The relationship between enzyme activity and neuroglia in the prodromal and demyelinating stages of cyanide encephalopathy in the rat

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In a previous communication (Ibrahim and Adams, 1963), we suggested that the increased enzymatic activity at the edge of the old yet active plaque of multiple sclerosis was due to the increase in oligodendrocyte population in this zone. Our observations on the multiple sclerosis plaque gave no indication whether this increase in neuroglial population and enzyme activity was a primary or secondary event in the demyelinating lesion. For this reason we have studied the neuroglial and enzyme changes in the prodromal stage of the experimental demyelinating disease produced by the injection of cyanide into the rat.

MATERIAL AND METHODS

In a pilot experiment carried out by the second author, large doses of cyanide were injected intraperitoneally into rats for periods up to five days: repeated daily injections were given and the maximum dosage achieved in the four-day period was 105 mg. Although no demyelinating lesions were produced by this regime, the oligodendroglia of the corpus callosum showed considerably increased oxidative enzyme activity as seen in histochemical enzyme preparations (see below). The very large doses of cyanide administered to the rats resulted in an impractically high mortality rate, so the following procedure, modified from Lumsden (1950), was used instead.

Twelve female rats with an average body weight of 275 g, and 13 male rats with an average weight of 350 g, were injected on five days a week with cyanide by the subcutaneous route. Solutions were prepared to contain 4 to 8 mg NaCN per ml. in distilled water. The initial dose was 2 to 3 mg. (0·8 mg./100 g. body weight). This level of dosage was maintained until no animal died following injection; the next day the survivors received an increment of 0·5 to 1 mg. in their cyanide dosage. The absence of injections on Saturday and Sunday led to increased cyanide sensitivity on Monday, so on the latter day the dosage was reduced by 1·0 mg. Injections were continued for three weeks and, by this time, the maximum dosage of cyanide was 6·0 mg. daily. All animals either died within two hours of injection or were killed by chloroform anaesthesia. Five animals survived the three-week period of injections and were killed at intervals from 25 to 92 days from the beginning of the experiment. Within 20 min. of death selected blocks from the brain were either placed in 15% formol-2% ammonium bromide or were frozen onto a cryostat chuck in a Séech chuck freezer. Three to four transverse blocks, 2 to 3 mm. thick, were usually prepared from each brain. Examination of control material established that autolytic and post-mortem changes had not occurred in the neuroglia during the 20-min. interval between death and fixation.

CYTOLOGICAL METHODS The upper and lower blocks of each brain were fixed in formol-ammonium bromide at room temperature for 24 to 36 hours and were transferred to fresh fixative at 50°C. for 10 min. before sectioning on the freezing microtome at 20 to 25 μ. These sections were then stained by Hortege’s methods for oligodendroglia (1956a, republished) and microglia (1954, republished). Penfield’s (1924) method was found especially valuable for staining oligodendroglia in the corpus callosum, where these cells are sometimes difficult to impregnate. Cajal’s (1913; 1916) gold-sublimate method and his (1920) modification of Bielschowsky’s (1904; 1909) silver method were used to demonstrate astroglia, and the unmodified Bielschowsky method was used for staining neurones and axones. Some sections from these blocks were stained by Sudan black and by the osmium tetroxide-a-naphthalamine (O.T.A.N.) method (Adams, 1959) for triglyceride fats, cholesterol esters, and phospholipids.

HISTOCHEMICAL METHODS The middle block of each brain was usually used for enzyme histochemistry. Unfixed sections were cut on the Séech cryostat at 6 μ. These sections were stained by adenosine triphosphatase (Padykula and Herman, 1955a and b), diphosphopyridine nucleotide (DPNH₂) tetrazolium reductase, and lactic dehydrogenase methods. The dehydrogenase and reductase methods were slight modifications (Adams, Davison, and Gregson, 1963) of the methods of Nachlas, Walker, and Seligman (1958a and b), Hess and Pearse

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1ATP phosphohydrolase (3.6.1.4).
2NADH₄ tetrazolium reductase = DPN diaphorase = NADH₄;
3cytochrome oxidoreductase (1.6.2.3).
4L-lactate : NAD oxidoreductase (1.1.1.27).
(1961), and Thomas and Pearse (1961); nitro blue tetrazolium (nitro BT) was used as the electron acceptor. The incubation times were: for lactic dehydrogenase one and a half hours, for DPNH₂ tetrazolium reductase one hour, and for ATPase two and a half hours. The sections stained for dehydrogenase and reductase were washed in water to remove substrate and were then briefly rinsed in acetone to remove the diffusible red monoformazan of nitro blue tetrazolium. All sections were mounted in glycerine jelly.

RESULTS

ESSENTIAL CHARACTERISTICS OF CYANIDE LESIONS

During the first few days of cyanide injections some animals develop necrotic lesions, usually seen in the corpus callosum, corpus striatum, posterior part of the cerebral cortex, hippocampus, medulla, and basal ganglia. These necrotic lesions are characterized by the relative absence of cellular reaction in the early stages of their development, by generalized softening, loss of neuroglial cytoplasm and processes, loss of neurones, pyknotic nuclei (Fig. 1), and early loss of axones (Fig. 2) throughout the lesion.

Demyelinating lesions usually appear from about the fourteenth day of injection onwards in the corpus callosum, and, sometimes, also in the callosal radiation, anterior commissure, optic chiasm and tract, hippocampal commissure, fimbria, thalamus, hypothalamus, substantia nigra, corpus striatum, hippocampus, cerebellum, medulla, and cerebral cortex. These demyelinating lesions are characterized by early oligodendroglial proliferation (Fig. 3),

Fig. 1. Necrotic lesion in corpus striatum. Note necrotic centre containing few cells and periphery showing neuroglial reaction. Hortega's silver method for microglia × 47.

Fig. 2. Degenerating axons (club and granular forms) in lighter-staining necrotic lesion (arrow). Bielschowsky's silver method for axons × 470.

Fig. 3. Early demyelinating lesion in corpus callosum. Note density of oligodendroglia in centre of lesion. Hortega's silver method for oligodendroglia × 118.
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followed by demyelination (Fig. 4) but with relative preservation of axones in the early stages (Fig. 5). The subsequent fate of these lesions is described below.

OLIGODENDROGLIA Our main interest was to observe changes in the oligodendroglia of white matter before the onset of demyelinating lesions. For this purpose, enzymatically active oligodendrocytes were counted in a transverse strip across the corpus callosum as far as the callosal radiations and in a further strip, at right angles to the first, across the junction between the corpus callosum and callosal radiation. In enzyme preparations the oligodendrocyte is recognized by its round, carmalum-stained nucleus and the crescentic or sometimes circular arrangement of enzymatically-active mitochondria around the nucleus (Adams, 1962; Friede, 1962; Yonezawa, Bornstein, Peterson, and Murray, 1962; Ibrahim and Adams, 1963).

The result of these counts is shown in Table I and they show some increase in the number of enzymatically-active oligodendrocytes in the corpus callosum during the progress of cyanide encephalopathy, even before demyelinating lesions appear. Furthermore, not only did the number of active cells increase, but the intensity of staining and size of their mitochondria also increased (Fig. 6). Likewise, the number of enzymatically-active mitochondria in the neuropil, which have been tentatively identified as those of oligodendroglia (Ibrahim and Adams, 1963), also show a considerable increase (Fig. 6). The number of enzymatically-active cells increases still further in the early demyelinating lesion (Fig. 7) but, subsequently, the number of active cells declines. These increases in enzymatic activity are not seen in necrotic lesions (Table I).

**FIG. 4.** More advanced demyelinating lesions in corpus callosum (top arrow) and hippocampal commissure (lower arrow). The dark area between the two is less affected white matter. Horte's silver method for oligodendroglia × 47.

**FIG. 5.** Partial preservation of axons in a demyelinating lesion of corpus callosum (white arrow). The rounded cells (black arrow) with dark nuclei surrounded by pale haloes are gitter cells. Bielschowsky's silver method for axons × 470.

<table>
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<tr>
<th>Days of Cyanide Injection</th>
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<td>56</td>
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1 Number of cells stained for lactic dehydrogenase per 0.1 sq. mm.
3 -- necrotic lesion elsewhere in brain

Table I

NUMBER OF ENZYMATICALLY-ACTIVE OLIGODENDROGLIA IN CORPUS CALLOSUM OF CYANIDE-INJECTED RATS

1 Number of cells stained for lactic dehydrogenase per 0.1 sq. mm.
3 -- necrotic lesion elsewhere in brain
3 -- demyelinating lesion elsewhere in brain
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To assess whether this increase in enzymatically-active cells was due to an actual increase in the number of oligodendroglia or due only to a greater proportion of cells becoming enzymatically-active, counts were made on the corpus callosum of two animals after three and 16 days of cyanide injections (Table II). The three-day animal, which had no apparent lesion anywhere in the brain, shows a slight increase in oligodendroglia while the 16-day animal, which had an early demyelinating lesion in another part of the corpus callosum, shows a further increase of these cells in normal areas. In the demyelinating lesion in the corpus callosum of this 16-day animal the number of oligodendroglia is strikingly increased even though the result is probably an underestimate on account of overlapping of cells. In silver-stained sections of late demyelinating lesions the number of oligodendrocytes declines but they do not disappear completely (Table I).

In Table III is shown the average of the mean of maximum and minimum diameters for 20 randomly selected oligodendroglia in normal areas of corpus callosum in cyanide-injected rats.
callosum in control, three-day, and 16-day animals. (Oligodendroglial diameters in the demyelinating lesion of the 16-day animal could not be accurately measured.) These results show that the oligodendroglia undergo hypertrophy, as well as hyperplasia (see above), in the pre-demyelinating phase of cyanide encephalopathy. Apart from hyperplasia and hypertrophy, the oligodendroglia in silver-stained preparations of the early demyelinating lesion show that form of intracellular oedema which is known as acute swelling (Fig. 8).

MICROGLIA These cells do not show cytological evidence of activation in silver preparations (see Ibrahim and Adams, 1963) until a definite necrotic or advanced demyelinating lesion develops. Gitter cells, containing sudanophilic and Marchi-positive (O.T.A.N.-black) granules, appear early in necrotic lesions but they are seen only in the later stages of demyelinating lesions (Fig. 9). An important difference between these two types of lesion is that the activated and gitter forms of microglia are found in the edge of the early necrotic lesion but, in the demyelinating lesion, they appear throughout the plaque, including its edge. In histochemical enzyme preparations, gitter cells show their usual characteristic oxidative activity (see Rubinstein and Smith, 1962; Smith and Rubinstein, 1962; Ibrahim and Adams, 1963).

ASTROCYTES In silver preparations of early necrotic lesions, hypertrophic and multinucleate forms of astrocytes are seen; activation of these cells leads on to the formation of a gliotic wall and, finally, to glial ‘scarring’. Silver preparations of early demyelinating lesions never show much evidence of astroglial activation, but hypertrophic and clasmatodendritic forms of astrocytes are present in the edge of older demyelinating lesions (Fig. 10). Gliosis and scar formation (Figs. 11 and 12) are seen in the last stage of these self-limiting, cyanide-induced, demyelinating lesions. Transformation of protoplasmic astrocytes into fibrous types is evident in lesions occurring in grey matter. Increased oxidative enzyme activity is seen in activated forms of astrocytes in both necrotic and demyelinating lesions (see Friede, 1962; Osterberg and Wattenberg, 1962; Rubinstein, Klatzo, and Miquel, 1962; Smith and Rubinstein, 1962).

SERUM BICARBONATE A small additional group of four animals was injected with a single dose of cyanide to assess the extent of changes in serum bicarbonate due to the drug. There was a substantial fall in the bicarbonate level (see Table IV) which was probably a result of depressed tissue respiration.

1Swelling and fragmentation of astroglial processes together with cytoplasmic granularity.
FIG. 10. Edge of a demyelinating lesion in corpus callosum. Note increased cell density at the edge and the reactive hypertrophied astrocytes (arrow) with their long dark processes directed towards the lesion. Rows of gitter cells are seen within the lesion. Cajal's gold-sublimate method for astrocytes × 118.

FIG. 11. Gliosis (arrow) in terminal stage of demyelinating lesion in corpus callosum. Cajal's gold-sublimate method for astroglia × 47.

FIG. 12. Higher power field of similar lesions to those seen in Fig. 11 to show astroglial fibres × 118.

### TABLE IV

<table>
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<tr>
<th>Hours after Injection of 2 mg. NaCN</th>
<th>Serum Bicarbonate Level (mM)</th>
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<td>5.5</td>
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<tr>
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</tr>
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### DISCUSSION

CYTOLOGICAL AND ENZYMATIC FEATURES OF CYANIDE LESIONS

Our observations have shown that both necrotic and demyelinating lesions occur during the course of cyanide encephalopathy in the rat. The necrotic lesion results in complete loss of structure, while the demyelinating lesion is characterized by loss of myelin but partial preservation of axons. In
the edge of the necrotic neuroglia, there is a moderate proliferation of microglia and astrocytes but the oligodendrocyte is not the predominant cell. Before the onset of demyelination, oligodendroglia of white matter proliferate, undergo hypertrophy, and become enzymatically more active; these changes become more prominent throughout the established demyelinating plaque. Proliferation and activation of microglia and astrocytes appear to be a secondary response to demyelination, as these changes follow but do not precede formation of the plaque.

These observations indicate that increased metabolic activity of oligodendroglia is a feature of the prodromal stage of cyanide demyelination. The initial increase in enzyme activity of the oligodendroglia does not appear to be due simply to increased activity in a greater proportion of these cells, for the 'silver' counts and cellular dimensions show comparable increases to those in the 'enzyme' counts (see Tables I, II, III). It is apparent, therefore, that the increased enzyme activity of the oligodendroglia is due to both hyperplasia and hypertrophy of these cells. It should be noted, however, that we have assumed that the initial increase in the number of oligodendroglia is due to hyperplasia (see Lewis and Swank, 1953; Smart, 1960; Koenig, 1960; Smart and Leblond, 1961; Adrian and Walker, 1962; Koenig, Bunge, and Bunge, 1962) but the possibility that it is due to infiltration or migration of these cells has not been entirely excluded.

Although the oligodendroglia proliferate and become enzymatically active in the early demyelinating lesion (the stage associated with myelin pallor) their numbers and enzyme activity subsequently decline during the later Marchi-positive and gliotic stages of the plaque. We are uncertain whether this subsequent fall in oligodendroglial population and enzyme activity indicates that cyanide demyelination is a non-progressive and self-limiting lesion or whether it is that the withdrawal of cyanide allows the demyelinating process to halt.

Previous studies have indicated that both necrotic and demyelinating lesions result from prolonged administration of cyanide to animals (Ferraro, 1933; Hurst, 1942; 1944; 1952; Lumsden, 1950; Levine and Stypulkowski, 1959) but a recent report has described lesions after brief exposure of animals to high concentrations of cyanide (van Houten and Friede, 1961). From our pilot studies it would appear that the latter technique produces predominantly necrotic and not demyelinating lesions. Necrotic lesions, whether produced by ischaemia (Macdonald and Spector, 1963), freezing (Rubinstein, Klatzo, and Miquel, 1962), or by cyanide result in little oligodendroglial proliferation and little increase in oxidative enzyme activity at the edge of the softened area. This circumstance may explain van Houten and Friede's observation of increased oxidative enzyme activity in astrocytes, which are characteristically seen at the edge of necrotic lesions and not in the oligodendroglia of their cyanide-injected rats. These authors, however, noted a diffuse increase in the enzymatic staining of white matter, which probably corresponds to the increased mitochondrial activity observed by us in white matter.

**MECHANISM OF CYANIDE DEMYELINATION**

It is known that cyanide is not a cumulative poison (Hurst, 1940; Wyndham, 1941) and it is not the products of cyanide metabolism that cause cyanide encephalopathy (Rose, Harris, and Chen, 1954). Van Houten and Friede (1961) and others have attributed the encephalopathy to the inhibition of cytochrome oxidase by cyanide but, if this explanation is accepted, it is difficult to explain the absence of lesions in the many other organs that depend upon oxidative metabolism. Moreover, cyanide inhibits a wide range of metal-dependant enzymes and it is, therefore, difficult to accept that cytochrome oxidase is the only enzyme to be impaired by this poison.

Gallagher, Judah, and Rees (1956) found that cyanide impairs the condensation of acetyl coenzyme A with α-glycerophosphate, resulting in diminished products of phosphatidic acid, a metabolic precursor of the phospholipids. However, failure of phospholipid synthesis in brain would not necessarily promote demyelination, for it has been shown that myelin phospholipids are metabolically rather inert and have, at most, only a slow turnover (Davison and Dobbing, 1960).

It is tempting to speculate that cyanide damages either vascular endothelium or the oligodendrocyte and that this results in local oedema of white matter (see Levine, 1960; Luse, 1960; van Houten and Friede, 1961). Such oedema has been observed in cyanide encephalopathy in the form of myelin pallor or reticulation and may itself, for reasons unknown, cause demyelination (Lumsden and Pomerat, 1951; Lumsden, 1957; Bunge, Bunge, and Ris, 1960; van Houten and Friede, 1961). The oligodendroglial changes we have observed in cyanide encephalopathy could be explained as hyperactivity of these cells in response to oedema. In this connexion the oligodendrocyte has been regarded as an osmo-regulator between the blood vessel and brain (Lumsden, 1957; Katzman, 1961).

**RELATIONSHIP OF CYANIDE DEMYELINATION TO MULTIPLE SCLEROSIS**

The main purpose of this experimental study has been to establish whether the increased oligodendroglial population and enzyme activity in the multiple sclerosis plaque (Ibrahim...
and Adams, 1963) is a primary or secondary phenomenon. Our observations on cyanide demyelination in the rat indicate that the oligodendroglial changes precede the onset of demyelination and, by analogy, we argue that such changes occur in the prodromal stage of the human lesion.

The experimental demyelinating lesion differs from the human plaque in that the former is not progressive. The active edge of the established plaque of multiple sclerosis is never seen in the older cyanide-induced lesion. The demyelinating process is, therefore, essentially the same in both conditions but the subsequent progress of the lesions is often different.

**SUMMARY**

After a few days of cyanide injections, rats commonly develop necrotic lesions in the brain but, after two weeks of injections, demyelinating lesions appear—predominantly in the corpus callosum. In the prodromal stages, before the onset of demyelination, the oligodendroglia of the corpus callosum show increased enzyme activity and undergo hyperplasia and hypertrophy. These changes become more marked in the early demyelinating lesion but they regress as the lesion matures and becomes inactive. The neuroglial reaction around necrotic lesions is predominantly astrocytic and microglial while the oligodendroglial element, described above, is not prominent.

It is suggested that the hyperactivity of the oligodendroglia in the prodromal stage of cyanide encephalopathy is a primary event in demyelination and is not a response to myelin breakdown. From this experimental evidence it is inferred that oligodendroglial activity at the edge of the multiple sclerosis plaque is a primary feature of the disease process in man.

**REFERENCES**


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