.88 M-sucrose. No peak of activity was observed in zones removed from a caesium chloride gradient and activity throughout remained strongly associated with particulate matter. Ultrasonic disruption had little effect on scrapie activity. Concentrated preparations of sufficient potency for characterization of virus particles by electron microscopy were not obtained. From various experimental evidence the scrapie agent, if a virus, appears to be of small size. The strong association of the agent with tissue debris suggests that the presence of a tissue component may be necessary in the experimental transmission of the condition.

INTRODUCTION

Recent experimental work on the condition of scrapie in animals has emphasized the strong association of the active agent with particulate cell fractions in brain homogenates (Mould & Smith, 1962; Hunter, Millson & Chandler, 1963; Hunter, Millson & Meek, 1964; Mould, Smith & Dawson, 1964). This has been further substantiated by chromatographic studies of tissue homogenates on DEAE-cellulose columns by Hunter & Millson (1965). It has also been pointed out that there may be species variations in the location and binding of the agent (Mould, Smith & Dawson, 1964), and that there are both definite histopathological (Zlotnik & Rennie, 1962, 1963) and possibly biochemical differences (Slater, 1965) in the brain lesions present in diseased mice according as to whether the source of inoculum was derived from a Suffolk sheep or from material passaged in goats.

The experimental procedures reported in this paper were all carried out with the 'Suffolk strain' of mouse scrapie (Zlotnik & Rennie, 1962, 1963) which produces histopathological lesions not identical with those produced by the 'drowsy goat strain' (Chandler, 1963) investigated by Hunter *et al.* Centrifugation studies of brain tissue homogenates in sucrose or caesium chloride density gradients have been made with the primary objective of location of the binding sites of the scrapie agent and concentration of scrapie activity. Some of the results have been reported in a brief communication (Mould, Dawson & Smith, 1964).

METHODS

Animal experiments. Moredun Institute stock Swiss white mice were used for all the experiments. The mice were 3–6 weeks old when inoculated. Groups of seven mice were inoculated (0.03 ml.) by the intracerebral route with serial tenfold dilutions of the various tissue preparations and each experiment terminated 5 or 6 months after the inoculation of the mice. Cases of scrapie were examined histopathologically for the presence of scrapie lesions and LD50 values were calculated according to the method described by Mould, Dawson & Smith, 1965.

Centrifugation of brain tissue homogenates in sucrose. Tissue was provided from the brains of mice in advanced clinical stages of mouse scrapie following the third passage in mice of the Suffolk sheep strain of scrapie. Crude particulate fractions from brain homogenates were prepared by differential centrifugation in 0.32M-sucrose. Brains from three clinically affected mice (1 g.) were homogenized in 0.32M-sucrose (10 ml.) and centrifuged for 5 min. at 500g to remove readily sedimentable tissue debris. The supernatant fluid was successively centrifuged for 10 min. at 1000g, 15 min. at 20,000g, and 75 min. at 100,000g to provide a nuclear, mitochondrial and ribosomal fraction respectively. The sediments were taken up in equivalent volumes of saline (0.85%) and serial dilutions in saline prepared from the suspensions and final supernatant fluid for inoculation in mice. The nuclear fraction was not tested.

A further experiment was designed to investigate the possibility of removing scrapie inactive material by a simple centrifugation procedure in sucrose density gradients. Sucrose solutions of increasing concentration were made up in a dilute phosphate-succinate buffer at pH 7.4 (Johnson, 1962; 0.004M- K_2HPO_4 , 0.001M- KH_2PO_4 , 0.004M-sodium succinate). Brains from three clinically affected mice (1 g.) were homogenized in 0.1M-sucrose (9 ml.) and centrifuged for 30 min. at 500g. The supernatant fluid was removed and maintained in a disperse state by gentle agitation throughout the remainder of the procedure. A sample (0.5 ml.) of the supernatant fluid was layered on 1.2M-sucrose (4.5 ml.) in a small centrifuge tube (5 ml.). Three tubes were prepared and centrifuged for 30 min. at 50,000g (Spinco Model L, SW39 rotor; 25,000 rev./min.). The whole of the supernatant was rejected and the small sediment from each tube pooled and resuspended in saline (2.5 ml.). Another volume (0.5 ml.) of the original supernatant fluid was layered on a density gradient consisting of 1.2M-sucrose (1.5 ml.), 1.0M-sucrose (1.5 ml.), and 0.88M-sucrose (1.5 ml.). Three tubes were again prepared and centrifuged for 30 min. at 50,000g. The cloudy zones that formed at the 1.2M/1.0M and 1.0M/0.88M interfaces were withdrawn through the side of the tube by a syringe and corresponding zones from each tube were pooled. The procedure was repeated in a preformed gradient of 0.88M, 0.75M and 0.60M-sucrose and finally in a gradient of 0.60M, 0.44M and 0.30M-sucrose. All the interfacial layers collected were dialysed against saline and saline dilutions prepared for inoculation into mice. Protein-N in each inoculum was estimated by a micro-Kjeldahl method.

Centrifugation of tissue homogenates in caesium chloride. Brains from four clinically affected mice (1.56 g.) were homogenized in 0.85% saline (15 ml.) and centrifuged for 30 min. at 500g. Equal volumes (5 ml.) of the supernatant fluid were centrifuged for 90 min. at 120,000g (SW39 rotor; 39,000 rev./min.). One sedimented

pellet was resuspended in saline for inoculation into mice and the other resuspended in saline (1 ml.) and agitated in an ultrasonic beam (90,000 cyc./sec.; 80 W.) for 20 min. The suspension was diluted (5 ml.) and a portion (1 ml.) layered on 40% (w/v) caesium chloride (4 ml.) in a 5 ml. centrifuge tube and centrifuged for 20 hr at 100,000g (SW39 rotor; 35,000 rev./min.). No attempt was made to remove visible zones from the tube after centrifugation. Equal volumes (1 ml.) were taken successively from the top of the gradient. The final sample included the small sediment packed at the base of the tube. The five fractions from the density gradient were dialysed against saline and serial dilutions prepared for inoculation into mice.

Separation in a caesium chloride gradient was repeated with a mitochondrial-sized fraction prepared from the brains of two scrapie-affected mice according to the method described by Hunter *et al.* (1964). The brains (0.72 g.) were homogenized in 0.25M-sucrose containing 5 mM-CaCl₂. The homogenate (7 ml.) was layered on 0.88M-sucrose (15 ml.), and centrifuged for 60 min. at 50,000g (SW25 rotor; 25,000 rev./min.). The sediment was resuspended in 0.25M-sucrose (7 ml.), a sample taken for inoculation into mice, and the remainder (5.5 ml.) layered again on 0.88M-sucrose and centrifugation repeated. The sediment was resuspended in saline (5.5 ml.), a further sample taken for inoculation into mice, and the remainder (4 ml.) agitated in an ultrasonic beam for 30 min. Equal volumes (1 ml.) were then layered on 40% (w/v) caesium chloride (4 ml.) and centrifuged for 24 hr at 100,000g. Successive layers of fluid of increasing density were withdrawn from the tube contents, dialysed against saline, and serial dilutions prepared for inoculation into mice.

Table 1. *Differential centrifugation of scrapie mouse brain homogenate in 0.32M-sucrose*

Titres are calculated as $-\log_{10}$ (LD50) or in terms of protein content as $-\log_{10}$ (specific LD50). The experiment was terminated 5 months after inoculation.

Inoculum	LD50 ($-\log_{10}$)	Protein (g./ml. $\times 10^{-3}$)	Specific LD50 ($-\log_{10}$)
Initial 10% homogenate	5.03 $\pm$ 0.21	11.14	5.84 $\pm$ 0.21
Mitochondrial fraction	4.73 $\pm$ 0.40	6.09	5.86 $\pm$ 0.40
Microsomal fraction	4.46 $\pm$ 0.32	1.86	6.17 $\pm$ 0.32
Final supernatant fluid	2.07 $\pm$ 0.22	0.87	3.86 $\pm$ 0.22

RESULTS

Centrifugation of brain homogenates in sucrose

Table 1 shows the results of the titration of activity in the crude particulate fractions obtained by differential centrifugation in 0.32M-sucrose. The LD50 of each inoculum is defined as that dilution of material which induced within the stated experimental period lesions in the brain consistent with those observed in mouse scrapie in half the animals inoculated. The specific LD50 of each inoculum is defined as the concentration of protein (g./ml.) which induced scrapie in half the animals inoculated. This remained essentially constant in the original homogenate and sediments. There was a significant fall in the scrapie activity of the final supernatant fluid. Complete removal of particulate material is improbable under the density conditions of the sucrose medium.

The distribution of scrapie activity after centrifugation in the pre-formed sucrose

density gradients is shown in Table 2 and Fig. 1. The data for each sucrose concentration refer to the scrapie activity present in the layer of tissue particles that penetrate a sucrose layer of the stated concentration. Although activity was present throughout all the various layers the highest recovery was in the material sedimenting through sucrose concentrations greater than 0.75 M. There was a small increase in the

Fig. 1. The recovery of scrapie activity after centrifugation of mouse brain tissue homogenates in pre-formed sucrose density gradients. (a) Relative activity of zones as function of sucrose concentration penetrated. (b) Recovery of activity in zones as function of sucrose concentration penetrated.

Table 2. *Centrifugation of scrapie mouse brain homogenate in layered sucrose density gradients*

The data refer to the scrapie activity in the layer of tissue particles that penetrate a sucrose layer of stated concentration. The LD50 values are corrected to the same effective dilution as the 10% homogenate. Specific LD50 is expressed as protein concentration (g./ml.). The experiment was terminated 6 months after inoculation.

Sucrose conc. (M)	Density of sucrose soln.	LD50 ($-\log_{10}$)	Recovery of activity (%)*	Protein (g./ml. $\times 10^{-4}$)	Specific LD50 ($-\log_{10}$)	Relative activity†
0.10	1.012	2.05 ± 0.35	0.6	15.76	3.57 ± 0.35	0.01
0.30	1.037	2.44 ± 0.52	1.6	0.56	5.48 ± 0.52	1.0
0.44	1.056	2.55 ± 0.60	2.0	0.99	5.32 ± 0.60	0.7
0.60	1.075	2.43 ± 0.55	1.6	0.96	5.14 ± 0.55	0.5
0.75	1.094	2.34 ± 0.65	1.3	0.48	5.39 ± 0.65	0.8
0.88	1.113	3.32 ± 0.27	12.0	1.21	5.92 ± 0.27	2.7
1.0	1.132	3.40 ± 0.54	14.5	0.67	6.26 ± 0.54	5.9
1.2	1.151	3.29 ± 0.19	11.2	2.25	5.77 ± 0.19	1.9
10% homogenate		4.24 ± 0.26	100	56.20	5.49 ± 0.26	1.0

* Compared with homogenate as 100%.

† Specific LD50 of the homogenate divided by that of sample.

relative activity, calculated from the numerical values for specific LD₅₀, in the more dense layers associated with a corresponding decrease in the less dense material. In Fig. 1 the relative activity and recovery of activity are plotted to a logarithmic scale with estimated deviations as an indication of the inherent error involved in the titration procedures.

Centrifugation of brain homogenates in caesium chloride

The appearance of the separated zones in the tubes after centrifugation are shown diagrammatically in Fig. 2. The experimental results from the complete sediment (Table 3) indicate that only about 0.01% of the activity was not sedi-

Fig. 2. Ultracentrifugation of scrapie mouse brain tissue homogenates in caesium chloride gradients. Appearance of zones of debris and light scattering zones with volumes of serial fractions (A-E; F-K) collected after centrifugation indicated. (a) Sonicated sediment from brain homogenate layered on caesium chloride. (b) Sonicated mitochondrial-sized fraction sedimenting through 0.88M-sucrose layered on caesium chloride.

mented under the centrifugation conditions used and that the activity was not affected by ultrasonic treatment of the resuspended sediment. Activity was scattered throughout the gradient but was highest in the low density region of the gradient where particulate matter predominated. The distribution was in general agreement with that already observed in the sucrose gradient. It has been reported (Mould, Dawson & Smith, 1965) that the potency of scrapie-active material was reduced by 1.8 log units by contact with caesium chloride for 24 hr comparable with the conditions applicable during the centrifugation procedure. A possible diminution to $10^{-3.7}$ of the effective LD₅₀ titre of the applied zone of active material would therefore be expected after centrifugation in caesium chloride, further decreased to $10^{-3.4}$ by the dilution factor (1/5). The spread of activity in the zones collected from the gradient (Table 3) agreed with this estimated value and substantiated the previously reported effect of the strong salt solution.

The experimental titres for the various fractions derived from the portion of mouse brain homogenate sedimenting through 0.88M-sucrose are listed in Table 4. There was good recovery of activity in the sediment with a high specific LD50. Ultrasonic treatment in this experiment reduced the total scrapie activity. After centrifugation in caesium chloride a number of well-defined zones were observed with a band of flocculated particles (fraction I) in the gradient over a density

Table 3. *Centrifugation of sediment from scrapie mouse brain homogenates in caesium chloride*

The experiment was terminated 6 months after inoculation.

Inoculum	LD50 ($-\log_{10}$)
10% homogenate	5.67 ± 0.17
Supernatant fluid above 100,000g sediment	1.50 ± 0.20
Material sedimenting at 100,000g	4.48 ± 0.56
Sediment after ultrasonic treatment	5.47 ± 0.31
Fractions from caesium chloride gradient as shown in Fig. 2a	A 3.76 ± 0.30
	B 3.79 ± 0.18
	C 3.35 ± 0.23
	D 3.08 ± 0.26
	E 2.79 ± 0.25

Table 4. *Centrifugation of material from scrapie mouse brain homogenate sedimenting through 0.88M-sucrose*

The experiment was terminated 6 months after inoculation.

Inoculum	LD50 ($-\log_{10}$)	Protein (g./ml. $\times 10^{-3}$)	Specific LD50 ($-\log_{10}$)
Material sedimenting through 0.88M-sucrose	5.49 ± 0.37	5.40	6.61 ± 0.37
Material after repeat sedimentation through 0.88M-sucrose	6.07 ± 0.35	7.53	7.23 ± 0.35
Sediment after ultrasonic treatment	4.97 ± 0.29	—	—
Fractions from caesium chloride gradient as shown in Fig. 2b	F 2.79 ± 0.55	—	—
	G 3.35 ± 0.23	—	—
	H 3.52 ± 0.35	—	—
	I 3.92 ± 0.26	—	—
	J 2.61 ± 0.50	—	—
	K 3.07 ± 0.24	—	—

range 1.16–1.22. A cloudy appearance and activity in the low density fraction (F) suggested that ultrasonic disruption of the particles must have taken place as low density material originally present in the brain homogenate had been removed by repeated sedimentation in 0.88M-sucrose. Contact with caesium chloride again reduced the overall recovery of scrapie activity which was spread throughout all the fractions removed from the gradient. The highest activity was observed within the density range 1.10–1.22.

DISCUSSION

Although there are histopathological and biological transmission differences (Zlotnik & Rennie, 1962, 1963, 1965) between the 'Suffolk strain' and the 'drowsy goat strain' of mouse scrapie the sedimentation behaviour of the activities from mouse brain homogenates appear to be similar (Hunter & Millson, 1964). Any improvement in the purification or concentration of the scrapie agent as indicated by the relative activity (Table 2) of the various zones of activity arising in the sucrose gradients was small, but the recovery of scrapie activity was higher in the material sedimented through 0.88 M-sucrose in agreement with the results reported by Hunter *et al.* (1963, 1964). Activity throughout remained strongly associated with particulate matter. No pronounced peak of activity was observed in the zones successively removed from the caesium chloride gradients. At this preliminary stage of attempted fractionation the protein content of the zones was not determined, but visible particles were scattered throughout tested zones. The effect of ultrasonic treatment can be explained either by the disruption of active particles into smaller fragments retaining activity or the release of an active agent followed by strong readsorption to the smaller particles. Prolonged ultrasonics treatment of the mitochondrial fraction tended to reduce the scrapie activity.

The zones isolated from such gradients have all been examined on the electron microscope for morphological characteristics, but except in the case of identifiable mitochondria (Hunter *et al.* 1964) were of heterogeneous appearance. This difficulty in the application of zonal gradient centrifugation to the separation of cellular fragments from brain tissue with its relatively complicated cytological differentiation has been recently discussed (Eichberg, Whittaker & Dawson, 1964). If it be assumed that the scrapie agent is closely associated with a single type of sub-cellular structure in brain tissue, then following homogenization, ultrasonic disruption and differential centrifugation, it would be certain to appear over a wide range of fractions with varying degrees of activity determined only by the amount of bulk material distributed within those fractions.

Many fractions of known biological activity prepared from sheep, goat and mouse scrapie brain homogenates have been examined by electron microscopy for the possible presence of characteristic virus-like particles. At an earlier stage in the investigation this was done with more varied scrapie preparations than could experimentally be subjected to biological testing in sheep or goats. When quantitative measurements of biological activity and purification were achieved with the mouse it became obvious from the low titres in supposedly purified material that sufficient potency had hardly yet been attained such that unequivocal recognition by electron microscopy might be possible. Within this limitation no virus-like particle has been observed. An extensive application by Drs Mackay & Vantsis (personal communication) of tissue-culture techniques has failed to demonstrate any cytopathogenic effects associated with the addition of scrapie-infected inoculum.

The biological activity, however, of an unfractionated scrapie-affected mouse brain, determined by titration inoculation of serial dilutions of the brain tissue in groups of mice, is high. At a period of 9 months after inoculation the end-point of the titre of scrapie activity is consistently 10^{-8} g. wet tissue/ml. (0.03 ml. inoculum). If the causal agent of this level of activity is a virus particle with a particle

infectivity ratio of 100:1, a concentration of 10^{10} particles/ml. might be expected in a 10% (w/v) brain homogenate. In thin sections of infected tissue an even higher local concentration of virus associated with lesions might be feasible and the possibility of characterization of a particle 50–100 m μ diam. greatly enhanced.

A proposition that the scrapie agent is a very small virus (< 25 m μ) as yet uncharacterized is substantiated by its adsorption on or occlusion by small tissue particles and the progressive loss of activity in the supernatant fluid from mouse brain homogenates after repeated centrifugation (Mould, Dawson & Smith, unpublished). The apparent absence of biological activity in free suspension in the absence of tissue particles, combined with the relative stability of the active agent to chemical and physical action in the presence of tissue particles could indicate almost a state of chemical combination with a tissue component. It is significant that in experimental work consistently high activities or titres have only been reached with brain homogenates under physiological conditions of pH and salt concentration. A change in pH, addition of salts, solvent action or heat have all decreased the potency of the original scrapie activity (Hunter & Millson, 1965; Mould *et al.* 1965). This has been, in fact, one of the difficulties encountered during investigations into the preparation of inocula of increased potency.

Although the transmission of scrapie to animals is most successful when tissue is also transferred, experiments have been reported where this was not the situation. Thus dialysates derived from goat brain homogenates and cerebro-spinal fluid were active in goats (Pattison & Sansom, 1964); extracts in buffer of sheep brain homogenates were active in sheep (Mould & Smith, 1962; Mould, Smith & Dawson, 1964); and scrapie was transmitted to the host animal by cerebrospinal fluid from sheep and goat (Stamp *et al.* 1959; Pattison & Millson, 1962).

As an addition to the proposition that scrapie activity may be passaged by small free virus particles occluded in fragmented tissue sediments two further conceptions are suggested for experimental verification. First, that the actual transfer of a tissue component itself in the inoculum is necessary and, secondly, that the nature of this component is of importance in the mechanism of adsorption of the inoculum by the recipient tissue.

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