Archiv für die gesamte Virusforschung 30, 224—237 (1970)

The Incorporation of [^{3}H] Thymidine and [^{14}C] Glucosamine into a DNA-Polysaccharide Complex in Normal and Scrapie-affected Mouse Brain

By

D. H. Adams, E. A. Caspary, and E. J. Field

Medical Research Council Demyelinating Diseases Unit, Newcastle General Hospital, Newcastle, England

With 3 Figures

Received August 4, 1969

Summary

Urea treatment of particulate material sedimented by prolonged centrifugation of brain cell sap has shown the presence of a complex of DNA-polysaccharide into which [^{3}H] thymidine is incorporated into DNA thymidylic acid ten times more rapidly in scrapie than in normal mice. Membrane containing, subcellular, fractions liberate apparently identical material after urea treatment which shows a similarly increased [^{3}H] thymidine incorporation in scrapie mouse brain. The evidence suggests that this material replicates in the cell sap of scrapie mouse brain and subsequently binds to membrane. First approximation calculations indicate that the quantity of DNA involved is of the same order as that which would be expected to be present in the scrapie agent present in mouse brain at peak titre.

1. Introduction

Adams and Caspary (1967) suggested that some of the unusual properties of the scrapie agent might be explained if the infective particle consisted of a small DNA core coated with polysaccharide rather than with protein. Investigations into the cell sap fraction of normal and scrapie mouse brain and spleen subsequently indicated the presence of particulate material with an approximate molecular weight of 5×10^5 which incorporated label from both [^{3}H] thymidine and uridine-diphospho-[^{14}C]-glucose or [^{14}C] glucosamine twice as rapidly in scrapie as in normal mice (Adams and Caspary, 1968; Adams, Caspary and Field, 1969). However, although the incorporation data could be interpreted as evidence for the DNA-polysaccharide agent suggested by Adams and Caspary, (1967) the

situation appeared in many ways to be more complex. Firstly both the cell sap fraction of scrapie brain, and the particulate matter sedimented from it by prolonged centrifugation have a very low infective titre (MOULD, SMITH, and DAWSON, 1965; ADAMS, CASPARY, and FIELD, 1969, and unpublished). Secondly the difference between normal and scrapie mice lay in a doubled rate of precursor incorporation into this material in scrapie mice, suggesting that it was present in both.

This and other considerations led ADAMS and FIELD (1968) to propose the linkage substance theory of the infective process in scrapie, which postulated essentially a three component system:

1. A DNA-polysaccharide complex found in cell sap and considered to be an incomplete sub-virus capable of self replication within the cell but not of producing the disease, present in both animals with active scrapie, and in normal susceptible animals.

2. Normal membrane to which the sub-virus cannot attach directly.

3. A linkage substance binding (1) and (2) by acting as a bridge between them.

This theory had the advantage of bringing together the experimental results already described, the well established fact that infective scrapie agent has so far proved to be inseparable from cellular membranes, and the heat and radiation stability of the agent. In susceptible animals the only obligatory component required to initiate the infective process would be the linkage material, which could be a small, stable polysaccharide or polysaccharide-protein complex.

The work described in the present paper was undertaken in an attempt to

a) establish more firmly the presence in cell sap of a small DNA-polysaccharide complex, and

b) to show that similar material was associated with membrane structures in scrapie mice.

2. Materials and Methods

2.1. Animals

Swiss albino strain mice were used, and infected with scrapie agent as described previously (ADAMS *et al.*, 1969). Groups of 10—15 scrapie and corresponding normal mice were used in each experiment.

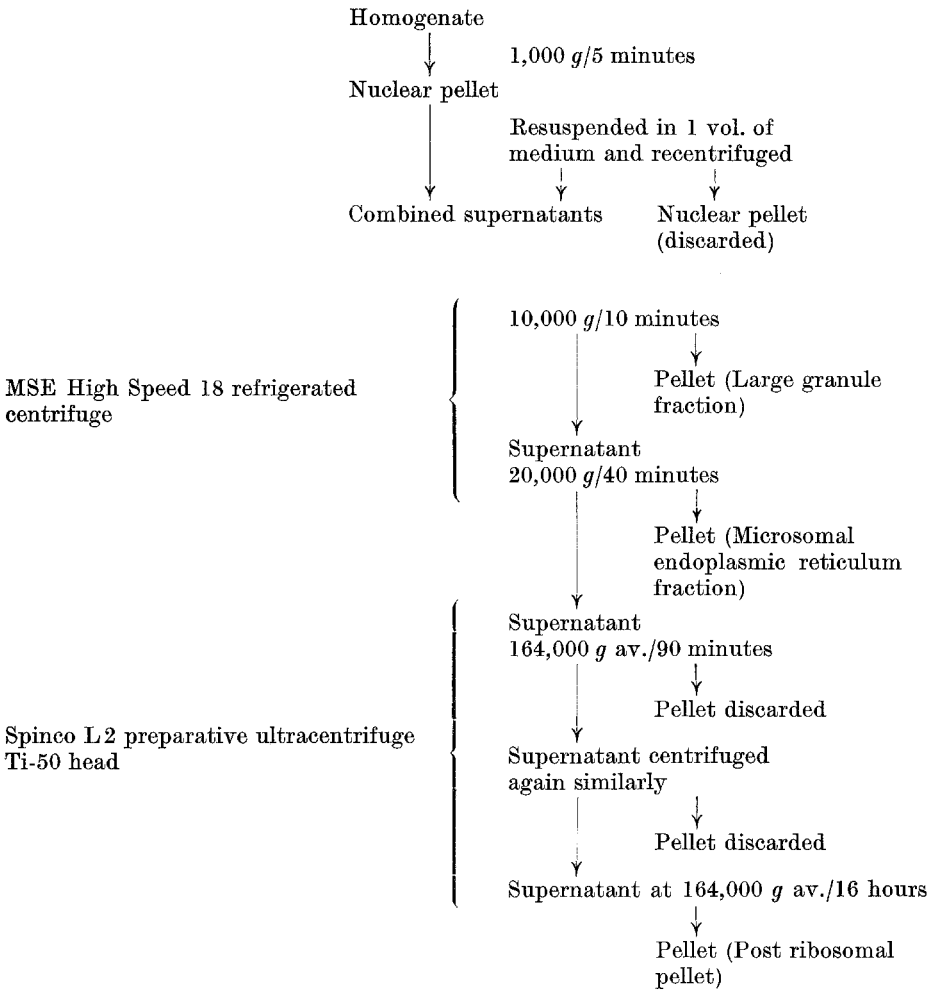
2.2. Isotopes

[^{3}H] thymidine (20 Ci/mM) and [^{14}C] glucosamine (3 mCi/mM) were obtained from the Radiochemical Centre, Amersham, and were injected by the intracerebral route 48 hours and 24 hours, respectively, before killing, as previously described. Each animal received 10 μCi of thymidine and 5 μCi of glucosamine.

2.3. Preparation of Homogenates and Fractions

Animals were killed by severing the cardiac blood vessels under light ether anaesthesia. All subsequent operations were done below 5°C. The brains were removed, weighed and homogenised in 10 vol. of 0.32 M sucrose + 0.5 mM-$MgCl_2$ by placing in a glass homogeniser fitted with a Teflon pestle, and rapidly moving the pestle up and down 12 times.

The homogenates were then centrifuged as follows:



The post ribosomal pellets were rinsed several times with 0.1 M phosphate buffer pH 7.5.

The separated fractions were then treated in various ways, as follows:

2.3.1. Post Ribosomal Pellets

In some experiments these were resuspended in 1 ml of phosphate buffer (0.1 M, pH 7.5) using a small tight fitting all glass homogeniser, and clarified by centrifuging at 3,000*g* for 5 minutes. In others the pellets (combined if necessary) were re-homogenised in 6 M urea pH 8.5 (5 ml per g wet wt. of original brain), and recentrifuged at 164,000*g* for 18 hours. The pellets were rinsed eight times to remove as much urea as possible, resuspended in phosphate buffer, and clarified as above.

2.3.2. Large Granule Fraction and Endoplasmic Reticulum Pellets

These were homogenised in approximately 5 ml of 6 M urea pH 8.5 per g wet wt. of original brain. The resulting suspension was centrifuged for 2 hours at 164,000*g*. The supernatants were removed, and recentrifuged for 18 hours at 164,000*g*. The resulting pellets were rinsed eight times, resuspended in buffer and clarified as described above.

2.3.3. Extraction of Nucleic Acids

Nucleic acids were extracted from whole brain or from pellets by the hot 10% sodium chloride method of TYNER, HEIDELBERGER, and LE PAGE (1953) modified by ADAMS (1965). This method extracts total nucleic acid, both RNA and DNA being precipitated together in the final step.

In some experiments whole brain was processed as far as the lipid extraction steps, and the resulting dried powder added to pellet material suspected of containing very small quantities of labelled DNA, to provide carrier DNA and thereby minimize loss.

When necessary, DNA and RNA were separated as follows. The mixed nucleic acids were dissolved in 0.1 N NaOH and incubated at 37° C for 24 hours to hydrolyse partially the RNA. DNA was then precipitated by adding 0.2 vol. of N HCl and recovered by centrifuging. The pellet was washed three times by resuspending in 95% ethanol and recentrifuging.

The amount of nucleic acid present in extracts was assessed spectrophotometrically on the basis that 1 mg nucleic acid/ml ≡ E 260 of 20—24 at a 10 mm path length.

2.3.4. Chromatography of Post Ribosomal and Urea Extracted Fractions

The clarified, resuspended pellet material was layered on to 20 × 1 cm columns of Sepharose 4B (Pharmacia Ltd., London), which had been previously equilibrated with 0.1 M — phosphate buffer, pH 7.5. Fresh columns were used for each sample. Elution was carried out using the same buffer, and 1 ml samples collected.

2.3.5. Chromatography of Nucleic Acid Hydrolysates

After separation, DNA and the partially hydrolysed RNA were heated at 100° C for 1 hour with N HCl. The acid hydrolysates were evaporated to dryness over flake KOH in a vacuum desiccator, and redissolved in 0.5 ml of 0.1 N HCl. They were then applied to 120 × 6 mm columns of DOW-50-8X resin (Sigma Chemical Co.) and eluted with 0.1 N HCl. After seven 1 ml fractions had been collected elution was continued for a short time with N HCl. Thymidylic and uridylic acids were eluted in 90—100% yield, with a peak at fraction 3. They were identified by spectrophotometric measurements at 260, 267 and 280 mμ, in comparison with those obtained from thymidine and uridine monophosphates (Sigma Chemical Co.).

2.3.6. Spectrophotometry and Radioisotope Counting of Column Fractions

The UV absorption of each fraction was measured at 260 and 280 mμ using microcells of 10 mm path length, and a Unicam SP 500 spectrophotometer. The fractions were assayed for radioactivity in a Packard Tricarb scintillation spectrometer, samples being prepared for counting as described previously (ADAMS, CASPARY, and FIELD, 1969).

2.3.7. Protein Estimation

This was done by HESS and LEWIN's (1965) modification of the technique of LOWRY, RANDALL, ROSEBROUGH, and FARR (1951).

2.3.8. Fraction Specific Activities

The optical density at 260 mμ of fractions eluted from Sepharose columns was used as a base for the assessment of their specific activity. Thus for each 1 ml fraction the total disintegrations/minutes and the E 260 was measured, the E 260 adjusted to a constant figure of 1.0, and the specific activity given as disintegrations/minutes/E 260 of 1.0. The same base line was used for [^{14}C] (polysaccharide) incorporation, for convenience and to provide the same base for comparison for both [^{3}H] and [^{14}C] incorporation.

3. Results

Chromatography of clarified, resuspended post ribosomal pellets on Sepharose 4B columns and collection of 1 ml fractions gave results identical with those previously described by ADAMS *et al.* (1969). Two peaks at E 260 mμ were ob-

tained, the first at about fraction 6 and the second at fractions 13—14. In animals injected with [^{3}H] thymidine and [^{14}C] glucosamine there was a peak of specific activity between the two absorption peaks. The specific activity was doubled in material from scrapie as compared with normal mice. Treatment of the post ribosomal pellets with 6 M urea resulted in the disappearance of a large part (90—95%)

Table 1. *Ratios of Absorption at 260 mμ/280 mμ of Fractions 8—12 (see Figs. 1 and 2) Eluted from Sepharose 4B Columns, and the Effect of Urea Treatment*

Fraction No.	Post ribosomal pellet material				Per cent nucleic acid[1]	Post ribosomal pellet (urea treated)				Per cent nucleic acid	Large granule fraction (urea treated)				Per cent nucleic acid
	Normal		Scrapie			Normal		Scrapie			Normal		Scrapie		
8	0.76	0.91	0.78	0.73	1.2	1.2	1.4	1.4	1.4	9.1	1.25	1.25	1.35	1.2	6.5
	0.80		0.85												
9	0.73	0.78	0.72	0.71	1.0	1.2	1.3	1.35	1.2	6.5	1.3	1.25	1.35	1.3	7.5
	0.75		0.82												
10	0.70	0.77	0.72	0.81	1.0	1.3	1.4	1.2	1.3	7.8	1.35	1.4	1.3	1.3	8.0
	0.79		0.70												
11	0.69	0.74	0.66	0.78	0.75	1.5	1.2	1.25	1.35	7.8	1.5	1.45	1.4	1.4	11.0
	0.77		0.66												
12	0.77	0.75	0.75	0.75	1.0	1.3	1.3	1.4	1.5	10.5	1.6	1.45	1.5	1.4	13.0
	0.74		0.78												

[1] Calculated from the data of WARBURG and CHRISTIAN (1941). Since there was no apparent difference in the 260/280 ratios of normal and scrapie mice all the values were averaged for the estimation of nucleic acid content.

Table 2. *[^{3}H]/[^{14}C] Incorporation Ratios Found in Post Ribosomal Pellet Material before and after Urea Treatment, and in Urea Treated Large Granule and Microsome Fractions. The Total Counts Recovered in the Specific Activity Peak Area (Fractions 8—12, Figs. 1 and 2) have been Used to Compute the Incorporation Ratios*

Source	[^{3}H]/[^{14}C] ratio				
	Normal		Scrapie		Average of all values ± SEM
Post ribosomal pellet	1.30	1.35	1.26	1.47	1.35±0.03
	1.26		1.45		
Urea treated post ribosomal pellet	1.71	2.60	1.52	2.60	2.11±0.25 (p 0.02—0.01)[1]
Urea treated large granule fraction	1.90	1.82	2.0	1.72	1.86±0.05 (p <0.001)[1]
Urea treated microsome fractions	1.63	1.80	2.24	1.88	1.89±0.11 (p 0.01—0.001)[1]

[1] Significance of difference between post-ribosomal pellet and urea treated fractions.

of the UV absorbing material, although a generally similar pattern of optical density was obtained by chromatography. There was also a change in the ratio of absorption at 260 mμ absorption at 280 mμ in the fractions after the urea treatment (Table 1). The specific activity of each fraction plotted for post ribosomal pellet material before and after urea treatment is shown in Fig. 1, and the figures are statistically evaluated in Table 5.

Taking the specific activity peak as occurring over fractions 8—12 inclusive, summation of the counts found before urea treatment and recovered afterwards, showed that the urea treated pellets contained only 5% of the [^{3}H] counts originally present. The ratio of $\frac{[^3H]\text{ counts}}{[^{14}C]\text{ counts}}$ recovered was also altered by urea treatment and the values obtained for this ratio averaged over fractions 8—12 for the total number of experiments conducted are given in Table 2.

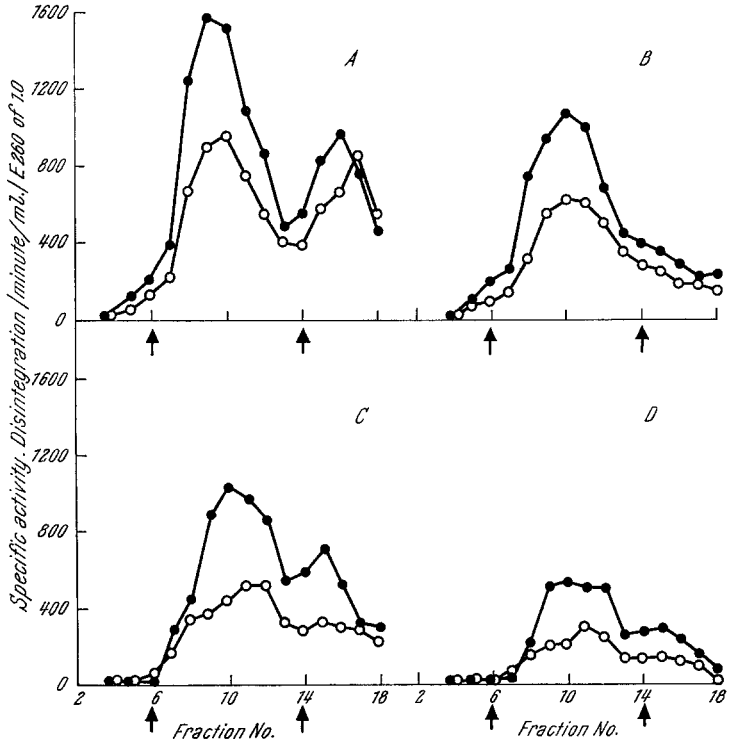


Fig. 1. Specific activities of Sepharose 4 B column fractions of post ribosomal pellet material, and of urea treated post ribosomal pellet material. Mice were injected with 10 μC of [^{3}H] thymidine and 5 μC of [^{14}C] glucosamine, as described. ● — ● Scrapie animals; ○ — ○ Normal animals. The broad arrows (Fractions 6 and 14) indicate the position of the E 260 absorption peaks. Post ribosomal pellet: Graph A Specific activity of [^{3}H]; Graph B Specific activity of [^{14}C]. Urea treated post ribosomal pellet: Graph C Specific activity of [^{3}H]; Graph D Specific activity of [^{14}C]

The results so far obtained, while confirming the presence of material incorporating [^{3}H] label and [^{14}C] label at a doubled or tripled rate in scrapie mice, also indicated quite clearly that, in particular, the [^{3}H] label from thymidine had been incorporated into non-nucleic acid material. The fact that urea treatment of the post ribosomal pellets released 95% of the [^{3}H] counts in a non-sedimentable state, while at the same time producing an 8—10fold enrichment of the nucleic acid content (Table 1), was strong evidence of this. Accordingly, counts were made of a number of sub-cellular fractions during the course of preparation, together with measurements of their protein content. These experiments showed that very large amounts of tritium label were incorporated into trichloroacetic acid precipit-

able material (Table 3) from which it was not released by heating, and which was presumably, therefore, of protein nature.

In further experiments post ribosomal pellet material was prepared from the same wet weights of normal and scrapie mouse brain. RNA and DNA were extracted together and the total yield of nucleic acids estimated by their absorption at 260 mμ. This material contained [^{3}H] counts, and the results are given in Table 4. From the centrifugation procedure the post ribosomal pellet would be expected to contain t-RNA, and indeed the previous data of ADAMS, CASPARY, and FIELD (1969) had shown the presence of [^{14}C] orotic acid incorporating material with a peak at a different place from the [^{3}H] thymidine incorporation peak in both

Table 3. *The Incorporation of Label from [^{3}H] Thymidine into Subcellular Fractions after Precipitation with 10% Trichloroacetic Acid*

Source	Disintegrations/min./mg protein		Per cent of counts released by heating with 10% TCA for 1 hour at 100°C
	Normal	Scrapie	
Supernatant remaining after centrifugation of the post ribosomal pellet	51,000	47,000	<1
Large granule fraction	55,000	145,000	<1
Microsomal fraction	9,500	13,000	Not tested

Table 4. *The Total Nucleic Acid Content of, and Radioactivity Associated with, Post Ribosomal Pellet Material*

Total nucleic acid (RNA and DNA) extractable from the post ribosomal pellet	Normal 25 μg		Scrapie 22 μg	
	Disintegrations/minute			
	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]
Radioactivity present in whole post ribosomal pellet residue after nucleic acid extraction	1800	620	3000	980
Radioactivity present in extracted nucleic acid	170	180	430	330
Radioactivity present in urea treated post ribosomal pellet	150	65	290	180

All results are brought to the equivalent of 1 gram wet weight of brain.

CsCl gradients and eluate from Sepharose 4B columns. It remained to show, therefore, that the post ribosomal pellet contained [^{3}H] thymidine incorporated into DNA. This was done by preparing a further batch of post ribosomal pellet material from the same amounts of normal and scrapie brain. During the procedure whole brain was added to provide carrier DNA as described in "Methods". Acid hydrolysates of the separated nucleic acids, prepared as described, were dissolved in 0.5 ml of 0.1 N HCl, layered on to DOW50 columns, and eluted with 0.1 N HCl. After fraction 7 the elution was continued with N HCl. Uridylic acid and thymidylic acid were eluted from the columns to which RNA and DNA hydrolysates were added respectively, the peak of the respective E260 readings being reached at fraction 3. Cytidylic acid was eluted from the RNA column shortly after chang-

ing to N HCl, but no clear elution of deoxycytidylic acid was obtained from the DNA columns. The fractions were counted for [^{3}H] and the results are given in Fig. 2. Thymidylic acid, obtained from scrapie material, contained about ten times as many counts as those from normal mice over the range of fractions (2—4) straddling the peak. Counts were also present in RNA uridylic acid, but the scrapie/normal ratio was only about 1.5:1, confirming the previous results that there was no great difference in [^{14}C] orotic acid incorporation as between normal and scrapie mice.

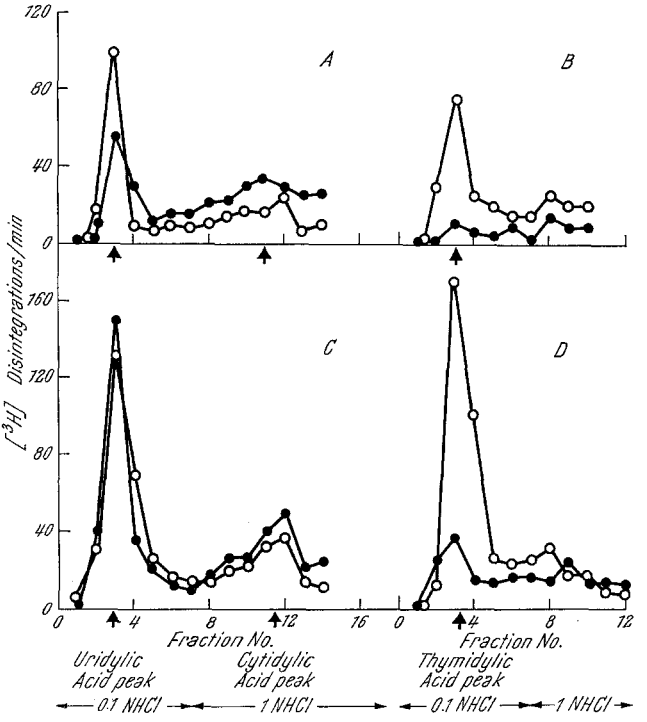


Fig. 2. [^{3}H] counts obtained by chromatography on DOW 50-8X columns of acid hydrolysates of DNA and RNA from post ribosomal pellet material and urea treated large granule fraction (for details see text). ○ – ○ Scrapie mice; ● – ● Normal mice. Post ribosomal pellet: Graph A RNA hydrolysate; Graph B DNA hydrolysate. Urea treated large granule fraction: Graph C RNA hydrolysate; Graph D DNA hydrolysate. The nucleotide absorption peaks (E 260/uridylic acid, E 267/thymidylic acid and E280/cytidylic acid) were at the points indicated by the broad arrows

3.1. Experiments with Membrane Fractions

Pellets were prepared from urea-treated large granule fractions, and endoplasmic reticulum fractions as described. The application of the clarified resuspended pellet material to Sepharose 4B columns produced two peaks in the same place as those in the resuspended post ribosomal material. The fractions were counted and the specific activities plotted in Fig. 3 and statistically evaluated in Table 5. Both show an area between the absorption peaks with a greatly increased specific activity peak in the material from scrapie mice. In the large granule membrane preparation from normal mice there was, in fact, no evidence of a peak of specific activity at this point. The ratios of the optical densities at 260/280 mμ averaged

over fractions 8—12 are given in Table 1 and the ratios of [^{3}H]/[^{14}C] incorporation in Table 2. In further experiments high speed pellets were prepared from urea treated large granule fractions made from the same wet weights of normal and scrapie mice. DNA and RNA were extracted from these after the addition of carrier brain powder as described for post ribosomal pellet material. Hydrolysates of the separated nucleic acids were chromatographed on DOW50 columns, and the fractions counted for [^{3}H]. The results are given in Table 3.

Table 5. *Statistical Analysis of the Observed Differences between Normal and Scrapie Mice in the Specific Activities of [^{3}H] and [^{14}C] Incorporated into Fractions Eluted from Sepharose 4B Columns. The Data are Taken from the Specific Activity Peak Fractions of Figs. 1 and 2*

Preparation	[^{3}H]			[^{14}C]		
	Normal	Scrapie		Normal	Scrapie	
	Mean specific activity ± SEM	Mean specific activity ± SEM	Significance	Mean specific activity ± SEM	Mean specific activity ± SEM	Significance
Post ribosomal pellet (10)[1]	744±52	1245±91	$P<0.001$	498±37	887±49	$P<0.001$
Urea treated post ribosomal pellet (10)[1]	435±29	835±76	$P<0.001$	226±20	455±41	$P<0.001$
Urea treated large granule fraction (10)[1]	192±16	626±44	$P<0.001$	96±9	338±18	$P<0.001$
Urea treated microsomal fraction (8)[1]	263±19	520±41	$P<0.001$	130±17	255±18	$P<0.001$

[1] Number of points computed. Four or five points from each of two experiments.

3.2. A Statistical Evaluation of the Observed Differences in Incorporation in Normal and Scrapie Mice

The results were analysed by comparison of idealised areas under the specific activity peak (Figs. 1 and 2). Table 5 shows the mean specific activity ordinate and its standard error for each peak and the statistical significance of differences between normal and scrapie values. The increase of specific activity in scrapie was highly significant in all subcellular fractions.

4. Discussion

The results have confirmed those already published (ADAMS and CASPARY, 1968; ADAMS, CASPARY, and FIELD, 1969) in showing that material can be sedimented from mouse brain cell sap by prolonged high speed centrifugation and that this incorporated label from [^{3}H] thymidine and [^{14}C] glucosamine twice as rapidly when derived from scrapie as that from normal brain. It is clear, however, from the ratio of absorption at 260/280 mμ (Table 1) that the material eluted from

Sepharose 4B at the [^{3}H] and [^{14}C] specific activity peaks contained only about 1% of nucleic acid, and it seemed doubtful whether all the [^{3}H] label found in this fraction had incorporated into nucleic acid.

It is often forgotten that [^{3}H] thymidine can be considered a specific precursor for DNA only under two conditions. These are (1) that a reasonably active DNA synthesis is occurring in the system under observation and (2) that short

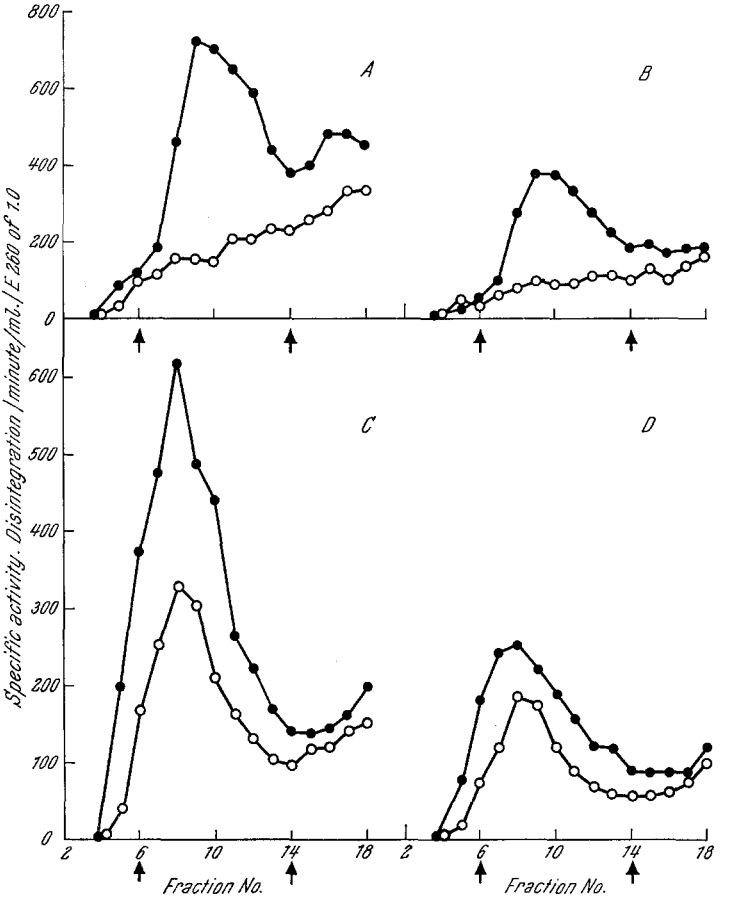


Fig. 3. As Fig. 1, but from urea treated large granule fraction. (Graph A [^{3}H], Graph B [^{14}C]), and urea treated microsomal fraction (Graph C [^{3}H], Graph D [^{14}C])

incubation times (2 hours or so) are used. The results of Table 3 show how rapidly and extensively thymidine is degraded in the brain to precursors which can be utilized for incorporation into non-nucleic acid material, such as protein. The use of very high specific activity thymidine has, presumably, accentuated the observed spread of label by allowing degradation products to pass through several precursor pools before the specific activity is reduced by dilution to negligible amounts. However, it seemed essential to use high specific activity thymidine over a long incubation period if there were to be any chance at all of detecting incorporation into scrapie agent nucleic acid.

Urea treatment of the post ribosomal pellets appeared to in fact remove a great deal of the non-specifically incorporated [^{3}H] and left a residual material in which, allowing for preparative losses, label was present in about the same quantity as that found in total nucleic acid extracted from the pellets (Table 4). The [^{3}H] specific activity found in total nucleic acid — *i.e.* RNA and DNA was increased 2—3fold (Table 4). When the nucleic acids were separated and degraded (Fig. 3) it became clear that the increased activity found in scrapie mice resided primarily in DNA rather than in RNA. Because of the prior addition of carrier nucleic acid the results in Fig. 3 cannot be calculated on a basis of specific activity, for direct comparison with those of Fig. 4. However from the same starting weight of tissue 5—10 times as many [^{3}H] counts were found associated with DNA thymidylic acid prepared from scrapie as compared with normal brain. Further, although urea treatment significantly altered the ratio of [^{3}H]/[^{14}C] labelling (Table 2), the change was not great, suggesting that polysaccharide was tightly bound to post ribosomal pellet DNA. These findings place on a much firmer footing the suggestion that there is a DNA-polysaccharide complex present in cell sap, and the results also suggest that the complex is replicating much more rapidly in the scrapie mice.

The evidence from the membrane fractions may thus be interpreted to mean that urea treatment removes a DNA-polysaccharide complex from scrapie large granule and endoplasmic reticulum membranes, and that this is identical with that produced by urea treatment of post ribosomal pellet material. This conclusion is based on four distinct lines of evidence:

1. the complexes sedimented under the same centrifugation conditions: and also

2. the complex eluted from membrane fractions showed a specific activity peak in the same place after elution through Sepharose 4B columns, and the peak fractions had the same ratio of absorption at 260 and 280 mμ.

3. The ratios of $\frac{[^3H]}{[^{14}C]}$ incorporation were not significantly different from those given by post ribosomal pellet material.

4. The results of nucleic acid extraction degradation and chromatography were almost identical with those from post ribosomal pellet material.

If then the same DNA-polysaccharide complex is obtained after urea treatment of large granule membrane fraction and of post ribosomal pellet material, the crucial questions are:

1. What is the nature of the relationship between the bound and unbound complex?

2. What is the significance of the tenfold, at least, increase in thymidine incorporation into the DNA moiety in scrapie mice?

So far as (1) is concerned, there are three possibilities:

a) The complex is formed independently in the cell sap and on membrane structures.

b) It is produced in the cell sap and then bound to membrane.

c) It is formed on membranes and released into the cell sap, either (i) as a physiological process, or (ii) as artifact of homogenisation.

'(a)' would seem unlikely on *a priori* grounds, and the constancy which has been seen in the relative amounts of bound and unbound material in a number of experiments would suggest that it is not released by simple homogenisation. Although both '(b)' and '(c)' then remain as possible explanations, the specific activities of the urea treated post ribosomal pellets and membrane fractions (Fig. 2 and 3) were higher in the post ribosomal pellet material. This would suggest alternative (b), that the complex is synthesised first in the cell sap and then bound to membranes. This is the process envisaged in the linkage substance hypothesis of ADAMS and FIELD (1968).

The evidence from labelled thymidine recovery in normal mouse preparations (Fig. 3) indicates that there may be a small content of the complex in either the bound or the unbound fraction. However, the recovered counts were so low in both cases that it is difficult to evaluate their significance and in any event this may be due to a low rate of replication in normal tissue. Nevertheless, the tenfold increase in incorporation into DNA thymidylic acid in both post ribosomal pellet and membrane fractions implies that this process of complex replication in the cell sap followed by linkage to membrane is at least greatly accelerated in scrapie mice. This again is consistent with the linkage substance hypothesis that the disease process in scrapie consists of binding of DNA-polysaccharide replicable material ("sub-virus") to membrane.

The results of Table 3 also indicated a twofold increase of incorporation of [^{3}H] label into membrane protein but an equal incorporation into cell sap protein, in scrapie mice. This suggests that there may be an overall increase in membrane metabolic activity associated with the disease. This point will be investigated in a subsequent publication.

4.1. An Attempt to Make Some Approximate Calculations

So far no calculations appear to have been made of the amount of scrapie agent DNA which could reasonably be expected to be present in scrapie brain at peak titre — assuming of course that the agent contains DNA. On the basis of reported titres of 5×10^8 infective doses/g wet wt. of brain and the estimate of target size indicating the presence of a nucleus of M.Wt. 2×10^5 (ALPER, HAIG, and CLARKE, 1967) the amount of DNA present would be about 0.2 ng/g wet wt. of brain assuming one particle per infective dose. Estimates of numbers of particles per infective dose for other viruses have varied between 10 and 100, (*e.g.* SCHWERDT and FOGH, 1957; DUMBELL, DOWNIE, and VALENTINE, 1957; KAPLAN and VALENTINE, 1959; RHIM, SMITH, and MELNICK, 1961). An additional factor with scrapie is the near certainty that homogenates of brain contain membrane fragments carrying multiple doses which will tend to raise the particles/infective dose ratio still further. However, if the above figures are accepted as a basis the probable concentration of agent DNA in scrapie brain would be between 2 and 20 ng/g wet wt.

It then seemed worthwhile to us to make an approximate estimate of the amount of DNA present in our urea treated preparations in the region of the [^{3}H] incorporation peak after Sepharose chromatography. In the present results, the optical densities found in the fractions 8—12 associated with the specific activity peak were at the limit of readability with the equipment available (E260

of 0.005—0.01/g wet wt. of brain). Since (Table 1) the E260/280 ratio of this material was in the region of 1.3, this indicated a nucleic acid content of 8—10%, there being little or no difference between normal and scrapie animals. The E260 readings of 0.005—0.01 must be reduced by a factor of two since nearly half this figure will be contributed by the protein content. In 1 ml samples this indicates the presence of approximately 100 ng/g wet wt., of total nucleic acid at the peak fraction, or say 250 ng over the whole peak area. The results have already shown the presence in post ribosomal pellets of 20—25 μg of RNA/g wet wt. of brain, and about half this quantity appears to be present in urea treated pellet material. The results of ^{14}C orotic acid incorporated (ADAMS *et al.*, 1969) also showed both in CsCl gradients and after Sepharose 4B chromatography of post ribosomal pellet material, that at least 2% of the RNA content was present in the fractions containing the DNA-polysaccharide complex. 2% of the 10—12 μg of RNA/g wet wt. present in the urea pellet, distributed through this area would mean that at least 90% of the 250 ng present in the column peak area was RNA. This indicates the presence of an amount of DNA of the order of 25 ng/g wet wt. of brain and the correct figure may well be less.

Apart from the uncertainties and speculation, involved in the calculations, those concerned with the post ribosomal pellet are related to free sub-virus, whilst those associated with membranes pertain to bound sub-virus (according to the linkage substance theory of ADAMS and FIELD, 1968). However, even supposing a transfer efficiency from cytoplasm to membrane of 100%, the theory still requires the synthesis of one sub-virus molecule for the production of one membrane bound complete particle.

The purpose of this calculation was to make an approximate assessment of the amount of DNA one might expect to be dealing with in scrapie brain, and to compare this with the amount of DNA which appears to be present in the material being produced by the fractionation procedures. The estimate of 25 ng/g wet wt. indicates at least that we are dealing with a DNA complex present in a quantity which is of the right order of magnitude, and makes it reasonable to suppose that the DNA under discussion is related to the replication of scrapie agent.

Acknowledgements

The authors would like to express their grateful thanks to Mr. D. Weightman for skilled technical assistance and to Miss Greta Joyce for help with the histological confirmation of scrapie infection.

References

1. ADAMS, D. H.: Some observations on the incorporation of precursors into the RNA of rat brain. J. Neurochem. **12**, 783—790 (1965).
2. ADAMS, D. H., and E. A. CASPARY: Nature of the scrapie virus. Brit. med. J. **3**, 173 (1967).
3. ADAMS, D. H., and E. A. CASPARY: The incorporation of nucleic acid and polysaccharide precursors into a post-ribosomal fraction of scrapie affected mouse brain. Biochem. J. **108**, 38P (1968).
4. ADAMS, D. H., E. A. CASPARY, and E. J. FIELD: The incorporation of [^{3}H] thymidine, [^{14}C] orotic acid, [^{14}C] uridine-diphospho-glucose and [^{14}C] glucosamine into a post-ribosomal fraction of normal and scrapie affected mouse brain and spleen. J. gen. Virol. **4**, 89—100 (1969).

5. Adams, D. H., and E. J. Field: The infective process in scrapie. Lancet **ii,** 714—716 (1968).
6. Dumbell, K., A. W. Downie, and R. C. Valentine: The ratio of the number of virus particles to infective titre of cowpox and vaccinia virus suspensions. Virology **4,** 467—474 (1957).
7. Hess, H., and E. Lewin: Microassay of Biochemical structural components in nervous tissue. J. Neurochem. **12,** 205—211 (1965).
8. Kaplan, C., and R. C. Valentine: The infectivity of purified and partially purified preparations of vaccinia and cowpox viruses. J. gen. Microbiol. **20,** 612—619 (1959).
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall: Protein measurement with the Folin phenol reagent. J. biol. Chem. **193,** 265—275 (1951).
10. Mould, D. L., W. Smith, and A. McL. Dawson: Centrifugation studies on the infectivities of cellular fractions derived from mouse brains infected with scrapie ("Suffolk strain"). J. gen. Microbiol. **40,** 71—79 (1965).
11. Rhim, J. S., K. O. Smith, and J. L. Melnick: Complete and coreless forms of reovirus (EiHO 10). Ratio of number of virus particles to infective units in the one-step growth cycle. Virology **15,** 428—435 (1961).
12. Schwerdt, C. E., and J. Fogh: The ratio of physical particles per infectious unit observed for poliomyelitis viruses. Virology **4,** 41—52 (1957).
13. Tyner, E. P., C. Heidelberger, and G. A. Le Page: Intracellular distribution of radioactivity in nucleic acid nucleotides and protein following simultaneous administration of ^{32}P and glycine-2-[^{14}C]. Cancer Res. **13,** 186—203 (1953).
14. Warburg, O., und W. Christian: Isolierung und Kristallisation des Gärungferments Enolase. Biochem. Z. **310,** 384—421 (1941).

Authors' address: Dr. D. H. Adams, MRC Demyelinating Diseases Research Unit, University of Newcastle upon Tyne, Westgate Road, Newcastle upon Tyne NE4 6BE, England.