Structural abnormalities in neurones

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A wide variety of neuronal alterations ascribed to the effect of hypoxia of different types has been described in man and in several species of experimental animals (Spielmeyer, 1922; Gildea and Cobb, 1930; Tureen, 1936; Hoff *et al*, 1945; Morrison, 1946; Grenell and Kabat, 1974; Krogh, 1952; Courville, 1951; Meyer, 1963). In a comprehensive review of the literature, Hoff *et al* (1945) concluded that the changes were remarkably alike whatever the cause of hypoxia. These changes, probably best seen with the Nissl stain, range from non-specific forms where the cells appear very pale or are undergoing swelling and loss of stainable substance to the classical descriptions given by Spielmeyer (1922). These latter changes, which include 'coagulation necrosis' or 'ischaemic cell change', 'homogenizing cell change' and 'liquefaction necrosis', have been discussed in terms of more modern views by Greenfield and Meyer (1963) and Brierley (1976). Nissl's 'acute cell disease' (Spielmeyer's 'acute swelling') and Nissl's 'chronic cell degeneration' must be added to the above neuronal alterations because they are so often mentioned as evidence of hypoxia and ischaemia.

From this wide range of neuronal alterations Spielmeyer's ischaemic cell change, the term originally used to describe the alteration in nerve cells after general or local circulatory arrest, is now recognized as the degenerative response that is common to all types of hypoxia. Thus it is also seen in substrate deficiency (hypoglycaemia), anaemic hypoxia represented by carbon monoxide intoxication, histotoxic hypoxia as exemplified by cyanide poisoning and the complex situation represented by status epilepticus. However, it is important to stress that the patterns of distribution of ischaemic cell change in the brain may differ widely among the categories of hypoxia.

The great diversity of the morphological alterations in neurones attributed to hypoxia is probably the outcome of postmortem autolytic changes unavoidable in human material, where there is a variable delay between death and necropsy coupled with slow penetration of the fixative, and to the use of immersion-fixation in experimental animals with the inevitable introduction of histological artefacts. Thus Hicks (1968) described diminished staining of Nissl

bodies and other eosinophilic changes as the earliest neuronal alterations in anoxic necrosis in human material but considered them to be indistinguishable from postmortem changes.

There are two common cytological artefacts encountered in human and experimental animal brains each of which has been interpreted as evidence of hypoxic damage and reported as such. The hyperchromatic neurone or 'dark cell' has been the subject of numerous studies, including those of Scharer (1938), Wolf and Cowan (1949), Koenig and Koenig (1952), Cammermeyer (1960, 1961, 1962) and Cohen and Pappas (1969). This artefact is most frequently observed after a fresh neurosurgical biopsy specimen is fixed in formaldehyde, when the brain of a normal animal removed immediately after death is immersed in a fixative, or when a perfusion-fixed brain is removed immediately from the skull. In paraffin and celloidin sections the dark cells appear heavily stained and unevenly shrunken (fig 1). It is generally agreed that dark cells result from postmortem trauma incidental to removal of the brain. The second type of artefact is known as 'hydropic cell change' or 'water change' (Jakob, 1927; Cammermeyer, 1960, 1961; Coimbra, 1964). In contrast to the hyperchromatic neurone the hydropic cell is swollen and the cytoplasm stains less intensively than normal (fig 2). It is sometimes seen in human material and particularly in infants. It occurs in immersion-fixed animal brain and is a particular hazard in those that are incompletely or inadequately perfusion-fixed.

Both the above artefacts and also artefactual perineuronal and perivascular spaces can be excluded in the experimental animal by the employment of carefully controlled *in-vivo* intravascular perfusion-fixation and delayed removal of the brain. We have employed this method of fixation for our light and electron microscopic studies of the evolution of ischaemic cell change in rodents and primates in the following experimental models (1-6).

1 The preparation developed by Levine (1960) in the rat which allowed ischaemic neuronal alterations to be produced unilaterally when ligation of a common carotid artery was followed by intermittent exposure to nitrogen was modified by Brown and

Fig 1 Immersion-fixed rat neocortex showing artefact of hyperchromatic or dark neurones. One neurone has corkscrew-like apical dendrite. Paraffin. Cresyl fast violet. $\times 530$.

Fig 2 Rhesus monkey. Inadequate perfusion-fixation of cerebral cortex showing artefact of hydropic cell change with swelling and peripheral vacuolation of the cytoplasm. Paraffin. Luxol fast blue and cresyl fast violet. $\times 800$.

Brierley (1966). The removal of a plastic 'carotid clasp' at the end of the exposure ensured the restoration of blood flow and subsequently the equal perfusion-fixation of both sides of the brain (Brown and Brierley, 1966, 1968, 1972, 1973).

2 Normal rats intermittently exposed to nitrogen (Brown and Brierley, 1966, 1968, 1972).

3 Squirrel monkeys (*Saimiri sciureus*) intermittently exposed to nitrogen with and without interruption of blood flow in the right common carotid artery (Brown, 1973).

4 In the rhesus monkey (*Macaca mulatta*) ischaemic neuronal damage resulted from profound arterial hypotension induced by a ganglion-blocking agent (Arfonad), head-up tilt and arterial bleeding (Brierley and Excell, 1966; Brierley *et al*, 1969). After respiratory arrest mechanical ventilation ensured normal arterial oxygenation. This model was used in a study restricted to mild cortical boundary zone lesions and the hippocampi (Brown, 1973).

5 Insulin-induced hypoglycaemia in *M. mulatta* without hypoxaemia, hypotension or epilepsy (Brierley *et al*, 1971a and b).

6 Unilateral and bilateral interruption of blood flow in the carotid arteries of gerbils (Brierley, Brown and Levy, in the press).

For our light microscope studies we employed formaldehyde, glacial acetic acid and absolute methanol (FAM), 1:1:8 (David, 1955), and for combined light and electron microscopy 6.5 per cent glutaraldehyde in cacodylate buffer, pH 7.4. After perfusion-fixation with FAM and glutaraldehyde the brains were left *in situ* for two to three hours and one hour respectively.

While it is recognized that the study of the very earliest stages of ischaemic cell change in the inevitably immersion-fixed human brain is made difficult by the presence of histological artefacts, the later classical stages leading to cell loss and eventual gliomesodermal reaction are readily identified provided the tissue is properly processed and stained. Thus our observations in the above experimental models have been combined with those of Dr Brierley in human brains in the following description of the evolution of ischaemic cell change.

Ischaemic cell change

This is a destructive process with a definite time course that begins with an alteration in a single type of organelle and ends with the disappearance of the neurone. Until 1966 the time course of this cellular change in the mammalian brain (including rat, monkey and man) remained unknown and it was regarded as a relatively slow process. Thus the view was widely held that examination of human and

experimental animal brain material where the survival period after a hypoxic stress was less than 12 to 24 hours would be unrewarding with regard to demonstrating unequivocal neuronal alterations (Polani and MacKeith, 1954; Richardson *et al*, 1959; Spector, 1961). In contrast, our investigations (Brown and Brierley, 1966, 1968, 1972, 1973) have shown that ischaemic cell change is an extremely rapid process. It is important to stress that its time course is not influenced by the severity of the hypoxic stress but only by the size of the cell, being more rapid in small neurones than in large. Differences in severity of the hypoxic insults are reflected only in the number of nerve cells involved. It is well established that ischaemic cell change is not randomly distributed in the brain but occurs in regions which exhibit 'selective vulnerability' to hypoxic stresses. Thus in all the types of hypoxia mentioned earlier there is more or less involvement of some of the following regions: (1) cerebral cortex, layers 3, 5 and 6; (2) hippocampus, Sommer sector and endfolium; (3) amygdaloid nucleus, central and basolateral portions; (4) cerebellum, Purkinje and basket cells; (5) brain stem; (6) thalamus; (7) striatum; (8) pallidum and (9) subthalamic nucleus. The spinal cord is the most resistant.

In all our experimental models brain damage was variable with the greatest range of severity in models 1, 2 and 6. In all models light microscopic examination showed that classical ischaemic cell change was always preceded by a stage we have called 'microvacuolation' (Brown and Brierley, 1966). Microvacuolation has also been observed in the primate brain after periods of status epilepticus (Meldrum and Brierley, 1973), and also in the brains of human subjects dying one hour after cardiac arrest and three to four hours after open-heart surgery (Brierley, 1976).

In a neurone showing microvacuolation (fig 3) the nucleus is either normal or slightly shrunken, the nucleolus appears normal and shrinkage of the cell body is appreciable only in the neocortex. The microvacuoles appear as apparently empty circular or oval spaces (0.16-2.5 μm diameter) within cytoplasm of normal or slightly increased basophilia and frequently extend into the proximal portions of the dendrites (fig 4). The electron microscope has shown that the majority of microvacuoles are swollen mitochondria (fig 5) which retain their double membranes despite progressive disruption of their internal structure (McGee-Russell *et al*, 1970; Brown and Brierley, 1972). Other microvacuoles correspond to dilated tubules and cisternae of the endoplasmic reticulum. There is an apparent accumulation of abundant free ribosomes and ribosomal rosettes coupled with an increase in cytoplasmic matrix

density. Some of the neurones are more or less surrounded by the electron-lucent expanded processes of astrocytes (perineuronal spaces of light microscopy).

Microvacuolation has been seen in hippocampal (fig 6) and neocortical neurones of rat 'Levine preparations' killed by perfusion-fixation immediately after exposures to nitrogen of only five and 15 minutes (Brown and Brierley, 1973). After five minutes a minority of the damaged neurones were surrounded by swollen astrocytic processes while this was true of the majority after 15 minutes' exposure. Microvacuolation observed 16 minutes after a period of hypotension and 30 minutes after one of hypoglycaemia in the neocortex of rhesus monkeys (Brierley *et al*, 1971a) and a similar alteration seen in the neocortex of a squirrel monkey killed immediately after 30 minutes' intermittent exposure to nitrogen (Brown, 1973) represent the earliest evidence of neuronal damage after a hypoxic stress in the primate. In all species examined microvacuolation can be seen earlier and persists for a shorter time in small neurones than in large in which it ceases to be recognizable after about four hours. It has been observed in Purkinje cells up to two hours after a period of hypoxia in experimental primates and in a human subject surviving one hour after cardiac arrest (Brierley, 1976).

There is a gradual transition from the stage of microvacuolation to that of classic ischaemic cell change. The cell body is variably shrunken, and, together with its processes, stains more or less intensively with aniline dyes (fig 7). The Nissl substance is dispersed and finely granular and the cytoplasm usually stains a vivid pink with eosin and blue or mauve with Luxol fast blue. The presence of a few microvacuoles represents a transitional stage from that of microvacuolation. The nucleus is shrunken, often triangular and darkly staining with aniline dyes and dark blue with Luxol fast blue so that the nucleolus may be difficult to distinguish. In electron micrographs vacuoles of various shapes, some derived from swollen mitochondria and others from the expanded cisternae and vesicles of both smooth and granular endoplasmic reticulum, contribute to the fenestrated appearance (residual microvacuoles) of the dense cytoplasm (fig 8). Remnants of cristae can be discerned in those vacuoles derived from swollen mitochondria while the expanded smooth membranes of the Golgi complex enclose very little condensed material. Occasionally undistended membranes of the Golgi complex participate in the sequestration of an area of cytoplasm reminiscent of the formation of an autophagic vacuole. Ribosomes can be identified on the expanded membranes of the granular endoplasmic reticulum and abundant free

Fig 3 Rat Levine preparation, survival 30 minutes after 40 minutes' intermittent exposure to nitrogen. Microvacuolation in ipsilateral hippocampus. The cell bodies are occupied by small vacuoles separated by strongly basophilic cytoplasm. The nuclei appear normal. Paraffin. Cresyl fast violet. $\times 800$.

Fig 4 As figure 3, survival one hour. Ipsilateral hippocampus showing evenly distributed microvacuoles in pyramidal neurones also extending into dendrites. Perineuronal spaces are subdivided by trabeculae. 1 μ m Epon section. Toluidine blue. $\times 2000$.

Fig 5 As figure 4. Electron micrograph of a microvacuolated hippocampal pyramidal neurone. Swollen mitochondria with disorganized cristae lie within cytoplasm of increased matrix density. The nucleus shows some distortion and an increase in electron density. $\times 5200$.

Fig 6

Fig 7

Fig 6 Rat Levine preparation perfusion-fixed immediately after five minutes' intermittent exposure to nitrogen. Electron micrograph showing swollen mitochondria in normal-looking cytoplasm of a pyramidal neurone of hippocampus. $\times 9500$.

Fig 7 Rhesus monkey, three-hour survival after hypotensive episode. Ischaemic cell change in large pyramidal neurones in parietal cortex. The cell bodies are shrunken and the nuclei are triangular and darkly stained. Celloidin. Luxol fast blue and cresyl fast violet. $\times 625$.

Fig 8 As figure 7. Electron micrograph showing ischaemic cell change with residual vacuoles in a neurone in parietal cortex. Swollen mitochondria with remnants of cristae and dilatations of the endoplasmic reticulum can be seen in the extremely dense cytoplasm. The nucleolus is visible in the dense nucleoplasm. Swollen astrocytic processes surround the damaged neurone. $\times 4400$.

Fig 8

ribosomes and ribosomal rosettes are scattered throughout the electron-dense cytoplasmic matrix. Breakdown of the parallel arrays of cisternae of the granular endoplasmic reticulum accounts for the dissolution of discrete Nissl bodies as visualized with the light microscope. In some neurones the increase in electron density of both nucleus and cytoplasm makes it difficult to identify the nucleolus and nuclear membrane. The damaged neurones are usually surrounded by the swollen processes of astrocytes containing few organelles. These processes frequently impart a distorted contour to the damaged neuronal cell membrane.

This stage has been identified in two rat Levine preparations and a single primate killed immediately after exposures to nitrogen of only 15 and 30 minutes respectively (Brown and Brierley, 1973; Brown, 1973). It evolves more rapidly in small than in large neurones where it can persist up to 24 hours. In the human brain this stage persists for at least six hours (Brierley, 1976).

In the subsequent stage of ischaemic cell change with incrustations further shrinkage of the neuronal cytoplasm and nucleus occurs. The cytoplasm and

apical dendrite continue to stain pink with eosin and blue with Luxol fast blue. Typical heavily stained small, spherical or irregular bodies (incrustations) lie on or close to the surface of the cell perikaryon and sometimes those of the dendrites (fig 9). At the ultrastructural level both nucleus and cytoplasm show a marked and comparable increase in electron density (fig 10). Within the electron-dense nucleoplasm areas of extreme density and of variable size and shape are present as well as clusters of dense (41–51 nm) granules. The cytoplasm is invaginated by swollen astrocytic processes. Frequently only a narrow rim of vacuolated dense cytoplasm remains around part of the shrunken nucleus (fig 10). Dense areas of cytoplasm occurring in the peripheral portions of the perikaryon and in the cell processes correspond to the typical incrustations seen with the light microscope. The many variable-sized vacuoles scattered throughout the dense cytoplasm probably represent dilated cisternae and vesicles of both smooth and granular endoplasmic reticulum as well as the remains of swollen mitochondria (figs 11, 12). Within the dense cytoplasm it is possible to identify ribosomes lying free and on the expanded mem-

Fig 9

Fig 9 As figure 3. Survival 24 hours. Ischaemic cell change with incrustations in hippocampal pyramidal neurones. Incrustations can be seen on the cell bodies and their processes. Paraffin. Cresyl fast violet. $\times 600$.

Fig 10 As figure 3. Survival two hours. Low-power electron micrograph showing ischaemic cell change with incrustations in a neocortical neurone. Vacuolated electron-dense portions of the perikaryon and dendrites around the dense nucleus correlate with the incrustations on light microscopy. $\times 3900$.

Fig 10

Fig 11

Fig 11 As figure 10. Electron-opaque profiles of neuronal cytoplasm (arrows) within an astrocytic process contacting an incrustated neurone. The occasional absence of the plasmalemmas suggests phagocytosis. $\times 12\,400$.

Fig 12

Fig 12 Normal rat intermittently exposed to nitrogen for 40 minutes, survival 45 minutes. Ischaemic cell change with incrustations showing a mitochondrion in association with vacuolated granular material and surrounded by a single membrane (arrow). Axon terminals appear distorted by adjacent expanded astrocytic processes. $\times 14\,300$.

branes of the endoplasmic reticulum. Extremely dense mitochondria, multivesicular bodies, dense bodies (small lysosomes), residual bodies (large lysosomes) and occasionally mitochondria in association with granular endoplasmic reticulum or vacuolated granular aggregates and surrounded by single membranes (autophagic vacuoles) can also be seen (fig 12). Many of the vesicle-containing axonal profiles in contact with the damaged neurones appear normal with recognizable synaptic contacts while others appear distorted by adjacent expanded astrocytic processes (fig 12). Some show depletion and dissolution of synaptic vesicles and other evidence of degeneration. Electron-opaque profiles of neuronal cytoplasm are occasionally seen within the expanded processes of astrocytes around the damaged cells (fig 11). The occasional absence of their respective plasmalemmas suggests a process of phagocytosis.

Ischaemic cell change with incrustations has been identified in the rat brain after a survival of 30 minutes (Brown and Brierley, 1968) and after 90 minutes in the rhesus monkey (Brierley *et al*, 1971b). It persists in large neurones of the rat up to about 24 hours and in those of the monkey brain up to about

48 hours (Brown and Brierley, 1968; Brierley, 1972, 1973).

The progressive disappearance of incrustations associated with increasing homogenization and loss of stainability of the cytoplasm represents the stage of homogenizing cell change (fig 13). The shrunken triangular nucleus, which frequently shows fragmentation after survivals exceeding 24 hours, is surrounded by remnants of uniformly eosinophilic cytoplasm (mauve staining with Luxol fast blue) lacking in Nissl substance. However, the appearance of this stage in semi-thin ($1\,\mu\text{m}$) plastic sections with their superior cytological detail differs from that seen in the thicker paraffin sections in that the remnants of cytoplasm around the shrunken nucleus stain less uniformly and present a heterogeneous rather than homogeneous morphology. This is confirmed in ultrastructural observations confined at present to the rat (Brown, 1973). There is a generalized decrease in electron density of both nucleus and cytoplasm. Thus the nuclear membranes are more readily identified around the shrunken nucleus and the variably sized clumps of dense nucleoplasm and denser (41–51 nm) granules are more clearly visual-

Fig 13

Fig 13 As figure 9. Homogenizing cell change in neocortex. The shrunken triangular nucleus is surrounded by homogeneous cytoplasm. Paraffin. Luxol fast blue and cresyl fast violet. $\times 1260$.

Fig 14 As figure 9. Homogenizing cell change in a neocortical neurone showing process of invagination (arrows) of peripheral areas of homogeneous cytoplasm by astrocytic processes sometimes leaving only a narrow rim of degenerating cytoplasm around the nucleus. $\times 5000$.

Fig 14

Fig 15 As figure 14. Expansion of the space between the two nuclear membranes has produced a bleb containing finely granular material. At this point the inner of the two membranes has disappeared and the outer one has ruptured. To the left of the bleb the nuclear membrane has disappeared with consequent fusion of nucleoplasm and degraded cytoplasm. $\times 16\,250$.

ized (figs 14, 15). Frequently the nuclear envelope disappears with consequent fusion of chromatic material with the surrounding degraded cytoplasm (fig 15). Occasional large intracytoplasmic blebs are formed by expansion of the space between the two membranes of the nuclear envelope (fig 15). These contain a homogeneous finely granular material which appears to be released into the cytoplasm by rupture of the outer nuclear membrane. Dense mitochondria are still recognizable organelles within otherwise totally degenerating cytoplasm where all organized structure has disappeared leaving a debris of many small membrane-bound vacuoles, coated dense vesicles and small dense bodies amid a heterogeneous granular matrix probably representing degraded free ribosomes (fig 15). Other areas of cytoplasm at the periphery of the degenerating perikaryon have a more homogeneous character comparable to the contents of the nuclear blebs (fig 14). The whole of the neuronal cytoplasm is invaginated by swollen astrocytic processes which occasionally appear to contain degenerating neuronal cytoplasm (fig 14). There is evidence (Brown, 1973) that such a process of invagination and phagocytosis by astrocytes and also the action of phagocytic microglia may be responsible for the subsequent stage of the 'ghost cell' where the cytoplasm is either absent or reduced to a minimally staining remnant around a shrunken dark-staining nucleus and eventual cell loss.

Homogenizing cell change and cell loss have been observed in neocortical boundary zone lesions after survivals of only four hours in the rat (Brown and Brierley, 1968). In regions of selective neuronal destruction in rat and gerbil brains homogenizing cell change and ghost cells are visible with the light microscope up to 21 days and in an infarct only up to seven to 10 days (Brown and Brierley, unpublished results). They have been seen to persist in the brains of primates for 10 days or more (Brierley, 1972, 1973). In general the stage of the naked nucleus is reached earlier in small than in large neurones.

No increase in extracellular space was observed at any stage in the evolution of ischaemic cell change.

Reversibility of the process of ischaemic cell change

This is a question of the greatest clinical importance. Ideally its assessment would require the study of large numbers of animals subjected to standardized insults and with minimal differences in susceptibility between animals. The appearance of larger numbers of damaged neurones at the shorter rather than the longer survival periods would then indicate reversibility. Our observations in a large number of experimental animals (rats, gerbils and monkeys)

never showed any appreciable difference between the number of nerve cells showing microvacuolation after survivals of 0 to one hour and the number showing later stages after survivals of more than one hour. Thus the assessment of reversibility must be tentatively based on ultrastructural criteria alone. It seems probable that the greatly swollen mitochondria showing loss of cristae are irreversibly damaged. This alteration, together with increasing density of the neuronal cytoplasm, a change that possibly represents protein denaturation, and the disorganization of other organelles present in the later stages of microvacuolation suggest that reversibility might be possible within at most the first half hour after the hypoxic-ischaemic stress.

However, there is evidence for a second type of neuronal cell change which may be reversible. Previously this type of alteration in rat brain was referred to as 'severe cell change' (Brown and Brierley, 1968). This term was considered inappropriate in view of the minor alterations observed in the fine structure and 'scalloped cell' was substituted (Brown and Brierley, 1973). The slightly dense, mildly distorted, non-vacuolated neurones seen in four Levine preparations killed immediately after short exposures to nitrogen (Brown and Brierley, 1973) probably represent the initial stages of this type of neuronal alteration that can attain its final form after a survival of 45 minutes. In paraffin sections the cytoplasm and nucleus show a progressive increase in staining intensity which is accompanied by distortion of the contours of the cell by expansion of the surrounding perineuronal spaces (fig 16). Its maximum development is seen as a marked 'scalloping' of the perikaryon, a slightly distorted nucleus and a moderate increase in staining intensity of both. There is no vacuolation of the cytoplasm or pyknosis of the nucleus. In semi-thin plastic sections the superior cytological detail confirms the relative normality of the nucleus and cytoplasm (beyond a progressive increase in staining intensity) even in the most heavily scalloped neurones. The maximally distorted neurones occur most frequently in arterial boundary zone lesions in the neocortex. Electron micrographs reveal a progressive increase in density of the cytoplasmic matrix and nucleoplasm accompanying the expansion of astrocytic processes indenting the perikaryon (fig 17). Where this expansion produces marked indentations, an end stage seen after survivals of 24 hours as well as of 45 minutes, the nucleus may be slightly distorted. Breakup of polysomes frequently occurs to give an even dispersion of ribosomes throughout the cytoplasm and this is sometimes accompanied by slight swelling of the granular endoplasmic reticulum cisternae while the remaining cytoplasmic organelles appear to be normal. The

Fig 16 As figure 12. Neocortical neurones exhibiting varying degrees of scalloped cell change. Darkly stained cytoplasm is indented by swollen astrocytic processes. Paraffin. Cresyl fast violet. $\times 1000$.

Fig 17 As figure 12. Electron micrograph showing scalloped cell change in a neocortical neurone. The perikaryon is distorted by expanded astrocytic processes. Note the normality of the cytoplasmic organelles and the trabeculae bridging the perineuronal 'spaces'. $\times 3600$.

expanded astrocytic processes indenting the neurone sometimes contain flocculent material of medium electron density but few organelles. The trabeculae which can be seen by light microscopy traversing the perineuronal spaces, consist of apposed astrocytic membranes and the extended profiles of other cellular processes including axon terminals (fig 17). There is no increase in the extracellular space associated with this cell change.

As these scalloped cells do not show the disorganization of mitochondria and other organelles that is typical of all stages of ischaemic cell change, it is tempting to speculate that this alteration might be reversible if the indenting astrocytic processes returned to their normal dimensions. This type of cell change has been described in epileptic brain damage in adolescent baboons following seizures induced by allylglycine (Meldrum *et al*, 1974) and in the focal and geographical brain lesions ascribed either to air embolism or hypotension after open heart surgery (Brierley, 1963). It is also seen in oedematous human cortex (Long *et al*, 1966). Thus the application of therapeutic measures which would reverse astrocytic swelling might bring about clinical improvement.

Neuronal artefact

Light (Jakob, 1927; Scharrer, 1938; Cammermeier, 1960, 1961, 1962; Coimbra, 1964; Brown and Brierley, 1968) and combined light and electron microscope studies (Cohen and Pappas, 1969; Brown, 1973; Brown and Brierley, unpublished observations) have defined the considerable differences between the appearances of the artefacts of dark neurones and hydropic cell change and those of all the stages in ischaemic and scalloped cell change. In paraffin and plastic sections the contour of the hyperchromatic or dark cell is frequently irregular and indented, the apical dendrite being occasionally corkscrew-like (fig 1). Both cytoplasm and nucleus are shrunken and stain more intensively than normal. Nissl bodies cannot be identified in the cytoplasm and 'clear' perinuclear spaces are sometimes seen (fig 18). The nucleolus appears to be of normal size but is occasionally difficult to identify in the dark-staining nucleus. Perineuronal spaces around these neurones are rarely seen except in the hippocampus (Brown, 1973). Electron micrographs of the dark neurones show a great increase in electron density of both the nucleoplasm and cytoplasmic matrix (fig 19). The cytoplasmic organelles appear normal with the exception of the Golgi complex where the membranes are frequently expanded producing electron-lucent arrays which contrast with the dense cytoplasm (fig 19). These electron-lucent areas

Fig 18

Fig 18 Rat brain removed immediately after perfusion-fixation with glutaraldehyde and subjected to deliberate trauma. Fascia dentata. Hyperchromatic or dark neurones with 'clear' perinuclear cytoplasm. Epon. Toluidine blue. $\times 1000$.

Fig 19

Fig 19 As figure 18. Electron micrograph showing the great increase in electron density of the nucleoplasm and cytoplasm in the shrunk dark neurones of the fascia dentata. Note the electron-lucent perinuclear arrays of expanded Golgi membranes and occasional swollen perineuronal astrocytic processes. $\times 3300$.

correlate with the clear perinuclear spaces observed with the light microscope. Cohen and Pappas (1969) reported the additional dilatation of the granular endoplasmic reticulum. It is important to stress that no expansion of the mitochondria occurs and that swollen astrocytic processes in contact with these dense neurones are rarely observed. In contrast to the dark cell the hydropic cell is pale, swollen and vacuolated (fig 2). The periphery of the soma frequently has a 'fuzzy' outline and may contain one or more vacuoles. Electron microscope observations of this artefact in the cortical neurones of inadequately perfused rhesus monkeys (Brown, 1973) and in rat neurones (Brown and Brierley, unpublished observations) show clumping of the nucleoplasm, swelling and rarefaction of the granular endoplasmic reticulum, and ballooning of the mitochondria, especially in the peripheral zones of the swollen perikaryon (fig 20).

It has been previously emphasized (Brierley *et al*, 1973) that the frequency of both artefacts in the brain of a human subject or experimental animal dying after

hypoxia is not proportional to the intensity of the hypoxic stress. Their distribution does not coincide with the selectively vulnerable regions. Thus both, but particularly dark cells, are more numerous over the crests of cerebral gyri and cerebellar folia whereas neurones undergoing ischaemic cell change are more numerous in the floors of sulci. Further, neither of these artefacts has a time course.

Cammermeyer (1972) has suggested that the early microvacuolated appearance of the neurone is an artefact caused by the superimposition of spongy neuropil upon the neuronal cytoplasm. He attributes the sponginess to poor perfusion-fixation resulting from 'no-reflow', the phenomenon described by Ames *et al* (1968) who observed that after a period of circulatory arrest exceeding five minutes blood flow is not restored in particular regions of the brain. We have frequently observed microvacuolated neurones in areas of tissue showing no spongy state (Brown and Brierley, 1966, 1968, 1972, 1973). Levy *et al* (1975b) described microvacuolation in 16 rats subjected to 10 or more minutes of a controlled hypoxic-

Fig 20 *Rhesus monkey. A neurone exhibiting hydropic cell change in inadequately perfused occipital cortex. Note rarefaction of the granular endoplasmic reticulum, swelling of mitochondria and membranes of the endoplasmic reticulum, more pronounced at periphery of the perikaryon, and clumping of the nucleoplasm. $\times 3000$.*

ischaemic stress. There was only one instance of localized spongy neuropil and one of impaired reperfusion. Careful examination of thin paraffin and in particular extra thin ($1\ \mu\text{m}$) plastic sections (fig 4) in our own material show that microvacuoles are unequivocally situated in the neuronal cytoplasm. Further, Cammermeyer's hypothesis, based upon light microscopical observations only, totally disregards the evidence of the electron microscope.

Cammermeyer (1973) also regards the classic ischaemic cell change of Spielmeyer as an artefact and suggests it 'is the homologue of the dark neuron in intact tissue'. As emphasized above, both light and EM appearances permit the differentiation between the dark neurone and ischaemic cell change. Their frequency in relation to the severity of the hypoxic stress and their distribution are in no way comparable. Ischaemic cell change is a process with transitional stages from the earliest microvacuolated neurone through to the naked nucleus and glial-mesodermal reaction and is not a 'steady state' as characterized by the dark neurone.

Other studies

The histological and histochemical techniques employed by others on invariably immersion-fixed material from Levine preparations failed to identify the earliest neuronal alterations. Thus Becker and Barron (1961) described the earliest light microscopic evidence of ischaemic neuronal alterations as 'focal neuronal damage' in the caudate nucleus after three hours, in the hippocampus and neocortex after four to six hours and Becker (1961) demonstrated mitochondrial swelling after 12 hours. They considered that histochemical methods were necessary to detect the earliest ischaemic neuronal damage. Zeman (1963) reported 'loosening of tissue texture with minimal shrinkage of nerve cell bodies' in the neocortex after one hour and Spector (1963) described 'alterations of staining reaction and shrinkage of nerve cell bodies' in the hippocampus after 10-18 hours. This latter author concluded that a 'biochemical lesion', as defined by histochemical techniques, must occur before injury to the neurone can be detected by light microscopy (Spector, 1963; MacDonald and Spector, 1963). Clendenon *et al* (1971) described histological alterations in the ipsilateral hemisphere after one hour which 'consisted of pallor of the striatum and lateral cortex of Nissl staining with evidence of swelling and loss of stainability of subcortical white matter'. In all the reports typical ischaemic cell change was neither described nor illustrated.

Some of the earlier studies of the fine structure of hypoxic-ischaemic brain lesions have been limited by the use of immersion or inadequate perfusion-fixation. Hills (1964), employing immersion-fixation in osmic acid in rat Levine preparations, described swelling of astrocytic processes in the cerebral cortex after 90 minutes and degeneration of neuronal and oligodendroglial cytoplasm after three hours. The author reported difficulty in interpreting some of the initial changes because of the possibility of fixation artefact.

Scholz and Hager (1959), Hager *et al* (1960) and Hager (1963) used perfusion-fixation with osmic acid in their studies of the neocortex of the Syrian hamster killed immediately after repeated asphyxiation with nitrogen for 12 to 15 minutes. The nature of their findings and their frequency (discussed by Brown and Brierley 1972, 1973), together with their reports of incomplete perfusion-fixation, suggest that poor preservation accounts for the greater part of their observations. Their fig 2 (Hager *et al*, 1960) appears to be the ultrastructural equivalent of the artefact of hydropic cell change and is similar to the electron micrograph of the same artefact illustrated

Brierley *et al* (1973) (their fig 2) and fig 20 in the present communication.

In a combined light and EM study, Olsson and Hossmann (1971) subjected cats to transient cerebral ischaemia with or without rinsing of the intracerebral vessels during the ischaemia and reported changes in the neurones when ischaemia (without rinsing) lasted more than eight minutes and the survival period was longer than 30 minutes. Although typical microvacuolation was illustrated it was neither described nor recognized as such. It is also unfortunate that the artefact of the dark neurone seems to have been accepted as evidence of ischaemic cell change. The overall interpretation of their findings is difficult as the evidence was based upon cortical biopsies fixed by immersion and brains removed immediately after perfusion-fixation.

More recently Shay and Gonatas (1973) produced circulatory arrest for 45 minutes in the lumbar spinal cord of the cat by occlusion of the descending aorta and concurrent arterial hypotension. The spinal cord was fixed by perfusion after survivals of 75 minutes and its removal was delayed. In the anterior horn neurones they observed swelling of the mitochondria and Golgi apparatus, dispersal of ribosomes and an increase in electron density of the cytoplasm. The lysosomes were unchanged in appearance and size.

Means *et al* (1976), using perfusion-fixation and delayed removal, described microvacuolation in anterior horn cells of the cat as early as seven minutes after compression of the spinal cord. The microvacuoles corresponded to swollen mitochondria. They also reported ischaemic nerve cell change and incrustations in 15-minute, 30-minute, one-hour, four-hour and eight-hour survivals.

The neuronal alterations in developing cortical infarction in squirrel monkeys, and, in particular the role of lysosomes in the production of ischaemic cell change, were studied by Little *et al* (1974a and b). Cortical ischaemia, ranging in duration from 45 minutes to 24 hours, was produced by occluding the right middle cerebral artery with a clip which was removed at the commencement of perfusion. They detected no changes with the light microscope at 45 minutes but observed slight swelling of the rough endoplasmic reticulum at the EM level. After 90 minutes swelling of mitochondria was also seen. They described swelling and fragmentation of large lysosomes at 12 hours and concluded that 'lysosomal enzyme release is not related to the early cellular changes' but probably contributes to the final destruction of the ischaemic neurone. Their findings that lysosomes appear to be less susceptible to ischaemia than other neuronal organelles correspond with our own observations and those of Clendenon *et al* (1971)

in the rat.

Microvacuolation and its later stages, depending on the survival times employed, have also been observed with the light microscope in rat Levine preparations with physiological control (Salford *et al*, 1973; Levy *et al*, 1975b), in status epilepticus in baboons induced by bicuculline (Meldrum and Horton, 1973; Meldrum and Brierley, 1973; Meldrum *et al*, 1973) and in gerbils subjected to one hour of unilateral carotid artery occlusion (Levy *et al*, 1975a). The later stages only (shortest survival 18 hours) were seen after subatmospheric decompression (hypoxic hypoxia) in baboons and rhesus monkeys (Brierley and Nicholson, 1969; Nicholson *et al*, 1970).

General conclusions

It has been possible by the employment of perfusion-fixation and standard neuropathological techniques in experimental animals to define the nature and time course of ischaemic cell change and to endorse the long established conclusion from human neuropathological studies that this cell change is the neuronal alteration common to all types of hypoxia.

Microvacuolation of the neuronal cytoplasm is the earliest cytological response to a hypoxic stress in rodent, monkey and man and the majority of microvacuoles correspond to swollen mitochondria. Swelling of the mitochondria, together with a slight increase in density of the cytoplasmic matrix and nucleoplasm (fig 6), were the only ultrastructural alterations in the damaged neurones of rats killed immediately after intermittent exposure to nitrogen of five minutes (Brown and Brierley, 1973). Since similar changes were accompanied by other ultrastructural alterations such as dilatation of the endoplasmic reticulum and progressive condensation of the cytoplasm and nucleoplasm (described above) when longer exposures to nitrogen and longer survivals were employed (McGee-Russell *et al*, 1970; Brown and Brierley, 1972, 1973), it must be concluded that the mitochondrion is probably the most sensitive organelle to a hypoxic stress. This is to be expected as the enzyme complexes responsible for respiration and oxidative phosphorylation are localized within these organelles.

The ultrastructural finding that microvacuolation of the neuronal cytoplasm precedes any alteration in the astrocytic cytoplasm (Brown and Brierley, 1973) is in keeping with the light microscope reports of damaged neurones but undamaged astrocytes and blood vessels in physiologically controlled rat Levine preparations (Salford *et al*, 1973; Levy *et al*, 1975b) and underlines the vulnerability of the neurone to even brief hypoxic insults. Both these latter groups of

investigators, using a physiologically controlled Levine preparation which excluded the systemic variables of apnoea, hypotension, bradycardia and acidosis, convincingly demonstrated that impaired vascular reperfusion was not a factor in the genesis of the neuronal alterations.

The report by Salford *et al* (1973) that existing biochemical methods lacked the sensitivity to detect the presence of small numbers of neurones undergoing ischaemic cell change after brief periods of hypoxia, while neuropathological techniques demonstrated such changes in the brains of similarly treated animals, endorses the view that these latter techniques, when applied to optimally perfused tissue, remain the most sensitive and reliable indicators of early hypoxic brain damage.

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