erably enriched) when stimulated (J. S. de Belleroche, H. F. Bradford & D. G. Jones, unpublished work), providing a tool of considerable potential for the study of dopamine synthesis, storage and release. Hypothalamic synaptosomes may be prepared in some quantity (4 mg of protein/g wet wt.) from sheep hypothalamus. These preparations respire, glycolyse, and show many of the properties outlined above. They should provide a valuable preparation for the study of endogenous serotonin, or noradrenaline, metabolism and release. They have already proved most useful in investigations of the secretion of hypothalamic tropic-hormone-releasing factors (Bradford et al., 1972; Edwardson et al., 1972; Bennett & Edwardson, 1973) and are forming the basis of a combined endocrinological approach in vivo and in vitro in demonstrating how feedback mechanisms may control the secretory activities of the anterior pituitary gland through target hormones acting on releasing-factor secretion or synthesis (G. W. Bennett & J. A. Edwardson, unpublished work).

With this approach, an agent acting on neurotransmission or neurosecretion is administered to the living animal. After an appropriate interval, in which the agent is manifestly effective, the nerve endings are isolated and their properties examined under conditions in which the metabolism is maintained. By using this experimental design, it has been shown that tetanus toxin administered in vivo, but not in vitro, greatly decreased the ability of spinal cord or medullary synaptosomes to release, in particular, the proposed inhibitory transmitters, glycine and γ-aminobutyrate (Osborne & Bradford, 1973).

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Biochemical Dissection of the Cerebellum

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A knowledge of the biochemical properties of the structural units of the brain is essential for understanding the complex function of this organ. In this respect the cerebellum offers many advantages: knowledge of the morphology and physiology of the cerebellum is relatively advanced and the ultrastructure of the various constituents has been described in detail (Eccles et al., 1967). Further, certain properties of the cerebellar architecture facilitate the isolation of specific neuronal structures in a relatively pure state, and these advantages have been utilized in recent studies from several laboratories, including our own.
(1) Neurons constitute a relatively large proportion of the total number of cells in the cerebellum. Certain types of nerve cells clearly differ in size and ultrastructure, and they are well defined by their excitatory or inhibitory function in the neuronal circuits. The size of the different cell populations in the rat can be estimated approximately from the number of Purkinje cells \((3.2 \times 10^5; \text{Armstrong \\ & Shild, 1970})\) and the ratios of the various interneurons to Purkinje cells (e.g. Altman & Anderson, 1971) \((6.4 \times 10^7\) granule cells and about \(10^7\) inhibitory interneurons, i.e. stellate, basket and Golgi cells). Since the total number of cells can be estimated as \(2.1 \times 10^8\) (Balázs et al., 1971) the number of glial cells and cells in the deep cerebellar nuclei can be obtained by subtraction. In the separation of the various types of nerve cells, it is important that the volume of the cell body of the Purkinje cell is about 40 times greater than that of the granule cell.

(2) The neuropil also has a characteristic ultrastructure. It has an abundance of unmyelinated axons and this has led to the isolation of a fraction enriched in axon segments (Lemkey-Johnston & Larramendi, 1968). The rich dendritic tree of the Purkinje cells provides the skeleton of the molecular layer. The most abundant nerve terminals in this layer are those of the parallel fibres of the granule cells, and they can be readily recognized by the small dendritic spines embedded in their structure. The most conspicuous structure in the internal granular layer is the cerebellar glomerulus. The giant terminals (diameter approx. 20 \(\mu\)m) of the mossy fibres (one of the major afferents to the cerebellum) constitute the core of the glomerulus. The mossy-fibre ‘rosette’ makes synaptic contact with the dendritic digits of the granule cells and the descending dendrites of the Golgi cells whose axons also terminate in the glomerulus on the periphery of the dendrites of the granule cells. The granule cells are excited by the firing of the mossy-fibre terminals, whereas they are inhibited by the Golgi cells.

(3) Developmental changes in synaptic organization in the cerebellum are well documented (e.g. Hajós et al., 1973); this may permit the isolation of synaptosomal fractions enriched in specific types of terminals (Kornguth et al., 1972).

Fractions enriched in neuronal perikarya

These are considered in another contribution to this Colloquium (Rose, 1974). It is sufficient to mention that preparations have been obtained which are enriched in perikarya of Purkinje cells or of ‘granule’ cells (Bocci, 1966; Cohen et al., 1973; Yanagihara & Hamberger, 1973). The structural preservation and the biochemical properties of these preparations have not yet been studied in detail, but interesting results are already forthcoming. For example, it has been established that, contrary to claims based on cytophotometric studies (e.g. Lentz & Lapham, 1970) Purkinje cells contain only a diploid DNA complement (Cohen et al., 1973). Further, dissociated stem cells from the cerebellum of newborn mice can be partially separated according to differences in size during the cell cycle (Barkley et al., 1973).

Subcellular fractions containing characteristic structures at a more complex level of organization than the organelles usually encountered in conventional fractionation

This is the main topic of the present contribution. First, some of the procedures which seem to be essential for obtaining these preparations will be considered mainly on the basis of our experience gained during the development of a method for the isolation of large fragments of the cerebellar glomeruli.

Tissue disruption. When the cerebellum was dispersed by using conventional motor homogenization techniques (Whittaker, 1969) large fragments of the glomeruli were not detected in significant numbers, but good yields were obtained with a Dounce type of manual homogenizer. Since no previous information was available, homogenizations by hand and by motor were compared in terms of the shear stresses developed under these conditions (Coakley, 1974).

Dispersion and isolation media. The morphological appearance of the complex structures, such as the glomerulus particles, were greatly improved by including \(\text{MgSO}_4\) (1 mM) in the sucrose media. In the absence of \(\text{Mg}^{2+}\) the nuclei were frequently ruptured, and
Table 1. *Relative specific activity of enzymes in subcellular fractions of cerebellum*

Relative specific activity is defined as enzyme activity as a percentage of the value in the homogenate/protein content as a percentage of the value in the homogenate. The experimental procedures were described before (Hajós *et al.*, 1974; Tapia *et al.*, 1974; Baláz *et al.*, 1972; Reijnierse, 1973). All sucrose solutions contained 1mM-MgSO₄, except those used for separating fractions 4–6. Fractions 1–3 were separated from the crude nuclear fraction by centrifugation on a discontinuous sucrose gradient (fraction 3, 0.3 M/0.8 M interface) followed by the fractionation of the 1.2 M/1.4 M interface layer on a linear gradient (1.2–1.35 M) with a 1.64 M-sucrose cushion (fraction 1, 1.35 M/1.64 M interface, and fraction 2 obtained in the upper half of the gradient). Fractions 4–6 were separated from the crude mitochondrial fraction on either a linear or a discontinuous gradient (only the latter results are given). Fraction 4 was obtained at the 0.8 M/1.2 M interface, fraction 5 was the material penetrating 1.2 M-sucrose but remaining above 1.35 M-sucrose, and fraction 6 was the pellet. The data for fraction 1–3, excluding protein, were each analysed as a two-factor (subcellular fraction and enzyme) factorial experiment with replication. The data for protein and for each enzyme in fractions 4–6 were analysed in a randomized block. The results are the means ± S.E.M., with numbers of replicates in parenthesis.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Choline acetyltransferase</th>
<th>Glutamate decarboxylase</th>
<th>Glutamate dehydrogenase</th>
<th>Succinate dehydrogenase</th>
<th>Protein (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Glomerulus particles</td>
<td>1.05 ± 0.14 (5)</td>
<td>2.54 ± 0.35 (5)</td>
<td>1.15 ± 0.16 (5)</td>
<td>1.91 ± 0.31 (4)</td>
<td>1.16 ± 0.14 (4)</td>
</tr>
<tr>
<td>2. Molecular layer fragment</td>
<td>0.82 ± 0.13 (4)</td>
<td>1.09 ± 0.17 (4)</td>
<td>1.21 ± 0.18 (4)</td>
<td>1.77 ± 0.27 (4)</td>
<td>5.80 ± 0.49 (4)</td>
</tr>
<tr>
<td>3. Myelinated axon</td>
<td>0.26 ± 0.03 (6)</td>
<td>0.16 ± 0.02 (5)</td>
<td>0.51 ± 0.07 (4)</td>
<td>0.43 ± 0.06 (5)</td>
<td>10.1 ± 0.96 (6)</td>
</tr>
<tr>
<td>4. Conventional synaptosomes I</td>
<td>3.14 ± 0.26 (3)</td>
<td>0.72 ± 0.11 (2)</td>
<td>0.43 ± 0.02 (3)</td>
<td>0.83 ± 0.23 (2)</td>
<td>3.14 ± 0.20 (3)</td>
</tr>
<tr>
<td>5. Conventional synaptosomes II</td>
<td>1.64 ± 0.14 (3)</td>
<td>0.98 ± 0.15 (2)</td>
<td>1.02 ± 0.05 (3)</td>
<td>0.81 ± 0.22 (2)</td>
<td>2.14 ± 0.14 (3)</td>
</tr>
<tr>
<td>6. ‘Free’ mitochondria</td>
<td>1.09 ± 0.09 (3)</td>
<td>1.14 ± 0.17 (2)</td>
<td>5.62 ± 0.27 (3)</td>
<td>2.24 ± 0.61 (2)</td>
<td>2.31 ± 0.15 (3)</td>
</tr>
</tbody>
</table>
the concentration of synaptic vesicles in the mossy-fibre rosettes was greatly decreased. The favourable influence of Mg$^{2+}$ may be attributed to a stabilization of the existing membrane structures and to a facilitation of functionally efficient resealing processes (Allfrey, 1959; Cotman et al., 1971; Dawson & Hauser, 1970). Further, Mg$^{2+}$ also improved the resolution of the various subcellular particles from contaminants, in part by preventing agglutination of particles caused by the release of sticky nucleoplasmic material, and in part by decreasing the amount of myelin figures in the preparation (see 'Myelinated axon segments').

Electron microscopy as a monitor of procedures for the isolation of complex structures. Often the only indicator of the structure concerned is its morphological appearance. Previous observations by Cotman & Flansburg (1970) were confirmed in our studies (Hajós et al., 1974): when sections were cut from a sufficiently thin osmium-fixed pellet in the direction of the sedimentation, the various constituents of the sample, which sedimented according to size, could be surveyed in one single section. Thus electron micrographs can provide guidance for establishing new procedures. It seems, however, that after the preparations have been exposed to high concentration of sucrose solution the outlines of membranes are often vague and even discontinuous. It was observed that the appearance of membrane structures can be greatly improved by glutaraldehyde-osmium fixation of the samples (Tapia et al., 1974). The fixation of the samples must be carefully studied: methods which give good fixation of tissue sections are often not suitable for subcellular fractions or for preparations containing cell bodies (e.g. Raine et al., 1971).

So far the following characteristic morphological structures have been isolated in relatively high purity from the cerebellum: large fragments of the glomeruli (glomerulus particles), molecular layer fragments, myelinated axon segments (Hajós et al., 1974; Tapia et al., 1974; Balázs et al., 1972), and segments of the parallel fibres (Lemkey-Johnston & Larramendi, 1968).

Glomerulus particles. The isolation of synaptosomes has greatly advanced the understanding of the processes of neurotransmission (Whittaker, 1969; De Robertis & Rodriguez De Lore Arnaiz, 1969), but further developments would be facilitated by the isolation of nerve terminals of a single type. Although certain differences in sedimentation properties have been noted, the separation of different populations of synaptosomes of conventional size has not yet been achieved (e.g. Iversen & Snyder, 1968; Kuhar et al., 1971). It has been considered previously that, because of their size, the mossy-fibre terminals may be isolated from the cerebellum (Israël & Whittaker, 1965). However, it was observed that various methods of tissue disruption failed to give recognizable 'naked' mossy-fibre terminals in sufficient numbers. Instead, an attempt was made to preserve and isolate large fragments of the cerebellar glomeruli including the mossy rosette (for experimental details see Hajós et al., 1974; Tapia et al., 1974; Balázs et al., 1972). The final preparation contained almost exclusively neuronal structures: the well preserved glomerulus particles constituted about 90% of the particulate material, about half of their volume being occupied by the mossy rosette and the other half predominantly by the amputated dendritic digits of the granule cells. The mossy terminals accounted for more than 90% of the presynaptic area, but biochemical data indicated that terminals of the Golgi cells were also present (see below and Table 1).

Molecular layer fragments. The preservation of these structures was not as good as that of the glomerulus particles. The identification was therefore tentative: it was based on the orientation of rows of mitochondria and other darkly stained structures along a longitudinal axis, and on the complete lack of myelinated profiles in the preparation. This pattern is characteristic of the territory, in the middle and outer zones of the molecular layer, of the Purkinje dendrites whose primary branches apparently resisted complete fragmentation.

Myelinated axon segments. The high purity of this preparation was attributable to the swelling of the myelin envelope, which acted as a balloon and made the axon segments float at a lower sucrose concentration than the other particles, including axon segments with an intact myelin sheath. De Vries et al. (1972) have also reported that myelinated
axon fragments can be preserved by a combination of relatively mild homogenization conditions and the inclusion of ions in the sucrose media.

Parallel fibre segments. These preparations were described by Lemkey-Johnston & Larramendi (1968) and certain biochemical estimates were obtained by Dekirmenjian et al. (1969) and Simon et al. (1971).

Conventional subcellular fractions
A partial resolution of the crude mitochondrial fraction obtained from hand-homogenized cerebellum was achieved by using sucrose-density-gradient centrifugation to separate fractions containing 'free' mitochondria and apparently two different populations of synaptosomes (Reijnierse, 1973, and unpublished results from our laboratory). Further, relatively pure preparations of nuclei in exceptionally high yield can be obtained from the cerebellum (McEwen et al., 1972; Tapia et al., 1974). McEwen et al. (1972) have found that, in comparison with nuclei from other parts of the brain, the cerebellar nuclei contained small amounts of protein and RNA, and the activities of the RNA polymerases were also low. Since the cerebellar preparations contained predominantly small nuclei the results are consistent with the observations by Austoker et al. (1973) and indicate the relative inertness of small, condensed nuclei.

Biochemical characterization of subcellular fractions
Work in our laboratory is still at the initial stages, but some interesting observations were obtained by studying the distribution of certain enzymes in the subcellular fractions (Table 1).

(a) 'Transmitter enzymes'. The glomerulus particles contained a high concentration of glutamate decarboxylase. It is unlikely that the excitatory mossy fibre terminals would function with γ-aminobutyrate as the transmitter. Thus it seems that glutamate decarboxylase activity is very high in the inhibitory Golgi terminals, which occupy only a small fraction of the total volume of the particles [see also Fonnum (1973) on the high concentration of glutamate decarboxylase in other inhibitory terminals].

The low relative specific activity of choline acetyltransferase is consistent with the view, emerging also from electrophysiological and histochemical studies (Crawford et al., 1966; Silver, 1967; McCance & Phillis, 1968), that acetylcholine may be a transmitter only in a relatively small fraction of the mossy terminals. These observations indicate therefore that identical morphological structures may have a different chemical composition even when they play a similar role in a neuronal network.

A subfraction obtained from the crude mitochondrial fraction contained high choline acetyltransferase activity (fraction 4, Table 1). Since only 10% of this enzyme activity was free, it seems that this subfraction was enriched in cholinergic synaptosomes. A similar subfraction has been obtained by Dekirmenjian et al. (1969) and they believe that its synaptosomes are mainly derived from the parallel fibres.

(b) Mitochondrial enzymes. The enzyme composition of mitochondria recovered in the various fractions showed characteristic differences. In comparison with glutamate dehydrogenase, the activity of succinate dehydrogenase was similar in the axon segments, it was almost double in the glomerulus particles, whereas it was about 40% in the 'free' mitochondrial fraction. A great proportion of mitochondria in the glomerulus particles is derived from the mossy terminals, whereas, under the present experimental conditions, the contribution of glial mitochondria in the 'free' mitochondrial fraction must be substantial. These results therefore confirm and extend previous observations on mitochondrial heterogeneity and are consistent with the hypotheses that have been recently developed about metabolic compartmentation in the brain (e.g. Van Den Berg, 1973; Baláz et al., 1973).

Although most of the work reviewed here related to the cerebellum, the techniques could be adopted for isolating complex structures from other parts of the brain.

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