The Incorporation of
\(^{3}H\)Thymidine, \(^{14}C\)Orotic Acid, \(^{14}C\)Uridine-diphosphoglucone and \(^{14}C\)Glucosamine into a Post-ribosomal Fraction of Normal and Scrapie-affected Mouse Brain and Spleen

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SUMMARY

Centrifugation of post-ribosomal supernatant fluids of brain homogenates at high speed for a prolonged period yielded a transparent pellet material. Resuspension of this pellet followed by centrifugation on a caesium chloride gradient resulted in a sharp band at a density of 1.34 g./cm\(^3\). The band material sedimented as a single peak of approximately 5 S in the analytical ultracentrifuge and had scrapie activity. Although the position of the band and its S value are similar in normal and scrapie-affected mice there was an increased incorporation of \(^{3}H\)thymidine and \(^{14}C\)uridine-diphosphoglucone into the gradient fractions immediately above and into the band at a density of 1.3 to 1.34 g./cm\(^3\) in scrapie-affected mice. Chromatography of the resuspended post-ribosomal pellet on Sepharose 4B yielded two peaks. In scrapie-affected mice, incorporation experiments showed an area between the two peaks with increased incorporation of \(^{3}H\)thymidine, \(^{14}C\)uridine-diphospho-glucose and \(^{14}C\)glucosamine. Experiments on spleen homogenates yielded comparable results.

INTRODUCTION

Much stress has been laid on the strong association in affected mouse brain between the scrapie agent and cellular particulates. It has also been shown that standard procedures such as ultrasonic vibration and detergent treatment do not bring about the release of the agent (Mould & Smith, 1962; Hunter, Millson & Chandler, 1963; Hunter, Millson & Meek, 1964; Hunter & Millson, 1964; Mould, Smith & Dawson, 1965). However Mould et al. (1965) also demonstrated the presence of the agent in the post-ribosomal supernatant fraction of brain homogenates prepared in 0.32 M-sucrose although the specific ID\(_{50}\) of such supernatants, based on protein content, was only about 1% of that of the original homogenate. In view of the dialysis experiments of Pattison & Sansom (1964), and of experiments designed to test radiation stability (Alper, Haig & Clarke, 1966; Alper et al. 1967), the results of which suggest that the scrapie agent may be of exceptionally small size (3 to 7 nm.), it seemed possible that a proportion of the agent in post-ribosomal supernatants might be unattached to particulates.

The unusual stability and properties of the scrapie agent have led to suggestions that it is a replicating agent without nucleic acid (Alper et al. 1966), a small basic
protein (Pattison & Jones, 1967b) or a replicating polysaccharide (Field, 1966). However, as these suggestions represent a radical departure from currently accepted concepts of virus replication, Adams & Caspary (1967) suggested that the agent might consist of a small nucleic acid core associated with a largely polysaccharide coat. The present paper describes some studies made on material prepared from post-ribosomal supernatants of normal and scrapie-affected mouse brain and spleen, after the intra-cerebral injection of precursors of nucleic acids and polysaccharides.

**METHODS**

**Animals.** Albino mice of the Swiss strain, bred in this laboratory, were used. Inoculation with scrapie agent was done at about 4 weeks of age as follows. Brain was removed from mice showing clinical signs of scrapie and homogenized in an all-glass homogenizer in 0·9 % NaCl to give a suspension of 10 % (w/v) brain tissue. The suspension was clarified by centrifugation at 2000 g for 10 min. and 0·05 ml. of the supernatant fluid injected intracerebrally into the recipient mice. The inoculated mice were killed either after 3 to 4 months (early scrapie) or when showing definite clinical signs (about 6 months). Samples of brain were examined histologically and shown to have the astrocyte hypertrophy characteristic of the disease (Hadlow, 1959; Field & Raine, 1964; Pattison & Jones, 1967a). The scrapie mice used in the experiments to be described were not inoculated specially but were derived from pools of affected mice maintained in this Unit.

**Isotopes.** [14C]Orotic acid (40 μc/μM), uniformly labelled uridine-5′:diphospho-glucose [14C], (UDPG) (190 μc/μM), [14C]glucosamine (3·1 μc/μM), and [3H]thymidine (20,000 μc/μM) were obtained from the Radiochemical Centre, Amersham. Orotic acid and glucosamine were dissolved in 0·9 % NaCl; UDPG was injected as received without further addition; and thymidine was diluted with 0·9 % NaCl to a convenient volume. Injections, by the intracerebral route under ether anaesthesia, were of 0·02 to 0·05 ml. Thymidine (10 μc) was injected 48 hr and the remaining substances (2 to 3 μc) 24 hr before the animals were killed. Other animals were inoculated into the spleen at open operation with 10 μc [3H]thymidine together with either 2·5 μc [14C]-glucosamine or [14C]orotic acid in 0·9 % saline as a single inoculum. These animals were killed after 48 hr.

**Preparation of homogenates and soluble fractions.** Animals were killed by decapitation and the brains or spleens removed rapidly and placed in ice. All subsequent operations were done below 5°. The brains were homogenized in 0·32 M-sucrose + 0·5 mM-MgCl₂ (10 to 20 ml./g.) by placing in a glass homogenizer with a Teflon pestle and rapidly moving the pestle up and down 30 times. The homogenate was centrifuged in the 50 head of a Spinco L2 ultracentrifuge at 25,000 rev./min. (30,000 g) for 45 min. The supernatant fluid was carefully transferred to fresh tubes and centrifuged for 75 min. at 144,000 g. The top four-fifths of the supernatant fluid were removed, made up to volume with fresh sucrose + MgCl₂ medium, and recentrifuged for 90 min. at 144,000 g. The top four-fifths of the supernatant fluid were again removed, made up to volume with 0·9 % NaCl and recentrifuged for 2 hr at 144,000 g. The top four-fifths of the supernatant fluid were once again removed, made up to volume with 0·9 % NaCl and recentrifuged for 18 hr at 144,000 g.

The transparent slightly pink pellets, which will subsequently be referred to as
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'post-ribosomal pellets', were rinsed several times with 0·1 M-phosphate buffer, pH 7·5, and resuspended in 3 ml. of phosphate buffer (for density gradient fractionation) or 1 to 1·5 ml. of buffer (for chromatography on Sepharose 4B columns) using a small tight-fitting all-glass homogenizer. In some experiments the resulting suspension was used without further treatment, but in most it was clarified by a short centrifugation at 3000 g for 5 min.

Caesium chloride density-gradient centrifugation. The final suspension (2·6 ml.) was mixed with 2·6 ml. of CsCl solution (pH 7·2, density 1·62 g./cm.3) and placed in tubes which were marked at 0·5 cm. intervals. The mixture was then centrifuged at 32,000 rev./min. for 65 hr in the SW.39 head of a Beckman–Spinco preparative ultracentrifuge. Gradients were sampled carefully from the top, using a Pasteur pipette, producing nine fractions of approximately 0·5 ml. The density of the gradient fractions was estimated from measurements of refractive index made with a refractometer.

Chromatography. The resuspended post-ribosomal pellet was layered on 10 x 1 cm. or 20 x 1 cm. columns of Sepharose 4B (Pharmacia Ltd, London), previously equilibrated with 0·1 M-phosphate buffer, pH 7·5. The same buffer was used for elution and 1 ml. samples were collected. Ferritin from horse spleen (batch no. 7448, Koch–Light Co. Ltd, Colnbrook) was used as a standard.

Spectrophotometry and radio-isotope counting of density-gradient and column fractions. The u.v. absorption of each fraction was measured at 260 and 280 nm. using micro-cells of 10 mm. path length and a Unicam SP.500 spectrophotometer, and the fractions were then assayed for radioactivity using a Packard Tricarb scintillation spectrophotometer. Samples were made up for scintillation counting in 10 ml. of a dioxane mixture containing 2,5-diphenyloxazole (7 g.) + 2,2-p-phenylene-bis (5-phenyloxazole) (0·3 g.) + naphthalene (100 g.), in 1 l. of solution. The measured efficiency of double label counting was 18 % from 3H and 61 % from 14C. Quench was determined by the addition of a known quantity of isotope to samples after initial counting and repeating the counts. In view of the very low isotope levels in these fractions counting was continued until the statistical error was less than 5 % (2 standard deviations).

Measurements in the analytical ultracentrifuge. Analytical ultracentrifugation was done in a Beckman Spinco Model E ultracentrifuge, according to the technique of Vinograd et al. (1963). Samples of material from post-ribosomal pellets which accumulated in the band region of CsCl gradients were dialysed against NaCl (0·15 M) for 18 hr and then layered on m-NaCl in the Vinograd cell. The boundary was observed with the photoelectric scanning system at 260 nm.

Infectivity studies. Successive tenfold dilutions of fractions 1 to 9 were made, using as diluent sterile normal saline, containing 200 µg./ml. of bovine serum albumin. It was hoped this might minimize possible loss of scrapie material (which in these preparations could well be present without the protection of tissue constituents) by adsorption on tube or syringe surfaces. Groups of six mice were inoculated intracerebrally (0·05 ml.) and the first dilution was 10⁻² (since the CsCl was toxic at 10⁻¹). Animals were observed over one year.

RESULTS

Resuspended post-ribosomal pellets were added to CsCl solutions and centrifuged as described. After centrifugation the gradients of both normal and scrapie material
showed a single sharp band at a density of 1.34 g./cm.³. Occasionally a second, but very much weaker, band appeared just above the main band at density 1.30 to 1.32 g./cm.³ (Plate 1). In preparations which had not received a preliminary centrifugation after resuspension some dense felted material was also present. This usually hung below the main sharp band but tended to be fragmented and to spread over two or three fractions. Its presence led to some difficulty in sampling, but it was removed from fractions before assay by allowing it to adhere to the sides of the tubes, the clear liquid being sucked out with a Pasteur pipette. Preliminary centrifugation of the resuspended pellet produced a deposit at the bottom of the tube and led to the absence of the felted material from the gradient, but the yield of band material was also reduced suggesting that some of this was carried down mechanically with the deposit.

The u.v. absorption rose rapidly to a maximum in the band containing fraction 6, then decreased, and increased again at the bottom of the tube. The top 7 fractions showed 260/280 nm. absorption ratios which lay between 0.85 and 1.05. In fraction 9 the ratio was about 2, suggesting a high proportion of nucleic acid. There were no obvious differences between scrapie affected and normal brain preparations (Fig. 1).

**Incorporation studies.** Normal and scrapie mice were injected intracerebrally with [³H]thymidine and [¹⁴C]UDPG, and the resuspended post-ribosomal pellets centrifuged on a CsCl gradient. This experiment was repeated twice with closely similar results. The specific activity of thymidine labelling in fractions 8 and 9 was low (Fig. 2). As already indicated (Fig. 1), these fractions showed a 260/280 nm. absorption ratio characteristic of nucleic acid. The incorporation experiment was therefore
CsCl gradients of resuspended post-ribosomal pellet from mouse brain, showing a single sharp band at density 1.34 g./cm.². A, Normal mouse; B, scrapie mouse.
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repeated using $^{14}$C orotic acid (Fig. 3), when fractions 8 and 9 had the highest specific activity and there was no great difference between the three groups of animals.

*Chromatography on Sepharose 4B.* Post-ribosomal pellets were resuspended in 1 to 1.5 ml. of 0.1 M-phosphate buffer, pH 7.5, layered on to columns of Sepharose 4B.
previously equilibrated with the same buffer, and the u.v. absorption and radioactivity of the fractions measured. In early experiments $10 \times 1$ cm. columns were used. On elution with more buffer two peaks of u.v. absorption at 260 nm, and two approximately corresponding peaks of radioactivity were obtained. A similar pattern of u.v. absorption was found whether or not the resuspended pellet was given a short preliminary

![Diagram](image)

**Fig. 5.** $E_{260}$ of the eluate obtained by passing a resuspended post-ribosomal pellet from normal and scrapie mouse brain through a $20 \times 1$ cm. Sepharose 4B column, the extinction being measured at 280 nm. ●, Normal brain; ○, scrapie brain; ▲, ferritin ($E_{280}$).

**Fig. 6.** Specific activities of fractions obtained by passing resuspended post-ribosomal pellets from normal mouse brain (A) and scrapie mouse brain (B) through $20 \times 1$ cm. columns of Sepharose 4B. Mice were injected previously with [3H]thymidine (48 hr) and [14C]glucosamine (24 hr). ●, Thymidine label; ○, glucosamine label; ▲, absorption peaks.

**Table 1. Statistical analysis of [3H]thymidine and [14C]UDP glucose specific activity in CsCl gradient fractions of normal and scrapie mouse brain**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>[3H]thymidine</th>
<th>[14C]UDP glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Mean specific activity</td>
<td>S.E.M.</td>
</tr>
<tr>
<td></td>
<td>4+5+6 (9)*</td>
<td>99.1 ± 13.3</td>
</tr>
<tr>
<td></td>
<td>7+8+9 (9)*</td>
<td>65.1 ± 8.9</td>
</tr>
</tbody>
</table>

* Not significant

* Number of points computed. Three points from each of three experiments.
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Table 2. Statistical analysis of [3H]thymidine and [14C]glucosamine specific activity in Sepharose 4B fractions of normal and scrapie mouse spleen

<table>
<thead>
<tr>
<th>Fraction</th>
<th>[3H]thymidine</th>
<th>[14C]glucosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Scrapie</td>
</tr>
<tr>
<td></td>
<td>Mean specific activity</td>
<td>S.E.M.</td>
</tr>
<tr>
<td>6+7+8 (9)*</td>
<td>80·6 ± 19·3</td>
<td>95·0 ± 18·0</td>
</tr>
<tr>
<td>10+11+12 (9)*</td>
<td>155·6 ± 19·5</td>
<td>348·3 ± 30·5</td>
</tr>
<tr>
<td>13+14+15+16 (12)*</td>
<td>80·1 ± 6·6</td>
<td>117·5 ± 20·1</td>
</tr>
</tbody>
</table>

Not significant Not significant P<0·001 P<0·001

* Number of points computed. Three or four points from each of three experiments.

Fig. 7. Ultracentrifuge pattern of the band material shown in Plate 1. A, normal mouse (S value 4·6); B, scrapie mouse (S value 5·4).

It was also found that resuspension of the deposit and passage through a similar column produced no u.v. absorption in the eluate. Accordingly, therefore, the resuspended post-ribosomal pellets were given the short preliminary centrifugation before chromatography in the experiments to be described. The specific activities of the fractions (disintegrations per min./E260 of 1·0) after the injection of [3H]thymidine and [14C]UDPG were determined (Fig. 4).

In order to produce a wider separation of the peaks, subsequent experiments were made using 20 x 1 cm. Sepharose columns (Fig. 5). To obtain an approximate estimate
of the size of the main components, ferritin was also passed through the same column. The optical density of the fractions shows that the ferritin peak lay between the two peaks obtained from the pellet. Ferritin, although having a molecular weight of \(8 \times 10^5\), behaves as if it had a molecular weight of \(5 \times 10^5\) on gel filtration (Andrews, 1965), and this smaller value has been assumed in the present study. There appeared to be little difference between the overall pattern in normal and scrapie mice, except

Fig. 8. Graph A: \(E_{260}\) of 1 ml. fractions obtained by passing resuspended post-ribosomal pellets from normal and scrapie mouse spleen through a column of Sepharose 4B; ■, normal spleen; △, scrapie spleen. Graph B: specific activities of the fractions plotted in graph A. Mice were injected 48 hr previously with \([^{1}H]\)thymidine and \([^{14}C]\)glucosamine. The positions of the two absorption peaks are shown by †. Normal spleen: ▲, thymidine label; △, glucosamine label. Scrapie spleen: ○, thymidine label; □, glucosamine label.

Fig. 9. Graph A: \(E_{260}\) of 1 ml. fractions obtained by passing resuspended post-ribosomal pellets from normal and scrapie mouse spleen through a column of Sepharose 4B; □, normal spleen; ■, scrapie spleen. Graph B: specific activities of the fractions plotted in graph A. Mice were injected 48 hr previously with \([^{1}H]\)thymidine and \([^{14}C]\)orotic acid. The positions of the two absorption peaks are shown by †. Normal spleen: ▲, thymidine label; △, orotic acid label. Scrapie spleen: ○, thymidine label; □, orotic acid label.
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that when similar amounts of starting tissue were used, the first peak was higher in scrapie mice than in normal, while the second peak gave the opposite pattern. This result was consistently obtained in five experiments. A further experiment using these longer columns was made after the injection of $[^3H]$thymidine and $[^{14}C]$glucosamine (Fig. 6).

Measurements in the analytical ultracentrifuge. The fraction containing band material, after centrifugation of the resuspended post-ribosomal pellet on a CsCl gradient, was dialysed against 0·15 M-NaCl for 18 hr. Both the normal and scrapie band material appeared to sediment as a single symmetrical component giving an $S_{20,w}$ value of 4·4 S for the normal and 5·5 S for the scrapie preparations (Fig. 7). The difference cannot be considered as significant in these preliminary measurements.

Closely similar findings were obtained from the spleen material (Figs. 8, 9).

The results of these experiments were analysed by comparison of idealized areas under the peaks of activity in the CsCl gradient fractions for brain (Fig. 2) and in the Sepharose elution fractions in the spleen experiments (Fig. 8). Tables 1 and 2 show the mean ordinate (specific activity) relating to each peak, its standard error and the statistical significance of differences between the results from normal and scrapie affected animals. Only in the area of maximum specific activity does the difference become highly significant and the proportional increase of specific activity is of the same order for both precursors and in both tissues.

Infectivity of fractions. One animal inoculated at a dilution of $10^{-2}$ with material from the band illustrated in Fig. 1 developed clinical scrapie which was histologically confirmed by the presence of vacuoles and astrocytic hypertrophy. One other animal inoculated with $10^{-8}$ dilution of the same band also developed the disease. All other animals remained normal and were checked histologically at the end of the experiment.

DISCUSSION

The present work was undertaken in an attempt to obtain experimental evidence bearing on the hypothesis that the scrapie agent may be a polysaccharide-nucleic acid complex (Adams & Caspary, 1967). The results do in fact suggest that there is a markedly increased incorporation of thymidine and polysaccharide precursors into a fraction of scrapie brain which appears to contain material of around 5 S and 500,000 molecular weight. This corresponds reasonably well with some of the estimates of the size of the scrapie agent (Alper et al. 1966; Pattison & Sansom, 1964). The results cannot of course be taken as definite evidence of incorporation into the agent, although this is obviously possible. The increased incorporation may be part of the process of hypertrophy of astroglial cells which appears invariably to accompany the development of the disease (Hadlow, 1959; Field & Raine, 1964; Pattison & Jones, 1967a).

Interpretation of the results is also complicated by the fact that very little work appears to have been done with the post-ribosomal pellet fraction used in the present study. Regarding the nature of the material banding in CsCl at a density of 1·34 g./cm.$^3$ (Gibbs, 1967), the recent report by Mills, Peterson & Spiegelman (1967) is interesting. These authors, working with phage Q$\beta$ RNA and its replicase $in vitro$, showed that if the newly synthesized RNA was used as template material in successive transfers, there was a progressive reduction in the size of the RNA genome. After 74 transfers 83% of the original genome had been lost, and the product was a replicating
RNA with a molecular weight of about $2 \times 10^5$ daltons. Although these authors stress that their studies were designed to place the RNA under selection pressure for fast growth under conditions free from the restrictions imposed by the cell, there seems no a priori reason why a similar process could not occur in vivo. This would raise the possibility that the band material found in the present study might be a residue of replicating material derived from the progressive loss of genome from what were originally conventional viruses. Further, although in the phage experiments of Mills et al. infectivity was lost early (at the 4th transfer) we think it not unreasonable to suppose that there may be agents which retain infectivity with much greater loss of genome. Part of the problem may well be the inability of the reduced genome to code for the normal protein coat material. Adams & Caspary (1967), in their nucleic acid-polysaccharide hypothesis concerning the nature of the scrapie agent, pointed out that the substitution of a polysaccharide for a protein coat would be expected to reduce the amount of coding information necessary for its construction.

The observations made on scrapie, as compared with normal, spleen were identical with those on brain material. The spleen experiments were undertaken since it seemed possible that brain changes might have resulted from glial fibril production by hypertrophied astrocytes known to occur early in scrapie (Hadlow 1959; Field & Raine 1964; Pattison & Jones, 1967a), rather than from a direct connection with the presence of scrapie agent. Thus Kimberlin & Hunter (1967) recently claimed that there was an increased turnover of DNA associated with nuclei in scrapie brain and that this was not of pathogenetic significance in the disease. It is known that the scrapie agent reaches a high titre in the spleen at an earlier stage in incubation than it does in the brain (Eklund, Kennedy & Hadlow, 1967) though there are no accompanying histological changes. Consequently the experiments reported in this paper were done in an attempt to exclude astrogial hypertrophy and its associated fibrilogenesis as a cause of increased precursor incorporation. In fact the results have shown clearly that, as in the brain, there was a peak of specific activity of $[^{3}H]$thymidine and $[^{14}C]$glucosamine label in an area between the two u.v. absorption peaks and that in spleen preparations from scrapie-infected mice the specific activity of such incorporation was approximately twice that found in similar preparations from normal mice.

In further experiments, the incorporation of $[^{14}C]$orotic acid was studied together with that of $[^{3}H]$thymidine. While the characteristic increased specific activity of thymidine label was still observed, there was no difference in the specific activity of orotic acid label in the two groups, and in this case the orotic acid specific activity curve closely followed the u.v. absorption curve from fractions 9 to 18. This strongly suggests that the post-ribosomal pellet contained both RNA and DNA and that the RNA component is associated with the second u.v. absorption peak. Further, infection with scrapie agent appeared to have no effect on the RNA-containing component. Apparently, therefore, the increased specific activity resulting from $[^{14}C]$glucosamine injection was associated only with an increased incorporation into DNA. It seems reasonable, therefore, to suggest that a new DNA-polysaccharide component is present in spleen at a time when the scrapie agent is being actively produced.

In both the $[^{3}H]$thymidine and $[^{14}C]$UDPG as well as the orotic acid experiments, the normal and scrapie curves follow much the same course though peaks in the former are considerably lower. In this as in so many other features (Field, 1967) scrapie appears to be an exaggeration of the normal. In fact it may sometimes be
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difficult to distinguish histologically the brain of a very old mouse from early scrapie of a 5-month-old animal. In view of this, an extension of the present incorporation studies to the changes accompanying ageing in the brain would be of interest.

The infectivity measurements of CsCl gradient fractions of brain did not yield as definite a result as had been hoped, but do suggest that infective virus was present in the post-ribosomal pellet and that it was concentrated in or near the band. There could be a number of reasons for the low titre. As already mentioned, Mould et al. (1965) reported that contact with CsCl reduces the infectivity of the agent, and in all preparations this contact occurred for about 70 hr. Secondly, it is possible that the scrapie agent may adsorb rapidly on to the surfaces of tubes and syringes despite the presence of bovine serum albumin. Thirdly, it is possible that the material present in our preparations contained latent or incomplete virus; for in view of the well established association of the scrapie agent with tissue constituents, it may be that such a combination is necessary for full activation.

It was concluded that what little scrapie agent was present in the gradient fractions was concentrated in the band shown in Fig. 1—that which showed thymidine and UDPG incorporation simultaneously. This strengthens the suggestion that the agent might comprise polysaccharide and nucleic acid.

The authors would like to thank Dr R. H. Pain for very kindly carrying out the analytical ultracentrifuge runs, Mr D. Weightman for skilled technical assistance, and Miss Greta Joyce for help with the bioassay and histological confirmation of scrapie infection. The photograph (Plate I) was kindly taken and printed for us by Mr D. Hughes.

REFERENCES


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