

## A COMPARISON OF ENZYME PATTERNS IN THE GRANULAR AND MOLECULAR LAYERS OF THE RABBIT CEREBELLAR CORTEX

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(Accepted January 6th, 1972)

### INTRODUCTION

The exploration of cerebellar function has been hampered by a disproportionately low emphasis on cerebellar biochemistry. While pathways of several cerebellar nerve fibers<sup>16-18</sup> and of the sites of their synaptic contacts<sup>15</sup> have been successfully traced by ultrastructural methods<sup>1,10,16,17,32,34</sup>, studies focusing on cerebellar enzyme activities<sup>2,3,13,14,23</sup> and subcellular organelles<sup>5,6,20</sup> have been relatively scarce. Although, as one isolated example, the  $\gamma$ -aminobutyric acid system has been studied in cerebella of several species<sup>9,14</sup> and the synthesis of  $\gamma$ -aminobutyric acid shown to occur in Purkinje axon terminals<sup>9</sup>, a comprehensive study of the intracellular localization of a complete set of 'marker enzymes' has not been reported. Kuriyama *et al.*<sup>11</sup> measured the levels of glutamic decarboxylase in the molecular and granular layers of the cerebellum of the rabbit while Robins *et al.*<sup>25</sup>, Hirsch<sup>13</sup> and, more recently, Shuter *et al.*<sup>31</sup> determined the levels of several hydrolytic enzymes in the granular and molecular layers of the cerebellum of several mammalian species. Recently, we carried out a similar study which we describe in the present report. A preliminary account of some of the findings has appeared<sup>28</sup>.

### MATERIALS

#### *Chemicals*

*O*-Nitrocatechol, *O*-nitrocatechol sulfate (K salt), acetylthiocholine iodide, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride (INT), pyridoxal phosphate, phenazine methosulfate and DL- $\alpha$ -glycerophosphate were from Sigma Co., St. Louis, Mo.; 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was a product of K and

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K Laboratories, Plainview, New York; dithiothreitol was purchased from Calbiochem., Los Angeles, California; L-glutamic acid was from Distillation Products, Rochester, New York; L-glutamine (ammonia free) and sucrose (enzyme grade) were from Schwarz-Mann, Orangeburg, New York; ADP from P-L Biochemicals, Inc., Milwaukee, Wisconsin; Biosolv-3 from Beckman Instruments, Fullerton, California and Triton X-100 from Rohm and Haas, Philadelphia, Pennsylvania. L-[<sup>14</sup>C]glutamic acid (U.L., s.a. 175 mCi/mole) was from ICN Industries, Irvine, California.

### *Animals*

Adult, male rabbits (1.5-2 kg body weight) were used. They were killed by air injection into the ear vein and immediately after death the skull was opened and the cerebellum excised and placed on an ice-cold surface.

## METHODS

### *Analytical*

Protein was determined according to Lowry *et al.*<sup>21</sup> with bovine serum albumin as standard.

### *Manual dissection of the granular and molecular layers*

The entire cerebellum was removed and placed on a prechilled, jacketed stainless steel plate which was continuously cooled to maintain a surface temperature of about 4 °C. The granular and molecular layers were hand-dissected using a stereo-view microscope and the separated samples placed into tared beakers containing ice-cold 0.25 M sucrose. No attempt was made to separate the Purkinje cell somata from the molecular layer (see Discussion for details). Fig. 1 illustrates the appearance of typical granular and molecular layer preparations. Generally, the granular layer was contaminated by the molecular layer elements more significantly (up to 20%,) than the latter by the granular layer elements (about 10%,).

### *Enzymes*

Arylsulfatase (EC 3.1.6.1) and succinate-INT-reductase (EC 1.3.99.1) were measured as previously described<sup>27</sup>. Acetylcholinesterase (EC 3.1.1.7) was determined according to Ellman *et al.*<sup>8</sup>. The mitochondrial  $\alpha$ -glycerophosphate dehydrogenase (EC 1.1.2.1) was assayed as follows: to 50  $\mu$ l of 0.125 M K-phosphate buffer (pH 7.5) containing 0.005 M KCN, were added 50  $\mu$ l of 0.23 M DL- $\alpha$ -glycerophosphate and up to 100  $\mu$ l of tissue extract and the mixture was incubated at 30 °C for 15 min. Then, 150  $\mu$ l of a solution containing 0.7 mg of phenazine methosulfate and 4 mg of INT/ml were pipetted into the incubating tubes which were vigorously buzzed

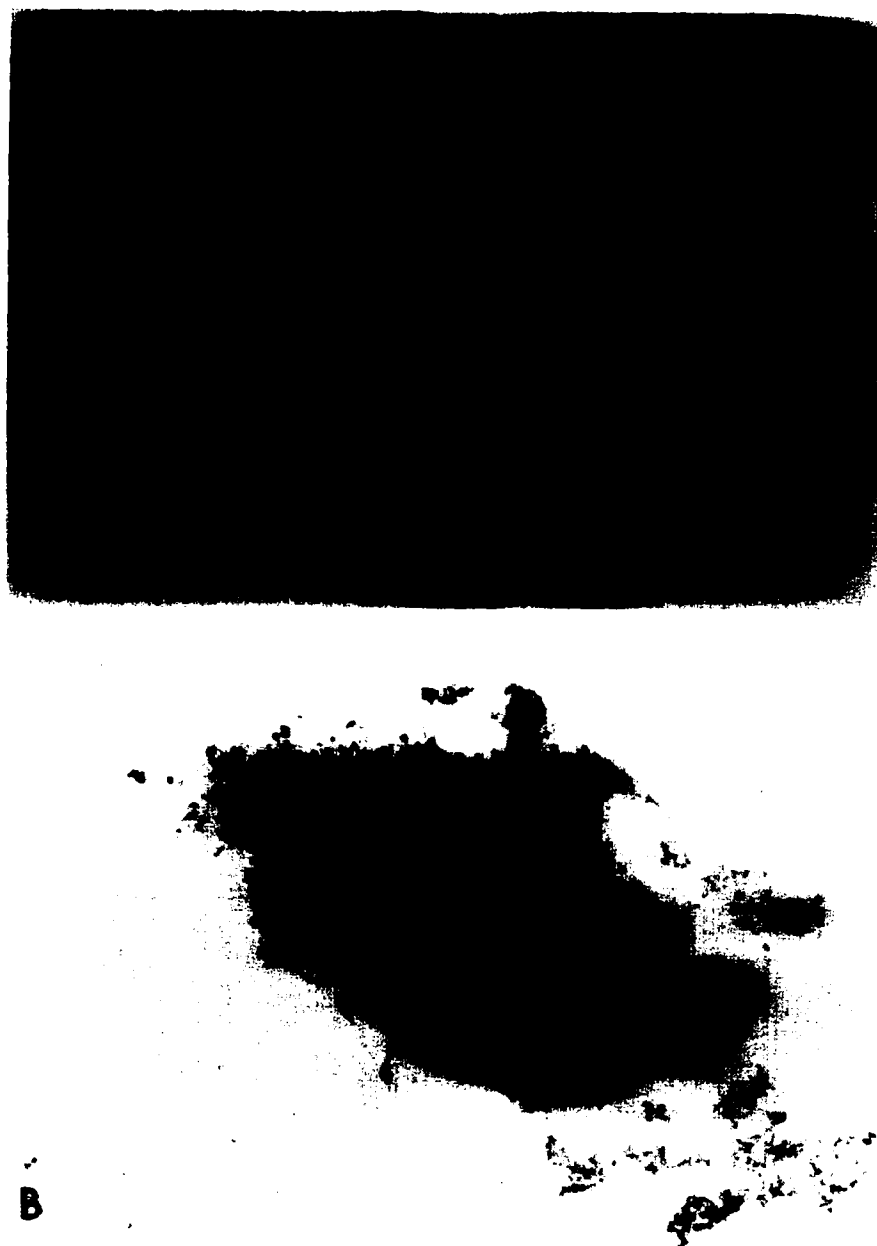


Fig. 1. A, Hematoxylin-eosin stained section through the hand-dissected molecular layer.  $\times 20$ . B, Hematoxylin-eosin stained section through the hand-dissected granular layer.  $\times 20$ .

and replaced in the bath for an additional 15 min of incubation. The reaction was terminated by the addition of 100  $\mu$ l of 10% (w/v) trichloroacetic acid followed by 3 ml of 95% (v/v) ethanol and vigorous mixing. The absorbancy of the resulting clear solution was determined at 500 nm. Glutamine synthetase (EC 6.3.1.2) was assayed according to Sellinger *et al.*<sup>26</sup> and glutamic decarboxylase (EC 4.1.1.15)

according to Susz *et al.*<sup>33</sup>, except that U.L. 1-<sup>14</sup>C]glutamate and dithiothreitol replaced L-[1-<sup>14</sup>C]glutamate and aminoethylisouronium bromide during the incubation and 10 N KOH replaced hyamine in the center well of the incubation flask. The solubilizer Biosolv-3 was used in conjunction with the toluene scintillation solvent for determination of radioactivity.

#### *Radioactivity*

This was determined in 10 ml of scintillation fluid containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene per liter of toluene. A Nuclear Chicago Unilux II spectrometer was used. Quenching corrections were applied using the channels ratio method and the counts/min were converted to disintegrations per min (disint./min). The counting efficiency ranged between 80 and 88%.

#### *Centrifugal fractionation of the granular and molecular cerebellar layers*

The dissected samples were weighed (609 ± 94 mg (S.D.) (n. 20) for the granular and 552 ± 100 mg (S.D.) (n. 20) for the molecular layer) and homogenized by 15 up-and-down strokes of a machine-driven stainless steel Teflon pestle rotating inside a glass vessel at an approximate speed of 500 rev./min. The homogenate (10–15% w/v) in 0.25 M sucrose was fractionated into 4 primary (p) and 5 gradient (g) fractions according to the procedure of De Robertis *et al.*<sup>7</sup>. Two of the gradient bands containing nerve endings (fractions 1.2 M and 1.4 M) were further fractionated by centrifugation in continuous, linear gradients of sucrose, 37–45% (w/v) for fraction 1.2 M and 42–50% (w/v) for fraction 1.4 M in the rotor SW-40 of the Spinco ultracentrifuge for 13 h at 20,000 rev./min. The gradients were pumped through a hole punched in the bottom of the centrifuge tube by means of a proportioning pump into test tubes resting in fraction collector racks. The effluent was collected at a rate of 15 drops/tube.

## RESULTS

#### *Determination of enzyme activities*

Two mitochondrial enzymes, succinate-INT-reductase and  $\alpha$ -glycerophosphate dehydrogenase, lysosomal arylsulfatase, two membrane-associated enzymes, acetylcholinesterase and glutamine synthetase and glutamate decarboxylase were determined. Since the main purpose of these determinations was the comparison of the enzyme activities in the granular and molecular layers, they are expressed in arbitrary units/g fresh tissue. The specific activities are expressed in units/mg protein. Although the protein content of a gram of granular and molecular layer tissue was quite similar (Table I), the activity of all enzymes, with the exception of  $\alpha$ -glycerophosphate dehydrogenase, was somewhat higher in the molecular than in the granular layer, the

TABLE I

PROTEIN CONTENT AND ENZYME ACTIVITIES IN THE GRANULAR AND MOLECULAR LAYERS OF THE RABBIT CEREBELLUM

All enzymes except glutamic decarboxylase in O.D. units/g of wet tissue : S.D. Glutamic decarboxylase in disint./min/g of wet tissue. The number of experiments is indicated in parentheses. Protein: mg/g; s.a., specific activity.

Component assayed	Granular layer			Molecular layer		
	Units	S.D./g	s.a.	Units	S.D./g	s.a.
Protein	41.3	8.3 (9)	—	46.8	12.8 (9)	—
Acetylcholinesterase	812	392 (5)	19.6	1092	502 (5)	23.3
Succinate-INT-reductase	554	256 (6)	13.4	700	158 (5)	15.0
$\alpha$ -Glycerophosphate dehydrogenase	354	69 (3)	8.6	324	38 (5)	6.9
Arylsulfatase	6.4	2.8 (4)	0.15	8.7	1.8 (4)	0.19
Glutamine synthetase	352	200 (4)	8.5	488	238 (4)	10.4
Glutamic decarboxylase	5044	(2)	122	5725	(2)	122

difference ranging from a low of 12.6% for succinate-INT-reductase to a high of 38.6% for glutamine synthetase. Moreover, although the higher molecular layer activities became less pronounced when expressed as specific activities, they matched the activity/g differences rather closely, in that they were again greatest for glutamine synthetase. Conversely, the slight trend toward higher values of the granular layer  $\alpha$ -glycerophosphate dehydrogenase became more pronounced and, as shown in Table I, its specific activity was 42% higher in the granular than in the molecular layer.

#### *The intracellular distribution of granular and molecular layer enzymes*

The centrifugation schedule employed in the present study was derived from one previously used quite effectively with various regions of the rat brain, including the cerebellum<sup>30</sup>. Centrifugation procedures subsequently published by others<sup>6,23</sup> differ from ours only slightly and, in our estimation, not crucially. Table II presents the results of the fractionation experiments. Of particular interest are the parallel distributions of protein in the primary fractions derived from the two layers as well as the rather sizable percentage of the total protein recovered in the nuclear fraction. In the gradient fractions, 24.8% of the total recovered protein distributed between fractions 0.8 M and 1.0 M of the molecular layer, as compared to only 18.8% for the corresponding fractions of the granular layer. This finding confirms the morphological evidence of a predominance of small parallel fibers and endings in the light gradient fractions of the molecular layer. Conversely, gradient fraction 1.4 M from the granular layer had about 18% more protein than the corresponding molecular layer fraction, a finding wholly consistent with the demonstrated presence of large-size, dense synaptic glomeruli in this fraction. The results of the subcellular fractionation ex-



(Table II continued)

Fraction	Succinate-INT-reductase (p2, g6)			$\alpha$ -Glycerophosphate dehydrogenase (p2, g3)			Arylsulfatase (p3, g5)			
	G (%)	M (%)	RSA	G (%)	M (%)	RSA	G (%)	M (%)	RSA	
Primary (p)										
Nuclear	20.3	25.1	0.54	29.6	33.1	0.78	16.7	10.1	0.44	0.29
Mitochondria	72.6	69.1	1.91	55.7	55.9	1.47	64.3	68.5	1.69	1.91
lysosomes	3.9	2.6	0.37	5.8	5.3	0.55	7.4	7.5	0.70	0.67
Microsomal	3.2	3.2	0.24	8.9	5.7	0.65	11.6	13.9	0.85	0.78
Soluble										
Gradient (g)										
0.8 M	0.4	2.7	0.06	6.2	2.4	0.98	2.1	2.0	0.33	0.22
1.0 M	2.1	4.7	0.17	12.1	10.3	0.97	5.3	5.3	0.42	0.33
1.2 M	24.1	32.6	0.72	26.8	34.7	0.81	23.1	22.1	0.69	0.70
1.4 M	66.9	52.1	1.79	45.2	45.1	1.21	53.8	52.4	1.44	1.65
Pellet	6.5	7.9	0.61	9.7	7.5	0.91	15.7	18.2	1.46	1.50

\* p, primary fraction, number of experiments; g, gradient fraction, number of experiments; G, granular layer; M, molecular layer; RSA (relative specific activity): % of component % of protein.

periments (Table II) confirm the validity of using succinate-INT-reductase and  $\alpha$ -glycerophosphate dehydrogenase as mitochondrial markers in both cerebellar layers, as no less than 55% and, for the former enzyme, up to about 73% of the total recovered activity, sedimented in the 'heavy particulate' fraction. Although the RSA of the lysosomal marker, arylsulfatase, was also highest in this fraction, the RSA of acetylcholinesterase was highest in the microsomal fraction and second highest in the fraction which contains the bulk of the synaptic elements ('heavy particulate'). Glutamine synthetase exhibited a diffuse intracellular distribution, its RSA value exceeding unity in all except the nuclear fraction. Moreover, its respective association with the cytoplasmic components of the granular and molecular layers was significantly different inasmuch as its RSA value was highest in the 'heavy particulate' fraction of the molecular and lowest in the corresponding fraction of the granular layer. Glutamic decarboxylase had the most complex pattern of intracellular distribution, for while similar percentages sedimented into the 'heavy particulate' and soluble fractions of the granular layer, the latter fraction contained more than 2.5 times the activity of the 'heavy particulate' fraction in the molecular layer.

Discontinuous sucrose gradient centrifugation (Table II) of the 'heavy particulate' fractions resulted in the concentration of the mitochondria in fraction 1.4 M rather than in the pellet. Lysosomes spread equally between fractions 1.4 M and the pellet, while glutamine synthetase peaked in the pellet fraction. The highest percentage of acetylcholinesterase was in fraction 1.2 M, yet fraction 1.0 M had the highest RSA values. Glutamic decarboxylase showed no peaks in fractions 1.0 M and 1.2 M of either layer; rather, in the granular layer it peaked in the pellet fraction while, in the molecular layer, fraction 1.4 M had the highest activity. An additional difference was its higher concentration in the lighter vs. its lower concentration in the heavier, fractions of the granular layer (RSA values of 3.11 and 0.80 vs. molecular layer values of 2.10 and 0.58 in fractions 0.8 M and 1.0 M, and of 0.50 and 0.80 vs. molecular layer values of 0.89 and 1.09 in fractions 1.2 M and 1.4 M). These findings agree with the morphological controls of molecular layer fractions 0.8 M and 1.0 M which revealed many fragments of parallel fibers with their (excitatory) synaptic contacts and of fractions 1.2 M and 1.4 M in which such profiles were rare<sup>20</sup>. Glutamine synthetase appeared to have a light component (fraction 0.8 M), especially in the granular layer (RSA: 1.38), yet the bulk of this activity was in the 1.4 M and the pellet fractions.

#### *Centrifugation in linear gradients of sucrose*

These experiments uncovered density differences between the mitochondria and the nerve endings of the granular and molecular layers. As shown in Fig. 2, the mitochondria of the granular layer fractions 1.2 M and 1.4 M (containing, respectively, 24.1% and 66.9% of the recovered succinate-INT-reductase) (Table II) equilibrated at higher densities than the corresponding mitochondria of the molecular layer. Similarly, the synaptic elements contained in fractions 1.2 M and 1.4 M of the granular layer and which accounted for a total of 63.7% of the recovered acetylcholin-



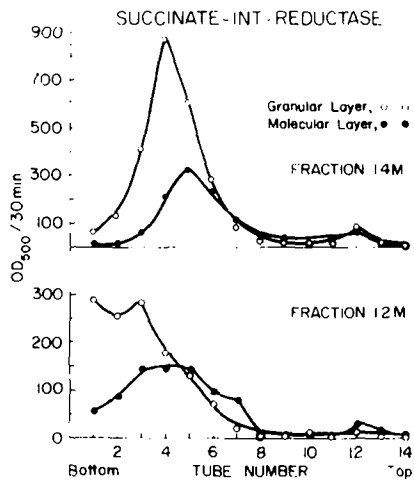


Fig. 2. Centrifugation of cerebellar nerve ending fractions in linear density gradients of sucrose. Fractions 1.2 M and 1.4 M were prepared by the procedure of De Robertis *et al.*<sup>7</sup> and, following pelleting and suspension in 0.25 M sucrose (1 ml), the bands from each layer were placed on 12 ml of a linear gradient of sucrose and the tubes were centrifuged as described in Methods. The enzyme activity was determined on portions of the effluent.

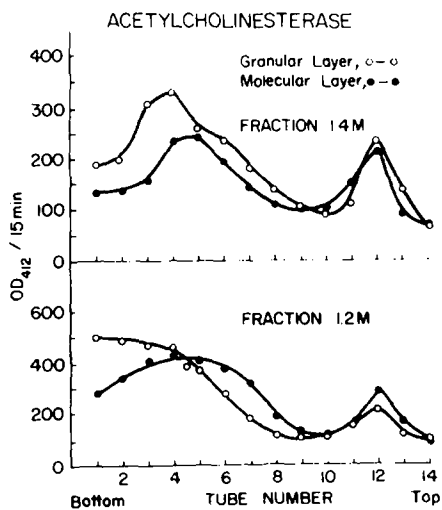


Fig. 3. Centrifugation of cerebellar nerve ending fractions in linear density gradients of sucrose. The procedure was as described in the legend to Fig. 3, except that acetylcholinesterase activity was determined.

esterase (Table II) activity also equilibrated at higher densities than the corresponding synaptic elements of the molecular layer (Fig. 3).

#### *Heterogeneity of the mitochondria in the granular and molecular layers*

Finally, we examined the possibility that granular and molecular layer mito-

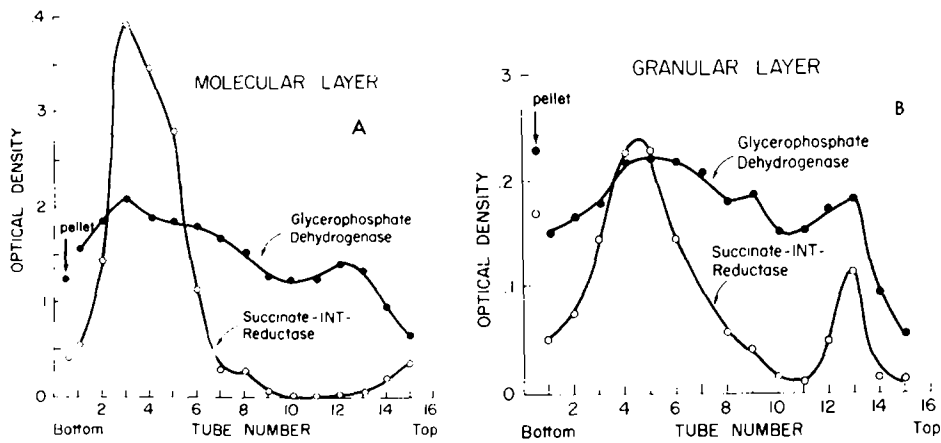


Fig. 4. A. The distribution of mitochondrial succinate-INT-reductase and  $\alpha$ -glycerophosphate dehydrogenase. Gradient fraction 1.4 M was isolated from the molecular layer by the procedure of De Robertis *et al.*<sup>7</sup>, and centrifugation in a linear density gradient of sucrose (42–50% w/v) was carried out as described in Methods. The activity of the mitochondrial enzymes was determined in portions of the effluent. The total activity of each enzyme in the gradient pellet is indicated by the arrow. Succinate-INT-reductase: ○—○,  $\alpha$ -glycerophosphate dehydrogenase: ●—●. B. As in A, except that the gradient fraction 1.4 M was prepared from the granular layer.

chondria may be heterogeneous in enzyme content<sup>11</sup> and hence in size<sup>1,24</sup> and density<sup>12</sup>. Evidence favoring this contention was obtained by centrifugation of fraction 1.4 M of the molecular (Fig. 4A) and granular (Fig. 4B) layers under identical conditions in a 42–50% (w/v) sucrose gradient overnight. A sharp peak of succinate-INT-reductase was obtained in the heavy portion of the gradient while  $\alpha$ -glycerophosphate dehydrogenase was distributed virtually throughout the entire gradient. Substantial amounts of both activities were also recovered in the pellets.

#### DISCUSSION

Although Purkinje cells have been separated previously from the molecular layer by free-hand dissection<sup>14</sup> and the levels of their glutamic decarboxylase compared to those of the granular and molecular layers, insufficient material was produced for subsequent fractionation. Still, as this procedure appeared promising, we investigated the possibility of scaling it up. However, after examining a number of cells by phase contrast (Fig. 5) and electron microscopy (Fig. 6), we soon desisted from this effort. Under low magnification (Fig. 5), many cellular shapes were noted with dendritic processes and axonal stumps often present together with shapeless debris and, rather frequently, contaminating granule cells. The damage to the Purkinje cells was severe and was localized mostly at the level of the cell membrane (Fig. 6A–C and F) so that, frequently, the cells were devoid of intracellular organization. As shown in Fig. 6D, cells with synaptic contacts were also found, but they invariably had glial

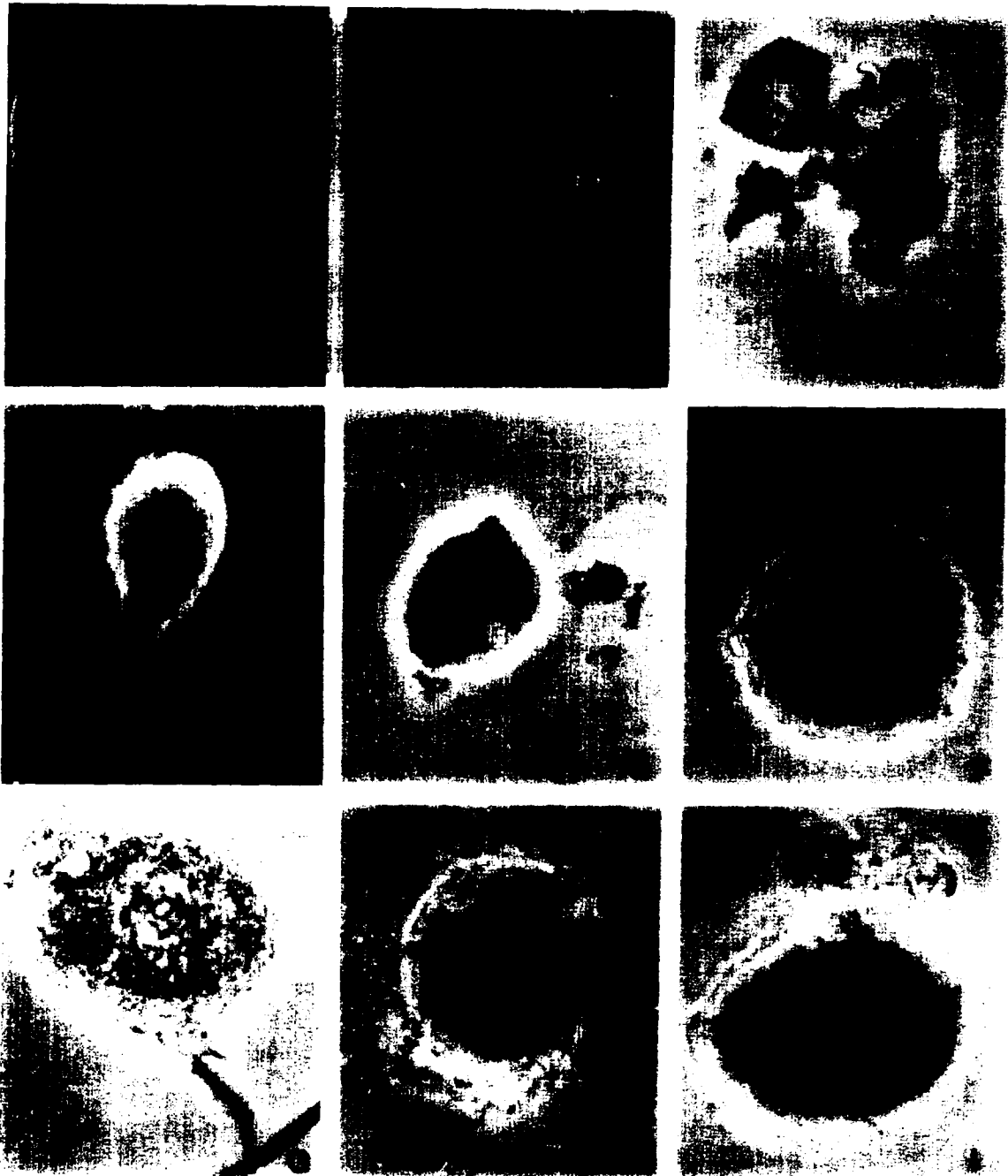


Fig. 5. Phase contrast photographs of hand-free dissected Purkinje cells. Differences in cell shape are remarkable and the presence of contamination is illustrated.  $\times 40$ .

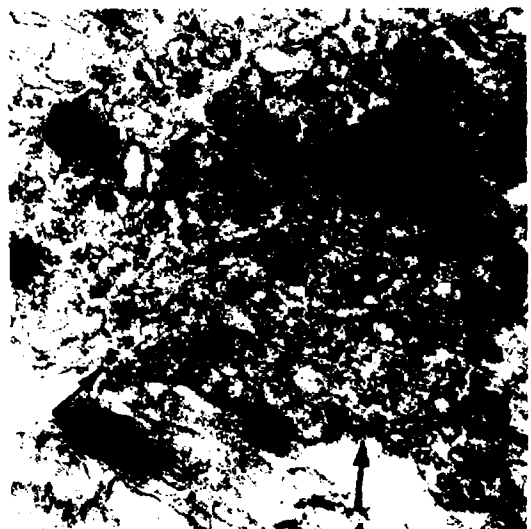
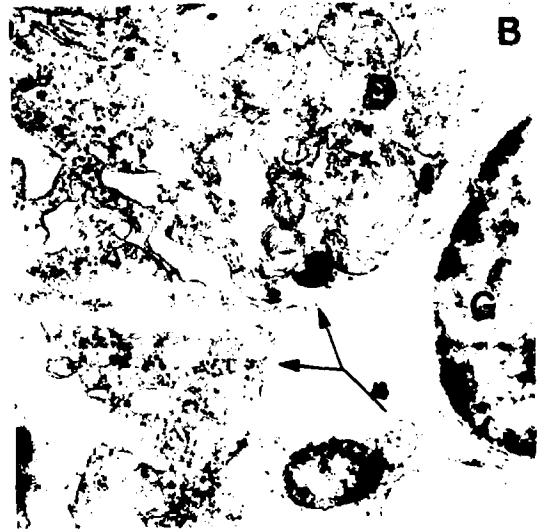
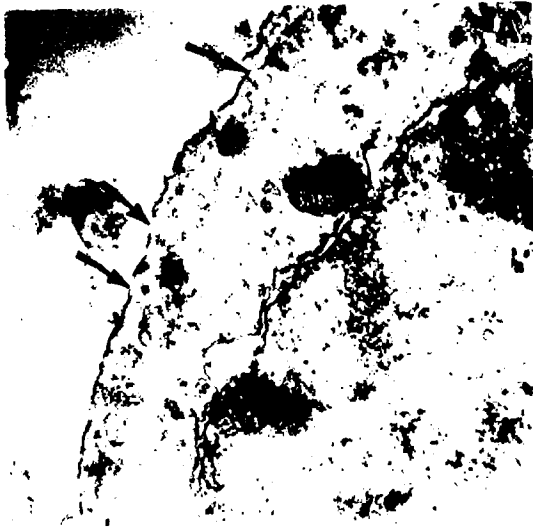


Fig. 6. Electron micrographs of hand-free dissected Purkinje cells: A, Arrows point at holes in damaged membrane of Purkinje cell. B, Arrows point at the damaged surface of Purkinje cell cytoplasm. G is a contaminating granule cell. C, Damaged surface of Purkinje cell at the arrow. D, In the circle, note preserved synaptic contact. Also, note the presence of glial processes surrounding the synaptic formation. E, Heavily damaged Purkinje cell. F, Heavily damaged Purkinje cell.

elements around them. In conclusion, we found free-hand dissection of Purkinje cells to yield greatly damaged and heavily contaminated cells, a finding which may be readily understood by realizing that their manual isolation involves their physical removal by a process of extirpation from within the midst of an extremely complex surrounding.

To our knowledge, the present study is the first in which granular and molecular layers were hand-dissected in sufficient amounts for fractionation of their subcellular and synaptic components. Fig. 1A shows the molecular layer as dissected together with the Purkinje cells, while Fig. 1B demonstrates that the dissected granular layer consists predominantly of granule cells, plus some contaminating molecular layer. Since the cellular composition of the molecular layer is thus more heterogeneous than that of the granular layer, it is admittedly rather difficult to identify unequivocally the cellular origin of all of the subcellular components isolated from it by centrifugal means. Yet, the recently accomplished quantitation of the vast differences in the ratio of neuropil to cellular volume between the two layers (ratio of 1:17 for the molecular layer and of approximately 1:1 for the granular layer; Z. Lodin, unpublished observations) should greatly facilitate this task.

In a previous publication<sup>20</sup>, we described the morphological appearance, visualized in the electron microscope, of most of the gradient fractions isolated from the granular and molecular layers by the centrifugal procedure employed in the present study. We were thus able to identify synaptic endings of parallel fibers in fractions 0.8 M and 1.0 M of the molecular layer, thus confirming similar observations of Lemkey-Johnston and Larramendi<sup>18</sup>. The heavier molecular layer fractions (1.2 M and 1.4 M) were shown to contain the large endings of the climbing fibers, while the corresponding granular layer fractions contained many large-sized glomerular polysynaptic endings. We concluded by stating that the centrifugal procedure led to the concentration of the excitatory synapses of the molecular layer in fractions 0.8 M and 1.0 M and of those of the granular layer in fractions 1.2 M and 1.4 M. The inhibitory synapses of both layers distributed throughout the remaining gradient fractions. The RSA values of glutamic decarboxylase in all of these fractions (Table II) fully support our morphological findings<sup>20</sup> since they were lower in the molecular than in the granular layer fractions 0.8 M and 1.0 M and were higher in the molecular than in the granular layer fractions 1.2 M and 1.4 M. Although these findings lend additional credence to our basic and previously stated contention that centrifugal fractionation of pre-separated granular and molecular layers can successfully accomplish a partial physical separation of excitatory and inhibitory synaptic endings<sup>20</sup>, they also show that this method alone is insufficient to achieve their respective purification.

Previous studies<sup>25</sup> in which enzyme activity levels were compared between the granular and molecular layers of the cerebellum showed that in febrile rabbits, aldolase and ATP-ase were somewhat higher in the molecular than in the granular layer whereas the reverse held true for glutamic dehydrogenase. A histochemical assessment of acetylcholinesterase activity in the molecular and granular layers dissected from 9 species revealed no differences in the rabbit<sup>3</sup>. Our results (Table I) for this enzyme indicate a consistent tendency toward higher values in the molecular

layer. More recently, Hirsch<sup>13</sup> compared the activity of several hydrolases in the molecular and granular layers of the cerebella of the monkey, the rat and the guinea pig and noted appreciably higher values for arylsulfatase,  $\beta$ -galactosidase and  $\beta$ -glucuronidase in the granular layer of all 3 species.

In a comparative study of the hexosaminidases of the molecular and granular layers of the cerebellum of the rat, the rabbit and the monkey, using *p*-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosyl- and galactosaminides as substrates, Shuter *et al.*<sup>31</sup> found more activity with either substrate in the granular layer of all 3 species, the excess over the molecular layer activities being the most striking in the rabbit. Our results with arylsulfatase (Table I) indicate opposite activity differences and are thus in contrast to the results of Shuter *et al.*<sup>31</sup>.

Lodin *et al.*<sup>19</sup> surveyed the staining pattern of the cerebellar cortex of the adult rabbit for a number of dehydrogenases and found that the activity of  $\alpha$ -glycerophosphate dehydrogenase was low in Purkinje cells, neurons and glial cells, but somewhat higher in the neuropil. Succinate dehydrogenase stained positively in the small neurons of the molecular layer and in the Golgi cells. The glomeruli cerebellosi of the granular layer also showed intensive staining. Our data (Table I) show barely significant differences between the  $\alpha$ -glycerophosphate dehydrogenase values in the two layers, but do reveal higher molecular layer succinate-INT-reductase activity, a finding compatible with the known presence of numerous mitochondria in the neuropil of the molecular layer<sup>11,15,17</sup>.

Only two of the previous fractionation studies reported any data on the subcellular localization of cerebellar enzymes<sup>5,23</sup>. Our enzyme fractionation data (Table II) show that the mitochondria of the granular and molecular layers contain equal proportions of succinate-INT-reductase and  $\alpha$ -glycerophosphate dehydrogenase. In view of our recent finding that, in the cerebral cortex of the rat, the latter enzyme is predominantly a marker of glial mitochondria<sup>29</sup>, the present results may reflect an equal extent of contamination of the granular and molecular layers by glial cells<sup>18</sup>.

'Mitochondrial heterogeneity' in brain was noted by Neidle *et al.*<sup>22</sup> and, more recently, by Pysh and Khan<sup>24</sup> who found that, in the rat, the mitochondrial volume fraction varied from a low of 3.5% in the granule cells to a high of about 28% in the cerebellar mossy fiber endings. Our results reveal that the buoyant density of granular layer mitochondria (Fig. 2) and nerve endings (Fig. 3) was always slightly higher than that of the corresponding molecular layer particles. The recently reported differences in buoyant density between neuronal and glial mitochondria<sup>12</sup> are also of interest in this regard since they may relate to the results depicted in Figs. 4 and 5 in which fraction 1.4 M succinate-INT-reductase and  $\alpha$ -glycerophosphate dehydrogenase show distinctly different buoyant density equilibration profiles. Succinate-INT-reductase peaked near the bottom of the gradient (molecular layer, tube 3) and at slightly lower buoyant densities in the granular layer (tubes 4 and 5). In the granular, but not in the molecular layer, a second peak of succinate-INT-reductase activity was noted; these mitochondria could be components of the synaptic elements of fraction 1.2 M which contaminated fraction 1.4 M or of lighter, glial elements. On the other hand, the diffuse profile of  $\alpha$ -glycerophosphate dehydrogenase in both

layers may reflect the dispersion of glial and neuronal mitochondrial fragments of continually increasing densities.

#### SUMMARY

(1) Homogenates of hand-dissected granular and molecular layers of the cerebellar cortex of the rabbit were prepared and the following enzymes assayed: acetylcholinesterase, succinate-INT-reductase, particulate  $\alpha$ -glycerophosphate dehydrogenase, arylsulfatase, glutamine synthetase and glutamic decarboxylase. Except for  $\alpha$ -glycerophosphate dehydrogenase which was higher in the granular layer, all of the other activities showed higher values in the molecular layer.

(2) Differential centrifugation of the homogenates into the nuclear, heavy particulate, microsomal and soluble fractions yielded peaks of relative specific activity of acetylcholinesterase in the microsomal, of glutamic decarboxylase in the soluble and the heavy particulate, of succinate-INT-reductase,  $\alpha$ -glycerophosphate dehydrogenase and arylsulfatase in the heavy particulate and of glutamine synthetase in no single fraction.

(3) Further separation of the heavy particulate fraction was accomplished by discontinuous gradient density centrifugation which yielded 5 fractions including a pellet. The distribution of glutamic decarboxylase and morphological evidence adduced previously<sup>20</sup> make it possible to state that the system of excitatory synapses sedimented predominantly into fractions 0.8 M and 1.0 M of the molecular layer and fractions 1.2 M and 1.4 M of the granular layer. The distribution of  $\alpha$ -glycerophosphate dehydrogenase, an enzyme predominantly associated with glial mitochondria<sup>20</sup>, further suggested an equal extent of contamination of the molecular and granular layers by glial cells.

(4) This study also provides a finding of general interest, inasmuch as it reveals that, by prior physical separation of the granular and molecular layers followed by their differential and density gradient centrifugation, it is possible to achieve a partial separation, although by no means a purification, of their excitatory and inhibitory synaptic complexes.

#### ACKNOWLEDGEMENTS

We should like to express our thanks to Drs. Faltin, Hartman and Sachá (Prague) for their valuable assistance in preparing the microscopic specimens and their photographs.

Supported by Grants NS-06294 from the U.S. Public Health Service.

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