GLYCOPROTEIN BIOSYNTHESIS IN NORMAL AND SCRAPIE-AFFECTED MOUSE BRAIN

By

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INTRODUCTION

Several studies of scrapie disease carried out in recent years have identified biochemical changes from the normal pattern in affected brain. Notable among these have been alterations in the levels of some lysosomal enzymes (Millson, 1965; Hunter and Millson, 1966), and an elevation of the rate of DNA turnover in scrapie-affected brain (Kimberlin and Hunter 1967). In contrast overall rates of protein synthesis (Millson and Hunter, 1968) and RNA synthesis (Kimberlin, 1968) are essentially the same in normal and affected brain. More recently, we have extended these observations to a study of the biosynthesis of glycoproteins in normal and scrapie brain, and this communication deals with some aspects of this work, which has already been reported in an abbreviated form (Hunter, 1972).

There are some special reasons for examining the metabolism of glycoproteins in scrapie brain, and in particular membrane glycoproteins. In the first place, scrapie activity is located almost exclusively in the membranous fractions of the cell (Hunter, Millson and Meek, 1964; Mould, Smith and Dawson, 1965; Millson, Hunter and Kimberlin, 1971) and there are some indications that this activity may be associated with polysaccharide or glycoprotein components. For instance, treatment with periodate of homogenates prepared from scrapie-affected mouse brain substantially reduces the biological titre of the material (Hunter, Gibbons, Kimberlin and Millson, 1969) under conditions that are fairly specific for the degradation of carbohydrate containing cis glycol linkages. Further, the lysosomal enzymes referred to above are almost exclusively glycoside hydrolases, and an increased amount of PAS staining material in histological preparations of affected brains has also been observed (Mackenzie, Wilson and Dennis, 1968). In addition, Adams, Caspary and Field (1969) have shown that there is a slightly increased incorporation of thymidine and polysaccharide precursors into a fraction of scrapie brain obtained after sedimenting most of the ribosomes. They have suggested that the scrapie agent may be a polysaccharide-nucleic acid complex. The biosynthesis of glycoproteins occurs in all subcellular membrane fractions (Zatz and Barondes, 1970) and particularly actively in the synaptosomes (Festoff, Appel and Day, 1969; Brunngraber, 1970).

The experimental techniques used in the present work have been similar to those of other workers, involving mainly measurement of the incorporation of radioactive precursors into the glycoprotein components of whole brain and subcellular fractions.

MATERIAL AND METHODS

Chemicals. L-[1-14C]Fucose (52 mCi/mM) was purchased from Calbiochem, Los Angeles, California, U.S.A.; L-[3H]Fucose (4.3 Ci/mM) was obtained from New England Nuclear Corporation, Boston, Massachusetts, U.S.A. and also from the Radiochemical Centre, Amersham (specific activity 920 mCi/mM). D-[1-14C] glucosamine (55.6 mCi/mM) and D-N-[3H]acetyl-mannosamine (500 mCi/mM) were supplied by the Radiochemical Centre, Amersham. NCS solubilizer (a commercial
preparation, containing quaternary organic bases) was purchased from G. D. Searle, Amersham, and NE233 and NE250 liquid scintillator from Nuclear Enterprises Ltd., Edinburgh, Scotland.

Animals. The mice used were BS VS (bacteria susceptible, virus susceptible) white mice (Schneider, 1959). They were inoculated i.c. with 0·02 ml. of a 10⁻² dilution of either normal or scrapie-affected mouse brain. Diagnosis of clinically affected scrapie mice was made as described previously (Hunter, Millson and Chandler, 1963). Injection of the radioactive glycoprotein precursors was performed using an Agla micrometer syringe. Between 10 µl. and 40 µl. were injected i.c. depending on the specific activity of the labelled compound.

Preparation of samples for liquid scintillation counting. Brains were homogenized in isotonic saline and a 1 : 10 homogenate prepared; one quarter of the brain provided an adequately sized sample for eventual radioactive assay. This amount was treated with an equal volume of 0·5 per cent. PTA in 6 per cent. TCA to precipitate the proteins, the rest of the sample usually being rejected. The precipitates were centrifuged to sediment the protein. The sediment was resuspended in PTA–TCA solution and washed twice by repeating the above procedure.

The washed protein from above (the equivalent of one quarter of a brain) was dissolved in 1 ml. of NCS solubilizer. The use of a whirlimixer (Fisons Ltd.) to resuspend the protein sediment greatly reduced the time required to dissolve the proteins. Ten ml. of NE233 liquid scintillation fluid was added to the samples which were then transferred to counting vials. The samples were left in the dark for 24 h. before counting in a Beckman DPM 100 liquid scintillation counter. The various subcellular components were prepared for counting in a similar manner. In a number of experiments, after sedimentation of the PTA–TCA precipitable proteins, an aliquot (usually 1 ml.) of the soluble fraction was added to 10 ml. of NE250 liquid scintillation fluid and radioactivity determined. This measurement was useful in assessing the efficiency of the i.c. inoculation technique used for administering the isotope. In the majority of experiments quenching effects were controlled by recounting samples after the addition of internal standard added to each counting vial.

Lipid extraction. Proteins were precipitated with PTA–TCA solution and pellets obtained by centrifugation at approximately 1000 g for 20 min. The pellets were treated and extracted with 19 vol. of chloroform–methanol, 2 : 1; this procedure was repeated. The pellets were finally resuspended in 5 per cent. TCA in 20 per cent. methanol, and again sedimented by centrifugation. The supernatant solutions were normally discarded and the pellets allowed to drain thoroughly before being dissolved in NCS solubilizer. In some experiments however, the lipid extracts were analyzed for radioactivity.

Cellular fractionation. The detailed procedure has been previously described (Millson, 1965). In brief, 1 : 10 homogenates of whole brain were prepared in 0·32 m-sucrose. A nuclear fraction was obtained by centrifugation at 600 g (av) for 10 min. This nuclear fraction was washed to remove contamination with cell debris by resuspending in 0·32 m-sucrose and centrifugation. This procedure was repeated 3 times. The crude mitochondrial fraction was obtained by centrifugation of the post nuclear supernatant at 14 000 g (av) for 20 min. A microsome and a soluble fraction were subsequently obtained by centrifugation of the post mitochondrial supernatant for 90 min. at 100 000 g (av). The crude mitochondrial fraction was separated into 4 subfractions by centrifugation on a discontinuous sucrose density gradient. The gradient tubes were prepared in the cold (4 °C.) by successive layering of 18 ml. of 1·2 and 10 ml. of 1·0 and 0·8 m–aqueous sucrose in 3 × 1 inch lusteroid tubes designed for the Spinco rotor No. 25.1. Three ml. of the crude mitochondrial fraction suspended in 0·32 m-sucrose was introduced on top of each gradient system. The gradients were centrifuged in a Spinco model L ultra-centrifuge at 53 500 g (av) for 120 min. The 3 clearly visible bands at the 0·32 m–0·8 m; 0·8–1·0 m and 1·0 m–1·2 m interfaces were isolated and designated myelin, synaptosomes 1 and synaptosomes 2 respectively and the resulting pellet the mitochondria. In some experiments synaptosomes 1 and
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2 were combined. Cell fractions were suspended in distilled water and protein precipitated by the addition of an equal volume of 0.5 per cent. PTA in 6 per cent. TCA. The precipitates were centrifuged to sediment the proteins and processed for radioactive counting as described above. Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using crystallized bovine plasma albumin as the standard.

**Determination of methylpentose and hexoses in acid extracts of brain.** Aqueous 10 per cent. homogenates were prepared at 2 °C. from groups of 12 mouse brains and 50 per cent. TCA was added to the homogenates to give a final concentration of 10 per cent. After standing at 2 °C. for 20 min. the protein precipitate was sedimented by centrifugation. The clear supernatant was removed and extracted 3 times with 5 vol. of diethyl ether to remove the TCA. The aqueous extract was reduced in volume to approximately 15 ml. by rotary evaporation at 60 °C. under reduced pressure. The sample was further extracted with diethyl ether and the aqueous extract reduced to 10 ml. or less by the above procedure. The final volume was made up to 10 ml. and 0.5 ml. was used for assay of hexoses and methylpentoses by the method of Dische and Shettles (1961). D-galactose and L-fucose were used as standards for hexoses and methylpentoses respectively.

**RESULTS**

**Incorporation of Radioactive Glycoprotein Precursors into Mouse Brain**

The incorporation patterns of L-[3H]fucose, L-[14C]fucose and D-\text{N}-[3H] acetyl-mannosamine into normal mouse brain were similar in that maximum incorporation is achieved between 12 and 24 h. after an i.c. injection of the isotope. The results of a more extended time course showed that this level

![Graphs showing incorporation of various glycoprotein precursors into mouse brain](image)

**Fig. 1.** Kinetics of incorporation of various glycoprotein precursors into whole normal (■) and scrapie affected (●) mouse brain. Mice were injected i.c. with 1 μCi of the appropriate compound and killed at the times indicated. The scrapie-affected mice were 18 weeks after inoculation and were showing typical signs of the disease. Groups of 6 mice were used at each time interval.
of activity was maintained for a further 24 h. In affected brain, the rate of incorporation was markedly elevated, the increase above the normal level being particularly pronounced in the period of rapid incorporation between 1 and 2 h. after the injection of the isotope. In contrast, the maximal incorporation of d-[14C]glucosamine was achieved between 12 and 15 h., and after a slight decline, the activity remained constant for 1 to 2 days. There was a slight increase in the incorporation of d-[14C]glucosamine into affected brain during the later incorporation times which was not evident before the maximum incorporation was reached (Fig. 1). The fucose label appeared to be associated

Fig. 2. The incorporation of L-[3H]fucose into scrapie-affected mouse brain during the development of the disease. Mice were injected i.c. with the isotope and killed after 1 h. Scrapie was first diagnosed clinically at 15½ weeks. The increased incorporation is expressed as a percentage of the normal controls. Groups of 6 to 10 mice were used at each time interval.

**TABLE 1**

<table>
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<th>Weeks after inoculation</th>
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Groups of 6 mice were used in each situation, and the animals were killed 2 h. after the injection of L-[3H]fucose. Subsequent manipulations are described in Material and Methods.
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almost exclusively with glycoproteins or polysaccharides as no more than 1 or 2 per cent. of this fucose label was found in lipid extracts from PTA-TCA precipitates of whole mouse brain.

The uptake of L-[3H]fucose into glycoprotein was further studied in mice affected with scrapie during the development of the disease. It was found that the increased fucose incorporation commenced approximately 10 weeks after inoculation, 5 weeks before the appearance of clinical signs (Fig. 2). The increase continued during the next 8 weeks reaching a level approximately double that of the control mice. In some experiments there was evidence that during the terminal stage of the disease there was a subsequent decline in the incorporation of L-[3H]fucose (Table 1).

Subcellular Distribution of [3H]Fucose in Mouse Brain

L-[3H]fucose was incorporated into TCA-precipitable material in all fractions of the cell (Table 1) with the highest activity occurring in the microsome fraction. During the development of disease the increased fucose incorporation was found in all fractions, the microsomes showing the highest absolute increase in specific activity. However, the mitochondria and soluble fractions of the cell displayed the highest percentage of 135 per cent. and 152 per cent. respectively and the myelin, the lowest with 63 per cent. at 10 weeks after inoculation.

Activity of L-fucosidase

The activity of L-fucosidase was elevated in affected mouse brain (Millson and Bountiff, 1972). Two experiments have thrown some light on the question of the relationship between the activity of this hydrolase and the increased fucose incorporation in scrapie brain.

In the first experiment normal and affected mice were injected i.c. with L-[3H]fucose and killed 2 h. later. The brains were removed, homogenized in isotonic saline and the nuclei sedimented; the resulting supernatant was centrifuged at 100,000 g for 90 min. to give a membrane fraction and a soluble fraction. The loss of radioactive counts from the acid precipitable material from both fractions was used as a guide to determine the effect of L-fucosidase on the degradation of endogenous glycoproteins. The effect was measured at pH 5.0 near the optimum pH of L-fucosidase and at pH 7.0 the physiological pH of the brain. No significant difference was observed between the membrane and soluble fractions from normal and affected mice at either pH value. In the second experiment the distribution of L-[3H]fucose in the subcellular fractions of control and affected mouse brain was studied at time intervals of 2 h., 1 day and 7 days. The rate of turnover of fucose containing glycoproteins in the subcellular fraction of affected mice was not significantly different from the turnover observed in normal mice.

Hexose and Methylpentose Pool Size in Mouse Brain

The major methylpentose found in mammalian cells is fucose and we have found that the methylpentose content in the brains of mice in the advanced
stages of scrapie was 41 nmoles per brain whereas 74 nmoles per brain was found in the brains of normal mice. In contrast, the total hexose content was 366 and 1222 nmoles per brain for normal and affected mice respectively.

**DISCUSSION**

We have made comparative studies of the rate of incorporation of 3 glycoprotein precursors $[^{14}C]$glucosamine, $N-[^{3}H]$acetyl-mannosamine and $[^{3}H]$-fucose into the acid precipitable fraction of whole brain from control and scrapie affected mice. Previous investigations have shown that $[^{14}C]$glucosamine is a precursor for hexosamine and sialic acid residues of brain glycoproteins and glycolipids (Barondes and Dutton, 1969; Holian, Dill and Brunngraber, 1971) whereas $N-[^{3}H]$acetyl-mannosamine is rather more specific and labels predominantly sialic acid (Kolodny, Brady, Quirk and Kanfer, 1970; Quarles and Brady, 1971). The increased rate of incorporation of $N-[^{3}H]$acetyl-mannosamine observed in scrapie brain probably occurs mainly into the sialic acid residues, but as no attempt was made in the present work to distinguish between the labelling of glycolipids and glycoproteins with these two precursors it cannot be claimed that the change in scrapie is specifically associated with glycoproteins.

The situation is much clearer with fucose since it is known that this precursor is incorporated as fucose into a terminal position in glycoproteins and there is evidence in this case that glycolipids are not labelled in brain (Zatz and Barondes, 1970); in the present work, the absence of fucose incorporation into brain glycolipids was confirmed by some studies involving the removal of lipids from the acid insoluble precipitate prior to the assay of radioactivity. It is, therefore, reasonable to conclude that the results shown in this paper represent an increased rate of fucose incorporation into the glycoprotein of scrapie brain.

After an experimental injection, the accumulation of scrapie agent in mouse brain proceeds logarithmically until about 15 weeks after inoculation, but after this time the situation is not clear (Haig and Clarke, 1965). However, in recent experiments we have found that the biological titre continues to increase at a reduced rate from 15 to 17 weeks. Thereafter it appears that there is no further increase in activity as 5 titration measurements made at 3 day intervals between 17 and 19 weeks after i.c. inoculation gave readings of 7.85±0.25 log$_{50}$/0.025 ml. We hope to publish these findings in greater detail elsewhere, but for the present they suffice to show that one would expect to find any chemical or biochemical changes, directly associated with the presence of the scrapie agent, to be maximal at about 17 weeks after an i.c. inoculation. The progressive increase in the level of fucose incorporation in affected mouse brain, just detectable 10 weeks after inoculation of the scrapie agent, does in fact reach a maximum at approximately 18 weeks. Thus it would be consistent with a phenomenon directly related to the presence of the scrapie agent.

On the other hand, we have reported previously (Millson, 1965; Hunter and Millson, 1966) that there is a progressive increase in the level of activity of $N$-acetyl-$\beta$-d-glucosaminidase and $\beta$-glucuronidase in scrapie mouse brain, first becoming detectable 8 weeks before the onset of clinical symptoms at about
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16 weeks after inoculation. Thus the increase in these glycosidases occurs approximately 2 weeks before increased \(^{3}\text{H}\)fucose incorporation is observed. Hence it is also possible that in the central nervous system of affected mice there is a progressive increase in the catabolism of glycoprotein which subsequently leads to a compensatory increase in glycoprotein biosynthesis. More recently we have observed an increase in L-fucosidase activity in the brains of affected mice (Millson and Bountiff, 1972), but the absolute amounts of enzyme are small and we have been unable to demonstrate either in vitro or in vivo any significant effect of this enzyme in cleaving radioactive fucose from previously labelled membrane and soluble fractions from normal and scrapie brain.

Preliminary studies of the fucose pool size in mouse brain indicate that it is, in fact, smaller in affected mice. Thus the specific activity of labelled fucose in the fucose pool of affected mice would be higher and this could lead subsequently to an increase in the incorporation of \(^{3}\text{H}\)fucose into fucose-containing macromolecules in scrapie brain. However, studies of \(^{3}\text{H}\)fucose distribution in various subcellular fractions during the development of the disease show that the increases are not proportionately spread between the normal and scrapie subcellular fraction. The highest percentage increase was found in the soluble and mitochondrial fraction eighteen weeks after inoculation.

It would appear that the situation can only be clarified by a more specific examination of the individual glycoprotein components of the various fractions. Such an investigation is in hand and we hope to make a detailed report shortly. It should then be possible to make a fuller assessment of the relationship between the presence of the scrapie agent in the central nervous system and the abnormalities of carbohydrates metabolism that are observed in the affected brain.

**SUMMARY**

The rates of incorporation of various radioactively labelled precursors into glycoproteins in the brain has been compared in normal mice and in mice inoculated intracerebrally with the scrapie agent. In the clinical stages of scrapie disease, there is a marked increase in the rates of incorporation of labelled L-fucose and D-N-acetylmannosamine into the glycoproteins of scrapie brain. More detailed studies using fucose as a tracer have shown that this increase first becomes detectable in scrapie brain about 10 weeks after inoculation of the agent and increases progressively thereafter until about 18 weeks after inoculation. Studies involving cellular fractionation have indicated that the increased incorporation is not spread uniformly among the different glycoprotein components of the brain.

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**REFERENCES**


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