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Some Effects of Methyl Mercury Salts on the Rabbit Nervous System

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Summary. Young adult rabbits have been given methyl mercury salts by subcutaneous injections or by gastric intubation. After 3 daily doses of 7.5 mg/kg by the 8th day moderate to severe ataxia developed, and after 4 doses, severe ataxia. Some of these latter animals might die. This species, therefore, seems to be about twice as sensitive to the neurotoxic properties of methyl mercury salts as the laboratory rat. With the light microscope extensive degenerative changes were seen in primary sensory ganglion cells, in both Purkinje and granule cells of the cerebellum, and in certain cells in several regions of the forebrain. The earliest changes became visible microscopically about the fourth day after commencing dosing, and reached a maximum of severity from the 7th to 10th day. The pattern of neuronal damage more closely resembled that found in the cat and in man than that seen in the rat. No evidence of changed vascular permeability was detected.

Key words: Methyl Mercury — Neurotoxicity — Spinal Ganglia — Cerebellar Cerebral Degeneration — Vascular Permeability.

Introduction

Since the early experimental studies of Hunter *et al.* (1940) with methyl mercury the rat has been extensively used, with only occasional investigation of the changes in the squirrel monkey (Grant, 1973), in the cat (Takeuchi *et al.*, 1962), in the chicken (Brown and Yoshida, 1965), and in the pig (Charlton, 1974). No extensive investigation of the rabbit nervous system has been made in this neurotoxic condition, although some early experiments of Swensson (1952) suggested that neuronal degeneration occurred in this species too, and Berlin *et al.* (1969) have used the rabbit for following the uptake of mercury into tissues after inhalation of mercury vapour.

Because of the usefulness of this species for many types of experimentation the following investigation has been made in order to delineate the pattern of neural damage.

Materials and Methods

Animals. Young adult New Zealand white rabbits of both sexes, weighing 1.0 to 2.5 kg were used. They were housed in metal cages on straw-covered wire mesh grids and fed diet S.G.1. (Oxo Ltd.) supplemented by greens and water given *ad lib.*

Chemicals. Methyl mercury chloride and methyl mercury acetate were obtained from Ralph N. Emanuel Ltd., Alperton, Middx. The chloride was suspended (5 mg/ml) in 50% polyethylene glycol 400 (B.D.H.) and injected subcutaneously, under light ether anaesthesia, at

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the base of the neck. The acetate was dissolved in tap water and given by stomach tube under light anaesthesia. The dose given was 7.5 mg/kg daily for 3 or 4 days making a total of 22.5 or 30 mg/kg respectively.

Assessment of Functional Disturbances. The animals were weighed daily and observed while sitting and walking.

The splay reflex in the hind limbs was elicited by lowering rapidly towards the floor. Their landing posture on dropping to the ground was observed. They were tested for sensation with pin-pricks.

Method of Killing. The animals were deeply anaesthetised with intravenous sodium pentobarbitone (approximately 45 mg/kg). The chest was rapidly opened and a cannula put through the left ventricle into the ascending aorta through which was pumped (120 mm Hg pressure), after a brief (1 min) saline washout, about 2 L of 10% formalin with 1% acetic acid over the course of 20 min. The carcass was left for 24 hrs before dissection and further post-fixation of tissues in 10% formalin.

Histological Methods. Coronal slices of cerebrum, coronal or sagittal and parasagittal slices of cerebellum, and transverse slices of brain stem and of spinal cord at levels C_1, C_2, C_7, C_8, Th_{12}, L_5, and S_1 were taken. Sensory spinal ganglia from the same levels were taken in longitudinal section and also the trigeminal (Vth cranial) nerve ganglia. Sciatic, peroneal, sural and median nerves were taken in transverse and longitudinal directions. These blocks were embedded in paraffin, sectioned at 7 μ or 5 μ and stained by the following methods: haematoxylin and eosin, cresyl fast violet, Luxol fast blue with cresyl violet, Glees and Marsland's silver stain for axons and occasional 20 μ sections were stained with the method of Nauomenko and Feigin (1966) for microglia.

In addition frozen sections (30 μ) were cut from tibialis anticus, psoas and foot muscles and stained with Sudan black B or with Glees and Marsland's stain for nerve fibres.

Vascular Permeability Studies. Evans Blue-albumen preparation was made by dissolving 2% Evans Blue in a 10% solution of bovine serum albumen (Fraction V. Sigma). After filtration, 20 ml was injected intravenously into the ear veins of 5 New Zealand white rabbits (1600 to 3000 g). Two hours later the animals were heparinised (250 I.U./kg) and anaesthetised with sodium pentobarbitone. After cannulation of the aorta from the left ventricle, 800 ml of Ringer's solution was run in at a pressure of 120 mm/Hg; immediately after this the tissues were fixed by perfusion of 1 L of 10% formalin under the same pressure. Following further fixation, but within 48 hrs, 25 μ frozen sections of cerebellum trigeminal and spinal ganglia, sciatic nerve and kidney were mounted in 1% glycerin and examined in a Leitz fluorescence microscope using an HBO 200 W burner with a BG/3 mm filter and a 530 k filter in the microscope tube.

One normal rabbit and 4 rabbits given methyl-Hg (7.5 mg/kg by gastric tube daily) for 1, 2 or 4 doses. They were killed 1 day after the 1st dose (day 1)—1 day after the 2nd dose (day 2), 1 day after 4 doses (day 4) and 4 days after 4 doses (day 8). One further poisoned rabbit (day 8) was given 2 ml of freshly filtered 2% Evans Blue in physiological saline intravenously and similarly washed out and perfused after 24 hrs.

^{131}I-Human serum albumen (Radiochemical Centre, Amersham) (specific activity 0.58 mc/ml Batch No. 201) was injected intravenously (100 μc/kg). Two control animals and 1 poisoned were killed 24 hrs later.

As with Evans Blue, under pentobarbitone anaesthesia the animals were perfused with 800 ml of Ringer's solution through an aortic cannula to remove as much blood as possible before 10% formalin (1 L) was perfused as fixative. After 24 hrs blocks of cerebrum (right and left), basal ganglia (right and left), cerebellum (right and left), spinal cord, dorsal root ganglia and sciatic nerve were cut out, weighed and assayed for gamma radioactivity in a scintillation counter (Nuclear Enterprises Ltd.). The radioactivity was assayed as counts/100s/mg tissue and expressed as ratio to the blood radioactivity taken at death, to normalize variations between animals.

Results

Functional Disturbances

All animals lost weight during dosing although some gained weight again temporarily between the last day of dosing and the onset of physical signs. The onset of the neurological

signs varied depending upon the route of administration. After subcutaneous injection the onset varied from the 7th to the 10th day and their severity varied equally. By contrast, after oral dosing the onset was regularly at the 7th or 8th day and the degree of severity was more constant. These differences were probably due to the occurrence of abscesses at the site of injection. Thus 5 animals were each given 4 oral doses of 7.5 mg/kg—4 were killed at 5, 7, 8 and 10 days respectively, 1 died and was discarded. Four were given 3 doses—2 were killed at 7 and 10 days respectively, 1 died and was discarded, and 1 recovered after mild functional disturbances. One animal was given 2 oral doses of 7.5 mg/kg—no signs developed and it was killed at 10 days. Two animals were given 3 daily oral doses of 6.0 mg/kg—both survived, 1 being severely, the other mildly affected.

Once begun, the development of abnormal physical signs was rapid and reached a maximum within 2 to 3 days. These features included pronounced tremor involving particularly the head and neck but often including the whole body, moderate to severe ataxia, decrease in muscle tone, and reduced splay reflex. No reduction in motor power was seen. Severely affected animals also showed diarrhoea at the onset of the functional disturbances.

Structural Changes

Primary sensory neurones—even at 4 and 5 days (day 0 is the day of the first dose) very occasional early degenerating nerve fibres could be found in longitudinal sections of sciatic nerves (Fig. 1 a), but by 7 days numerous ballooned and fragmenting axons were seen in all nerves studied (Fig. 1 b), particularly in the sural nerves. Occasional fragmenting axons were seen among fibres entering dorsal root ganglia at 7 days. Early activation of Schwann cells was visible at this time as shown by increase in nuclear and nucleolar size and increase in juxta-nuclear cytoplasm. By 8 days and even more markedly by 10 days (Fig. 1 c), there was the full picture of Wallerian-type degeneration in many fibres in all nerve bundles examined. Sections of muscles stained for nerve fibres did not show any degeneration of motor nerve fibres, although degenerating sensory nerve fibres were frequently seen in this material. At 10 days small focal necroses were commonly seen in many muscle fibres.

In dorsal root ganglia at 4 days a very few degenerating nerve cells were occasionally found (Fig. 2). At 7 days, however, many neurones were altered and early axon degeneration was present within the ganglia. A few neurones in each ganglion at this stage were necrotic and filled with phagocytic cells. Many neurones showed reduction in the amount of Nissl substance in the middle zone of the cytoplasm, the Nissl substance being well preserved at the periphery of the cell and around the nuclei (Figs. 3 and 4). The full picture of chromatolysis, with eccentric nucleus in a swollen pale cell, was rarely seen at this stage. All these cellular changes were more readily seen in the larger neurones; the state of the smaller cells was less easy to assess. By 10 days alterations in the sensory cells were maximal and loss of the middle zone of Nissl substance was common among the larger cells. Many cells in addition showed patchy focal loss of Nissl substance. Chromatolysis was still rare, but was more frequently seen at 13 days (Fig. 5). In such cells the cytoplasm was occassionally hyaline and largely free of Nissl substance, and the nucleus was sometimes markedly eccentric. A perinuclear basophilic ring was often a noteworthy feature of such cells.

Degeneration of nerve fibres accompanied by astroglial and microglial response was noteworthy in the dorsal columns throughout the spinal cord at the

10th and 13th days. There was a parallel and similar change in the spinal tract of the Vth nucleus in the medulla.

Loss of an occasional anterior horn cell was found in one 10 day animal, and in one 13 day animal that had abscesses at the subcutaneous injection sites. The normal appearances of

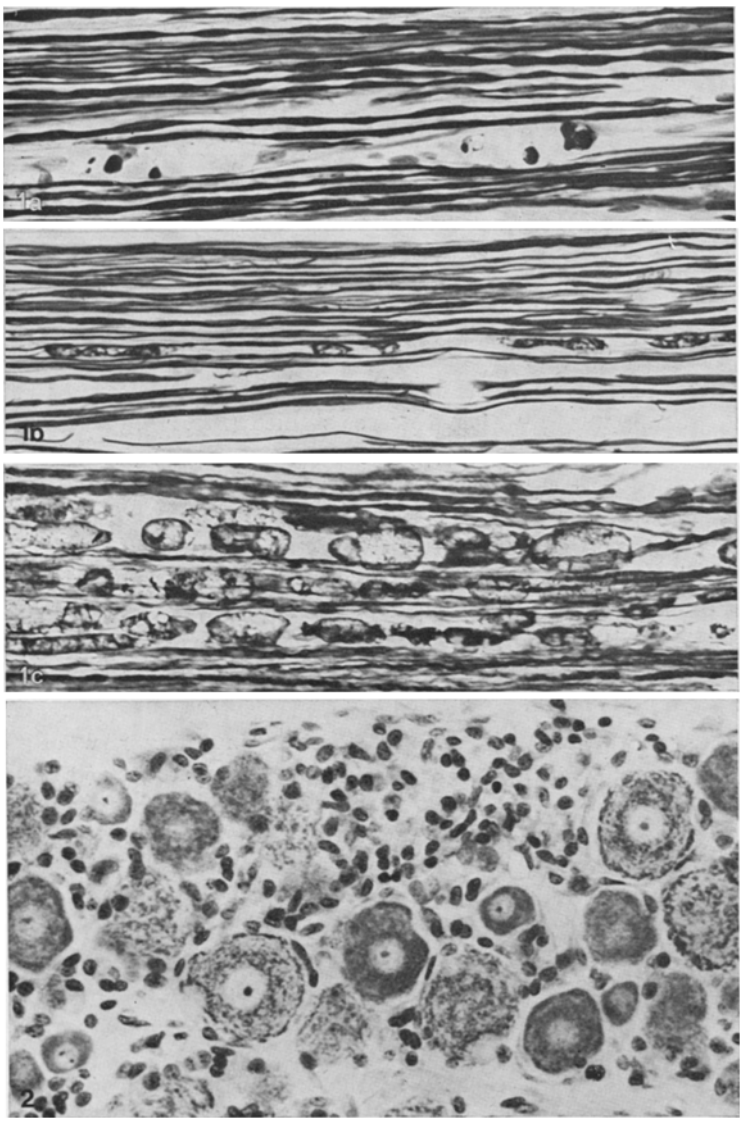


Fig. 1. (a) Sciatic nerve 4 days after 4 doses of 7.5 mg/kg. One degenerating fibre seen. Glees and Marsland. ×400. (b) Sciatic nerve 7 days after 4 doses of 7.5 mg/kg. Degenerating fibres present but scanty. Glees and Marsland. ×400. (c) Sacral spinal root 10 days after 4 doses of 7.5 mg/kg. Many fragmenting fibres present. Glees and Marsland. ×400

Fig. 2. Cervical root ganglion at 4 days after 4 doses of 7.5 mg/kg. One degenerated neurone seen at top. Cresyl fast violet. ×400

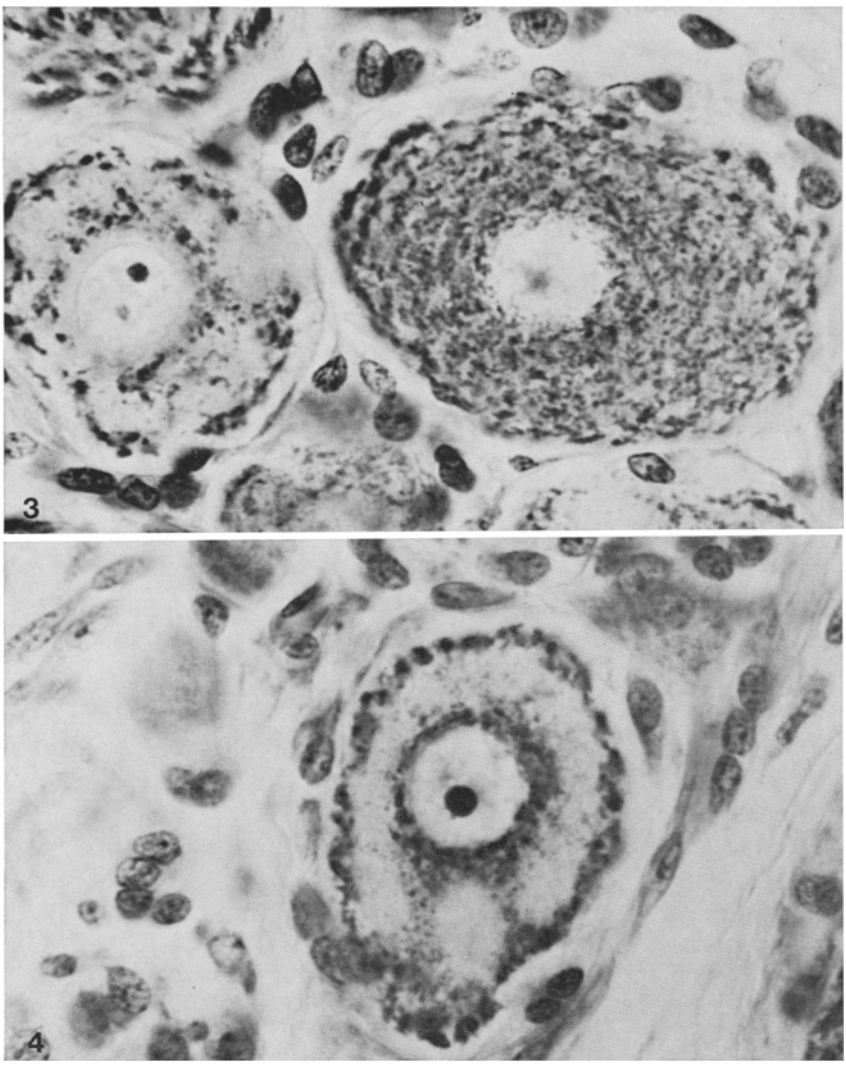


Fig. 3. 7th cervical ganglion 4 days after 4 doses of 7.5 mg/kg. A cell partly cleared of Nissl substance (left) and a normal cell (right). Cresyl fast violet. ×1000

Fig. 4. Cervical root ganglion 10 days after 4 doses of 7.5 mg/kg. Note clearing of Nissl substance from the middle zone of the cell. Cresyl fast violet. ×1000

these cells throughout the series contrasted strongly with the extensive damage to the primary sensory neurones.

Cerebellum

No changes were found in the cerebellum at 4 or at 5 days, but at 7 days there was striking widespread pyknosis of many granule cell nuclei almost everywhere (Fig. 6). This feature was less pronounced later than 7 days, but could still be seen at 13 days. None were seen in the cerebellum of animals killed at 19 and at 24 days.

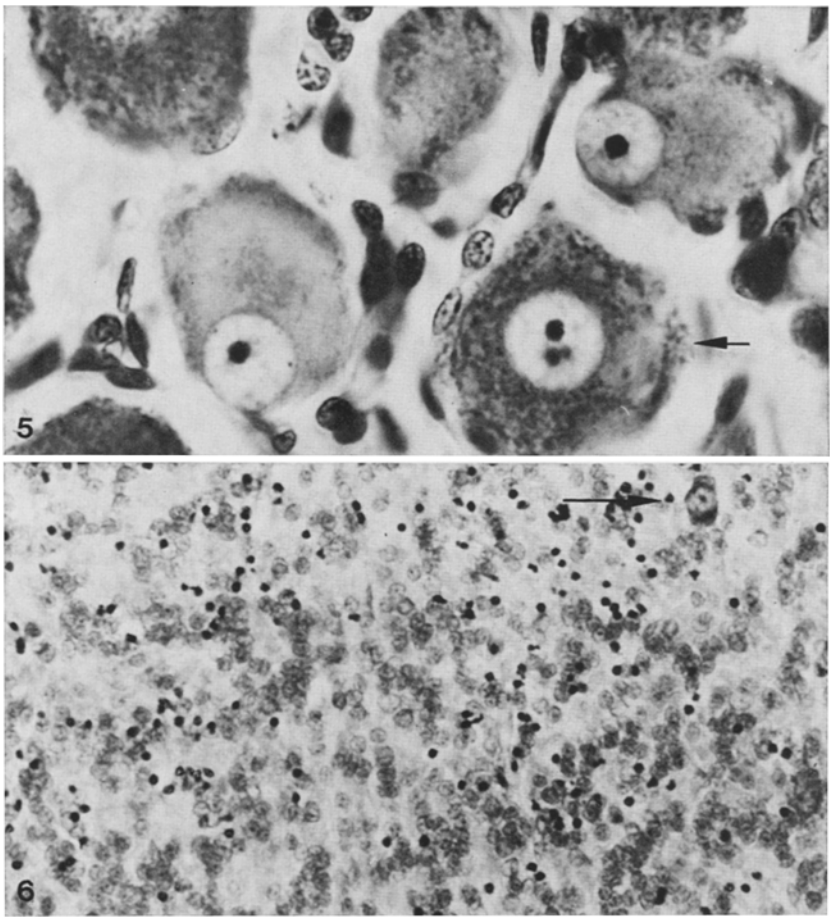
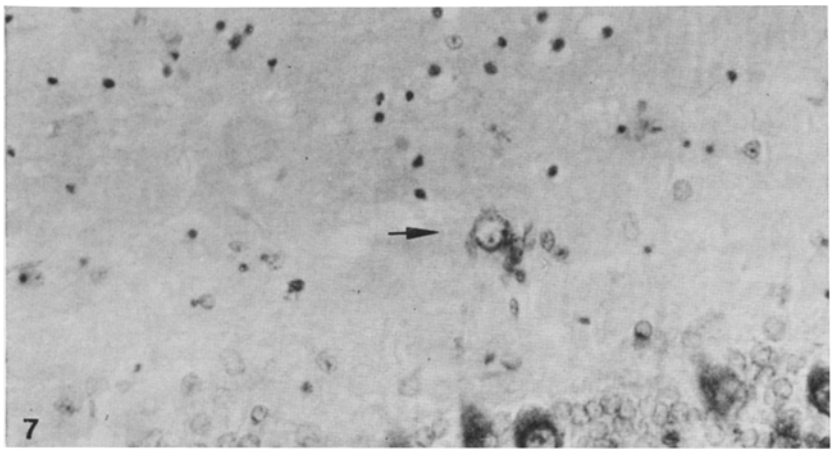


Fig. 5. 12th dorsal ganglion. Two chromatolytic cells 13th day after 4 doses of 7.5 mg/kg. Another cell (arrow) shows a cleared patch of Nissl substance. Cresyl fast violet. ×1000

Fig. 6. Granular layer of cerebellum 7 days after 4 doses of 7.5 mg/kg. Scattered pyknotic nuclei are seen. One normal Golgi cell present (arrow). Cresyl fast violet. ×400

Glial response to this feature was inconspicuous. In the molecular layer at 7 days numerous small pyknotic and karyorrhectic nuclei were to be seen scattered throughout (Fig. 7). Sometimes these degenerate nuclei were lying adjacent to stellate cells, but usually they lay on their own. They were probably microglial cells, for the granule cell clusters around ectopic Golgi neurones that are characteristic of the molecular layer of the rabbit cerebellum (Spaček *et al.*, 1973) were in general unaffected. Pyknotic nuclei in this site were also seen at 10 days but not thereafter.

Purkinje cells showed no alterations up to 7 or 8 days but at 10 (Fig. 8) and particularly at 13 days moderate numbers had randomly disappeared, leaving clusters of neuroglial cells in the molecular layer lying external to gaps in the Purkinje cell layer. Bergmann astrocytes showed reactive changes, especially at



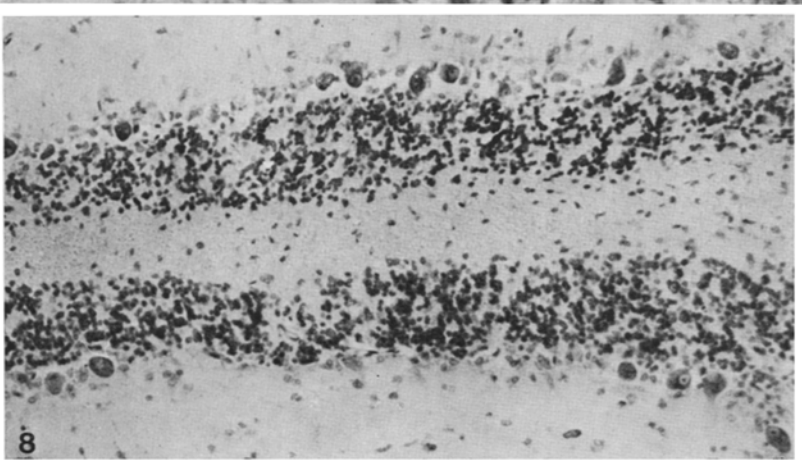


Fig. 7. Molecular layer of cerebellum 7 days after 4 doses of 7.5 mg/kg. Numerous pyknotic and karyorrhectic nuclei seen. Normal Purkinje layer below and an ectopic Golgi cell with small cluster of granule cells at arrow. Cresyl fast violet. ×400

Fig. 8. Ten days after 4 doses of 7.5 mg/kg. Cerebellar cortex showing loss of Purkinje cells and activation of glial cells. Cresyl fast violet. ×160

13 days when many were seen in mitosis near places where Purkinje cells were lost. No changes were seen in the roof nuclei of the cerebellum or in the cerebellar white matter.

Except for the degeneration of nerve fibres in the spinal tract of the Vth cranial nerve, no alterations occurred in the brain stem.

Cerebral Hemispheres

All the animals that had received 4 doses of 7.5 mg/kg showed symmetrical changes in the cerebral cortex. These were principally in the external granular, internal granular and in the external pyramidal layer (layer II, III and IV). They consisted of the presence of small pyknotic nuclei, and dividing astrocytes and microglial nuclei. In some areas, such as the area insularis, the dentate gyrus of the

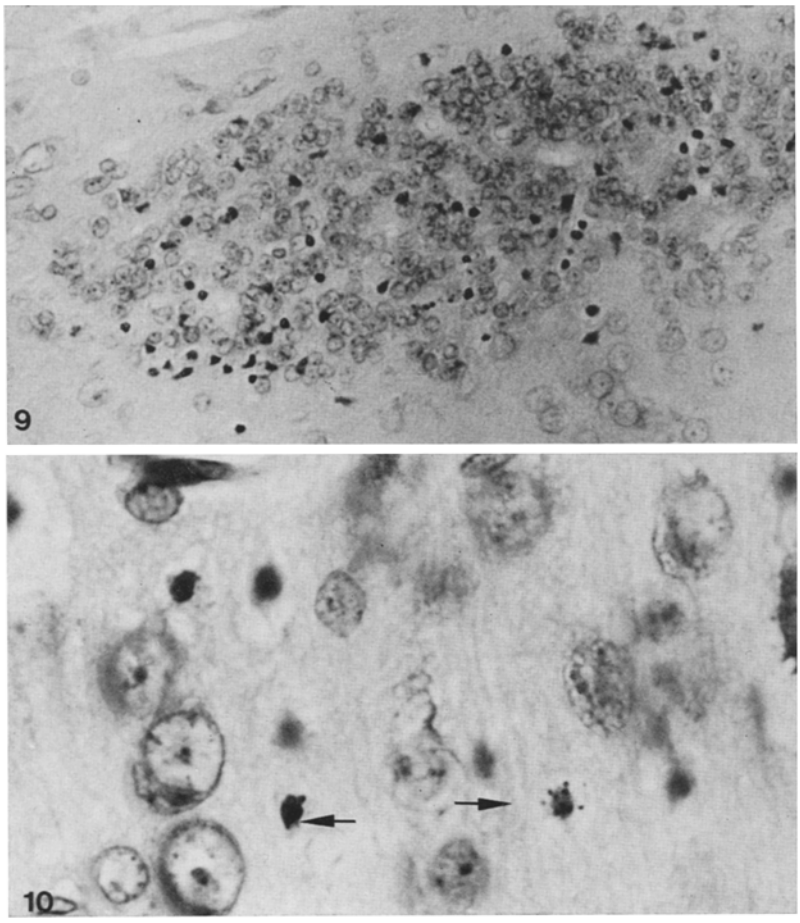


Fig. 9. Island of Calleja showing numerous pyknotic nuclei 7 days after 4 doses of 7.5 mg/kg. Cresyl fast violet. ×400

Fig. 10. Cerebral cortex showing small pyknotic nuclei 7 days after 4 doses of 7.5 mg/kg. Cresyl fast violet. ×1000

hippocampus and in the insula granularis of Calleja (Fig. 9), the changes were more extensive and striking, and scattered karyorrhexes were also present. In posterior cerebral regions (Fig. 10) the area retrosplenialis and the subiculum were notably affected. The changes in all these areas were maximal at the 10th day and appeared to involve principally small neurones, large nerve cells seeming to be unaffected. By the 13th day proliferating astrocytes and microglial cells were easily found in all these areas. The white matter was apparently unaltered.

Vascular Permeability Studies

24 hrs after ^{131}I-human serum albumen the radioactivity of the pieces of nervous tissue from the methyl mercury poisoned animal did not differ in any way from the control figures.

Fluorescence microscopy showed red fluorescence in the epi- and perineurium of the *sciatic nerves*, with less intense coloration of the endoneurium in the control and the poisoned animals equally. Sensory ganglia showed red fluorescent staining of nerve cells both centrally and peripherally. Some cells were intensely stained, some less so, and others not at all. Poisoned animals did not differ from controls. Sections of cerebellum showed no extravascular staining in either control or poisoned animals. The 8 day animals showed many brightly autofluorescent cells in the molecular and granular layers. These appearances coincided in time and situation with the pyknotic nuclei and necrotic cells seen in the conventional histological material. A few Purkinje cells were also showing bright auto-fluorescence. By neither method, therefore, was it possible to demonstrate any increase in leakiness of the vascular bed to the relatively large albumen molecule at any time between dosing and the appearance of necrotic cells.

Discussion

The rapidity of onset and the dose level needed to produce these effects were markedly different from that previously found in the rat (Cavanagh and Chen, 1971). In the latter species, 8 doses of 7.5 mg/kg produced a severe degree of functional disability, with a few ganglion cells dying in the spinal ganglia, but extensive sensory axonal degeneration. A few rats died after such a dose but no Purkinje cell loss occurred and little of note was found in the cerebrum in our experience. With half this dose (7.5 mg/kg $\times 4$) the rabbits were severely affected so that about half would have died of this series if they had been allowed to, and the disability more extensively involved the higher centres in a way not found in the rat. Moreover, in the rat little or no change was found in any region at 8 days, and microscopically definite axonal changes were not common until the 10th day, but reached a maximum at about the 13th day. By contrast, necrotic sensory neurones and fragmenting axons were beginning to be seen in these rabbits 4 to 5 days after starting the dosing schedule. As in the rat, however, axons were certainly affected more extensively than their cell bodies, which in general only tended to show patchy or diffuse loss of Nissl substance. Whether this in the early stages can truly be called chromatolysis before much axonal loss is present is debatable. Typical chromatolysis with eccentric nuclei in swollen pale cells devoid of Nissl substance was rare and occurred later. It is probable that the patchy loss of Nissl substance seen in many neurones at 4 and 5 days represents areas of ribosomal loss found at an early stage (2 days onwards) in the rat with the electron microscope, before the onset of axonal degeneration (Jacobs *et al.*, 1975). It will be necessary to confirm this for the rabbit by ultrastructural studies.

The loss of granule cells was essentially similar both in extent and in timing to that found in the rat for it followed the changes in the sensory ganglia by several days and appeared to be randomly scattered throughout the granular layer. The damage to Purkinje cells was unsuspected, but again occurred relatively late in the succession of events and appeared to be random. Purkinje cell damage was noted by Brown and Yoshida (1965) in the chicken, by Grant (1973) in the cat but not in the squirrel monkey, by Takeuchi *et al.* (1962) in the cat, and by Takeuchi *et al.* (1962) and Hunter and Russell (1954) in man. The earliest change in the cerebellar cortex was the finding of pyknotic and karyorrehectic nuclei in the

molecular layer in addition to the granule cell pyknosis due to death, possibly of microglial cells, although it was impossible to be certain of the cell type. Those damaged cells seemed to be lying close to capillaries in some instances and it seemed possible that some change in vascular permeability may have contributed to their destruction—Steinwall and Olsson (1969) showed that in rats given methyl mercury dicyandiamide intravenously, there followed a demonstrable increase in capillary permeability to Evans blue over the subsequent 24 hrs. Moreover, Jacobs *et al.* (1975) found some ultrastructural evidence of damage to capillary endothelial cells in the spinal ganglia of rats poisoned with 8 daily doses of 7.5 mg/kg of methyl mercuric chloride.

In this study, however, the rabbits were given doses that were compatible both with survival to 8 days and with the production of cell death in the cerebellar cortex. In such circumstances no accumulation of ^{131}I-labelled albumen, or the abnormal extravascular escape of Evans Blue—labelled albumen, was found in this or other regions of the nervous system. It should be recalled, however, that the dose of methyl mercury dicyandiamide given intravenously to rats by Steinwall and Olsson (1969) was 200 mg/kg. The LD_{50} by mouth of the substance for rats is in the region of 60 mg/kg and by intravenous injection is probably less than this. The accumulated dose in our animals receiving 4 doses was 30 mg/kg, a fraction of the amount given in a single dose by Steinwall and Olsson. It seems probable, therefore, that their observations are likely to be of little relevance to our experimental animals dosed chronically with small amounts of methyl mercury at least so far as permeability to large molecular weight substances such as albumen is concerned. It must be questioned, however, whether either of the 2 methods used here are sensitive enough to detect small, but perhaps significant permeability changes. It is for further studies to determine whether the permeability of the vascular bed to other substances is altered in chronic methyl mercury intoxication.

It should be noted that we found, as did Olsson (1971), that the sciatic nerve of rabbits is more permeable to the labelled albumen, than in the rat and that dye is consistently present in the endoneurium. We also found the dye stains a greater proportion of the rabbit ganglion cells than Olsson (1968) observed in the rat. It is conceivable that this natural difference in permeability to larger molecules may help to explain the marked difference between the species in their sensitivity to mercury.

Damage to the cerebral cortex varies markedly among the species examined. Cavanagh and Chen (1971) found virtually no changes in the rat, while Klein *et al.* (1973) using a more prolonged dosing schedule saw astrologial changes. In man damage may be very extensive, most often being seen in the visual cortical areas (Hunter and Russell, 1956; Takeuchi *et al.*, 1962), but occurring in other regions of the cortex as was clearly shown in the Minamata cases (Takeuchi *et al.*, 1962). Sometimes almost complete loss of nerve cells may be seen but lesser degrees of cell loss with astroglial reaction are more common. In the squirrel monkey only the cerebral cortex is affected, the maximum damage being in the visual cortical areas as in man (Grant, 1973). In the rabbits definite cell death of neurones was only apparent in the small neurones. The very small granular cells in the islands of Calleja and the granular cells of the dentate fascia were most

noticeably involved. The small neurones of the laminae II—IV, particularly in the retrosplenial region which is just anterior to the visual cortex, were next most markedly affected. Large pyramidal neurones did not appear to suffer greatly, if at all. The status spongiosus that accompanied extensive neurone loss in man and the squirrel monkey was not found in the rabbit. It is of interest that the smallest cortical lesions found by Grant (1973) in the squirrel monkey also involved small neurones of laminae II and III and even where damage was extensive in laminae V and VI, large pyramidal neurones remained preserved. The reason for this selective effect on small neurones in these regions is quite obscure at the present time, but it would appear that there is a quantitative rather than a qualitative difference between human and squirrel monkey lesions on the one hand and rabbit on the other.

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