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Preparation of Enriched Fractions from Cerebral Cortex containing Isolated, Metabolically Active Neuronal and Glial Cells

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1. A procedure has been developed for the separation of intact metabolically active neuronal and glial cells in bulk from rat cerebral cortex. Separation depended on dispersion of the tissue in a Ficoll medium followed by centrifugation on a discontinuous Ficoll gradient. Up to $1{\cdot}5 \times 10^7$ neuronal cells could be collected from 12 brains within 3hr. The morphological appearance of these cells seemed good, and the fraction was 8·5-fold purified in terms of dry weight. Average dry weight per neuron was 2300$\mu\mu$g. Maximum glial contamination of the neuronal fraction was 11% as determined by carbonic anhydrase measurements. The glial fraction was free from neurons but contained various subcellular contaminants. 2. Concentrations of nucleic acids, phospholipid, protein and phosphoprotein were determined in the separated fractions. The neuronal fraction was richer than the glial in all except phospholipid. Succinate dehydrogenase was equally distributed between neurons and glia but the neuronal fraction was 1·8-fold enriched in cytochrome oxidase. 3. Measurement of respiration by the cells showed an endogenous uptake of 117mμmoles of oxygen/mg./hr. in neurons, and 173mμmoles of oxygen/mg./hr. in glia. Addition of substrate at 10mM stimulated uptake to similar values in both fractions. With glucose it was 390, with pyruvate 355, and with glutamate 215mμmoles of oxygen/mg./hr. This represented a larger stimulation of neuronal than of glial respiration compared with the basal level. 4. Respiration in cell suspensions was 70–80% of that of slices, whereas fractionated tissue homogenates had respiratory rates of only one-third those of the cell suspensions. Lactate dehydrogenase content of cell suspensions was maintained during gradient centrifugation and washing. 5. The possible uses of isolated cell preparations are discussed.

Cerebral cortex contains two very different cell types, neurons and glia. Morphologically, as well as functionally, the two cell classes are distinct, despite variations between the several subgroups of both neurons and glia. It is therefore of relevance to attempt to separate the neurons from the glia and to study the metabolism of each cell type in isolation from the other.

Previous approaches to this problem have been made. Thus various brain regions differ in their relative content of neurons and glia, and such areas can be studied either on a macro-scale (e.g. Korey, Orchen & Brotz, 1958; Rose, 1962) or on a micro-scale (Chu, 1954; Robins, Smith & Eydt, 1956; Lowry, 1957; Pope & Hess, 1957). Hydén and co-workers have devised very elegant methods for the micro-dissection of single neurons and bundles of glia from the Deiter's nucleus and other regions of the brain, and have used micro-techniques for determining dry weight, RNA content and other variables (Hydén, 1960; Hamberger, 1963). Roots & Johnston (1964) have more recently described a method whereby neurons can be collected from suspensions of the ox thalamus at the rate of about 300/hr. by trapping them with a nylon loop under a binocular microscope.

These techniques, however, cannot provide a sufficient quantity of isolated cells for comprehensive biochemical investigation, and a new method has therefore been devised which enables enriched fractions consisting largely either of neurons or of glia to be prepared rapidly, in bulk, and reasonably intact, from the cerebral cortex. [In this work, the words 'neuron' and 'glia' are used in the same

sense as by Hydén and his co-workers (for review see Hamberger, 1963). Such neurons are largely shorn of their dendritic processes and are thus more precisely neuronal cell bodies, whereas the glia are more accurately bundles of adherent glial cells.] Up to $1{\cdot}2 \times 10^6$ neurons can be separated from each rat brain within 2hr., and cells so prepared remain metabolically active for at least 3hr. subsequently. This method, and some characteristics of the cells isolated by its use, are described here. A preliminary report of some of this work has already appeared (Rose, 1965*a*).

METHODS

Animals. Male rats of the hooded or Sprague–Dawley strains and of about 150g. body wt. were used throughout.

Reagents. Ficoll, a sucrose polymer with molecular weight 400000 (batch no. To 5990), was obtained from Pharmacia Ltd., London, W. 13, and was used directly without prior dialysis. Ficoll solutions were stored in the refrigerator and were not used after more than 10 days' storage.

Ludox HS 40%, a colloidal silica, was from Brown and Forth Ltd., London, N.W. 1.

Deuterium oxide was from Imperial Chemical Industries Ltd., London, S.W. 1.

Cytochrome *c*, 90–100%, molecular weight 12270, was from Koch–Light Laboratories Ltd., Colnbrook, Bucks.

All other reagents were of the best available analytical grade. All solutions were made in glass-distilled water.

Materials. Nylon bolting cloth, 110-mesh (i.e. 110 threads/in., aperture size 130μ) was obtained from J. Staniar Ltd., Manchester 3, and was washed with detergent and rinsed with distilled water before use.

Stainless-steel gauze, 350 mesh (i.e. 350 threads/in., aperture size 40μ) was from F. W. Potter and Soar Ltd., London, E.C. 2.

Preparation of neuronal and glial fractions. The method finally adopted was as follows. Rats were killed by a blow to the back of the neck and exsanguinated. The brains were removed, bisected, and each hemisphere was placed, cortex downwards, on a block of paraffin wax, all subsequent operations being carried out at 0–4°. All regions of the brain other than the cortex were removed with forceps and scalpel, the cortical 'shell' was crudely cleaned of white matter and placed on to 110-mesh nylon bolting cloth, about 8cm. square. When sufficient material had been collected (up to 20 cortices, each weighing 600mg., could be handled at a time), the bolting cloth was bunched together into a bag and the tissue gently 'teased' through it into an ice-cold medium containing 10% (w/v) Ficoll, 100mM-KCl and 10mM-potassium phosphate buffer, pH7·4 (3·5ml./cortex). 'Teasing' was performed by repeatedly stroking the bag containing the tissue with a thick glass rod. The entire disruption process took 5–10min., depending on the amount of tissue being handled.

When all the tissue had passed into suspension, leaving behind in the nylon bag only strands of white matter and capillaries, it was filtered once, under gentle suction, through a stainless-steel mesh of about 40μ pore size. The resulting filtrate is subsequently referred to as suspension (S).

For analytical experiments, 2·5ml. samples of the suspension were put on to a gradient, made by layering 1·5ml. of a solution containing 30% (w/v) Ficoll, 100mM-KCl and 10mM-potassium phosphate buffer, pH7·4, over 1·0ml. of 1·45M-sucrose, in the 5ml. tubes of the SW39 head of the Spinco model L2 ultracentrifuge. [This gradient differs slightly in composition from that previously described (Rose, 1965*a*).] The tubes were centrifuged at 39000rev./min. and 0° ($125000g_{av.}$) for 45min. Four fractions could then be removed from each tube by means of Pasteur pipettes. The first (A) was a floating layer of lipid and relatively undisrupted tissue. The second (B), at the 10–30% Ficoll interface, represented the enriched glial fraction. The third (C), at the 30% Ficoll–1·45M-sucrose interface, was the enriched neuronal fraction. The fourth fraction (D) was a pellet at the bottom of the centrifuge tube consisting largely of red cells, capillaries and other debris, and the clumped nuclei of disrupted neurons.

For metabolic experiments, the above procedure was scaled up. Samples (10 or 20ml.) of the suspension were layered on to suitable gradients in the 30ml. or 60ml. tubes of the SW25 head of the Spinco model L2 ultracentrifuge. Centrifugation was for 120min. at 25000rev./min. ($53000g_{av.}$). The A and D layers were discarded, and the B (glial) and C (neuronal) fractions washed and concentrated by resuspending in a medium of final concentration 10% Ficoll, 100mM-KCl and 10mM-potassium phosphate buffer, and centrifuging for a further 20min. at 10000rev./min. ($6000g_{av.}$) in the angle-50 head of the Spinco model L2 ultracentrifuge. The resultant pellets were finally resuspended once again in a small volume of the 10% Ficoll medium. The entire extraction procedure for metabolic experiments took about 3hr.

Microscopy. Samples were examined either by phase contrast or after light staining with methylene blue; photography was made by phase contrast with or without methylene blue staining. Electron micrographs were kindly made, after osmium fixation, by Dr J. A. Armstrong of the National Institute for Medical Research.

Cell counts. To obtain a criterion for the number of intact cells in each preparation, and to compare the efficiency of various disruptive methods examined, cell suspensions were examined by phase contrast or after light staining with methylene blue. The number of reasonably intact, isolated, freely suspended neurons was counted and compared with the numbers of free neuronal nuclei (recognized by virtue of their size and single nucleolus). Counting was continued until at least 50 nuclei or neurons or both had been observed. The measure of intactness of the isolated cell preparations was defined on the basis of the neuronal/nuclear ratio. In the worst preparations, this ratio was of the order 0·04; in the best, before purification by centrifuging, it was 0·30–0·60. Ratios defined in this way were reproducible. Thus in seven preparations of cell suspensions made by passing cortex through nylon bolting in a 10% Ficoll medium, the ratio was $0{\cdot}50 \pm 0{\cdot}05$ (S.E.M.).

Total cell population. The total number of isolated cells present in the suspensions was estimated by staining with toluidine blue and counting total neuronal nuclei on a haemocytometer. Numbers of intact neurons present were then estimated from the neuronal/nuclear ratio.

Analytical methods. Dry weights were determined by precipitating the cell suspensions with at least 10vol. of ice-cold 10% (w/v) trichloroacetic acid, chilling for 20min., centrifuging and washing the precipitate once with 10%

trichloroacetic acid. The precipitates were transferred to tared weighing bottles and dried overnight at 105° before weighing.

Phospholipid, 'nucleic acid', 'residual organic phosphate', phosphoprotein and 'acid-precipitable phosphate' were determined as inorganic phosphate by Rose's (1965*b*) modification of the method of Schmidt & Thannhauser (1945). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Enzyme determinations. For enzyme measurements the cell samples were diluted with 0·25M-sucrose and ground in a Teflon–glass homogenizer. All determinations were performed under regulated temperature conditions in a Unicam SP.800 recording spectrophotometer. Activities were expressed in arbitrary units/mg. of protein in the samples: each individual determination quoted in the Tables represents the mean of two to four separate assays at varying tissue concentrations. Methods used were as follows: carbonic anhydrase, Lindskog (1960); cytochrome oxidase, Smith (1955); lactate dehydrogenase, Bergmeyer, Bernt & Hess (1963); succinate dehydrogenase, Slater & Bonner (1952).

Metabolic methods. For metabolic experiments, Warburg flasks were set up containing 0·2ml. of cell suspension, 75μmoles of NaCl, 1·2μmoles of $MgSO_4$, 3·0μmoles of sodium phosphate buffer, pH7·4, and 6·0μmoles of substrate, in final volume 0·6ml. Ions present in the cell suspension raised the final concentration of Na^+ to 145–150μmoles/ml. and of K^+ to 26–31μmoles/ml. as determined by flame photometry, so that the final ionic composition of the medium resembled that of Elliott & Henderson (1948). The centre well contained 0·1ml. of 30% NaOH and a filter-paper wick. Flasks were gassed with O_2 and incubation was continued for at least 2hr.

RESULTS

Separation of neuronal and glial fractions

A variety of methods were examined for making cell suspensions and subsequently separating them into enriched glial and neuronal fractions. Preparation of suspensions was hampered by the fragility of the neurons; this fragility led to the adoption of the neuronal/nuclear ratio (described in the Methods section) to compare different disruptive procedures. With this ratio as a criterion, the following procedures were examined.

Media used to make the initial cell suspension included, amongst others, glucose–phosphate saline, 0·32M-sucrose, 0·88M-sucrose (with or without addition of salts and buffers), 5% Ficoll and 10% Ficoll (containing 100mM-potassium chloride and 10mM-potassium phosphate buffer, pH7·4). Addition of crystalline bovine serum albumin (1–5%) to the suspending medium was also tried. Of the media, glucose–phosphate saline was the least satisfactory and 10% Ficoll and 0·88M-sucrose–1% serum albumin were the best. However, after 90min. storage in ice, the neuronal/nuclear ratio in 0·88M-sucrose diminished from 0·32 to 0·25, whereas in 10% Ficoll the ratio remained constant at 0·52. Ficoll was therefore adopted for subsequent work.

Disruptive techniques included the use of a top-drive macerator at low speeds for periods varying from 30sec. to 10min., vigorous magnetic stirring, mincing of the tissue with scissors or scalpel, and passing through a hypodermic syringe. All these treatments resulted in very low neuronal/nuclear ratios (0·02–0·10). Shaking the minced cortex with glass beads, recommended by Chu (1954), failed to disrupt the tissue at all. Passing through nylon bolting cloth as described in the Methods section proved swift and efficient, in particular as much unwanted lipid material was held back by the bolting. Under the conditions described in the Methods section, a reproducible neuronal/nuclear ratio $0·5 \pm 0·05$ (S.E.M.) was consistently achieved. This ratio was not greatly altered by subsequent filtration through metal gauze.

Enzymic disruption of the tissue was also attempted. After shaking for 5min. in 0·1% papain containing 5mM-cysteine (McIlwain, 1954) or collagenase (0·025%) at 37°, chopped cortex began to break up, but both whole cells and nuclei soon disintegrated. Trypsin (0·05%) was less drastic, but few whole cells were released from the tissue even after 3hr. incubation at 37°.

Once disrupted, the suspension was readily separable by gradient centrifugation, and this, apart from preliminary experiments with the phase

Table 1. *Preparation of enriched neuronal and glial fractions from rat brain*

For preparation and nomenclature of fractions, estimation of neuronal/nuclear ratios and identification of individual fractions, see the text.

Fraction	Neuronal/ nuclear ratio	$10^{-6}\times$ Total free neurons	Total dry wt. (mg.)	$10^{-4}\times$ Neurons/ mg. dry wt.	Purification factor
S (suspension)	0·32	2·1	59·2	3·5	1
A (debris)	0·13	0·1	33·0	0·3	0·1
B (glial)	0·00	0·0	19·0	—	6
C (neuronal)	2·05	0·8	2·7	29·8	8·5
D (nuclei)	0·06	0·02	1·6	1·2	0·3
Recovery (%)		57	97		

Table 2. *Recovery of protein in density-gradient fractions from rat brain cortex*

Data from nine experiments are presented: for preparation and nomenclature of fractions, see the text.

	Protein recovered (%)*									
Expt. no.....	1	2	3	4	5	6	7	8	9	
Fraction										Mean
A	61·0	70·4	57·1	57·2	75·1	79·2	68·8	73·8	71·4	68·3
B	17·0	13·8	28·7	29·6	12·3	7·1	17·6	17·7	16·8	18·0
C	9·2	10·6	5·3	5·2	4·4	5·9	7·7	3·0	4·0	6·2
D	9·7	5·2	8·9	8·0	8·0	7·8	5·8	5·5	7·9	7·5

* Average recovery 103%.

Table 3. *Numbers of neurons in density-gradient fractions from rat cortex*

For preparation and nomenclature of fractions and identification of neurons see the text. Counts were performed under phase contrast, at 100× magnification and in random fields.

	Suspension		Fraction C		
	Total objects		Total objects		
Field	$>2\mu$	Neurons	$>2\mu$	$>5\mu$	Neurons
1	243	8	135	47	33
2	316	10	157	59	43
3	463	3	89	22	15
4	371	5	78	15	10
Total	1393	26	459	143	101
Neurons (% of total)	1·8		22	71	

system of Albertsson & Baird (1962), was the only method studied. The gradient finally adopted had the advantage of removing both lipid materials (in layer A) and broken nuclei and red cells (in pellet D). A typical protocol is shown in Table 1; variations in the distribution of protein through the gradient in a set of nine successive experiments is shown in Table 2.

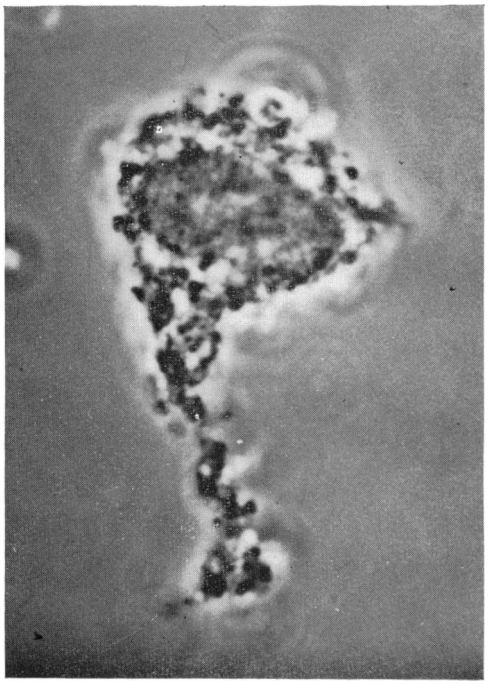
Although sucrose gradients were as effective as Ficoll in separating neurons from glia, the appearance of the cells after centrifugation was swollen, and many appeared to have burst. But it was not possible to replace sucrose for the bottom layer of the gradient by Ficoll, which could not be obtained in dense enough solution. Solutions of Ficoll in deuterium oxide instead of water, or the use of the colloidal silica Ludox HS, recommended by Mateyko & Kopac (1963), also proved unsatisfactory.

The behaviour of the cells on the gradient was not, however, exclusively a function of the density of the medium, as replacement of K^+ by Na^+ ions in the Ficoll media resulted in a different distribution of material on the gradient, with the disappearance of the A layer and increased material in the B and C layers.

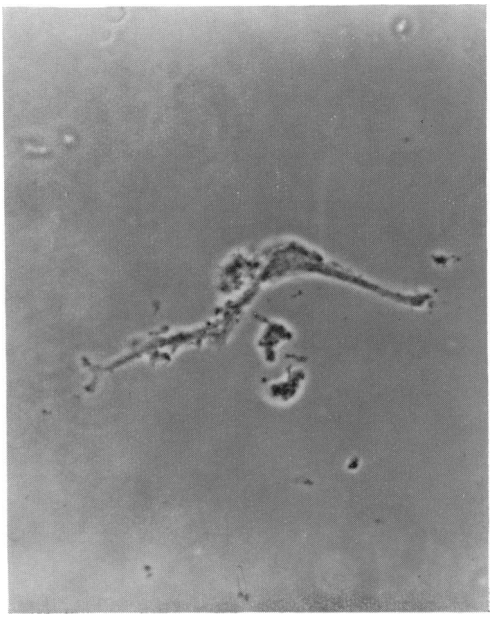
Microscopic observation of the isolated fractions

Neurons. Table 1 shows an overall 'purification factor' for the neurons in fraction C of 8·5, when calculated in terms of dry weight, whereas the neuronal/nuclear ratio improved by a factor 6·5. As a percentage of the total number of objects visible in the microscope field, the purification of fraction C was also marked. Thus Table 3 shows that whilst in the initial suspension neurons comprised some 1·8% of the total number of objects greater than 2μ in longest diameter, in fraction 3 22% of all objects greater than 2μ, or 71% of all objects greater than 5μ in longest diameter, were identified as neurons in varying stages of preservation.

The total yield of neurons/rat cortex was $1{\cdot}2 \times 10^6$. Estimates of the total number of neurons in cortex vary. Nurnberger (1958), on the basis of a mechanical disruption procedure, found approx. $2{\cdot}2 \times 10^7$ neurons/g. wet wt. Tower & Elliott (1952), on a histochemical basis, found $10{\cdot}5 \times 10^7$ neurons/g., but their figures were not corrected for shrinkage. Cragg's estimate (B. Cragg, personal communication), also histochemical, varied according to the cortical region from $2{\cdot}5 \times 10^7$ to $4{\cdot}8 \times 10^7$ neurons/g.



(A)



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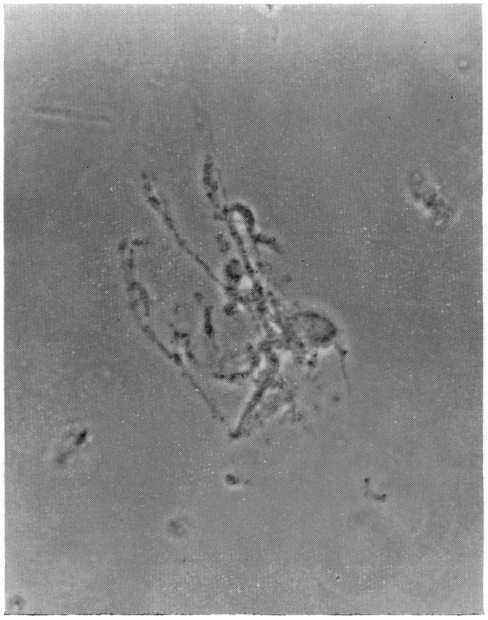
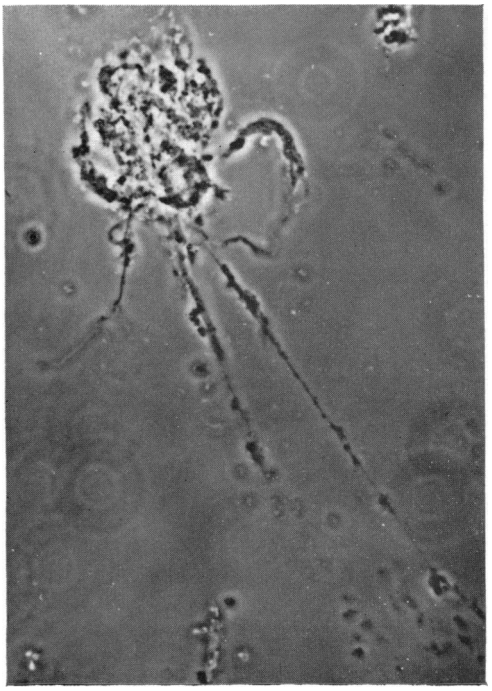
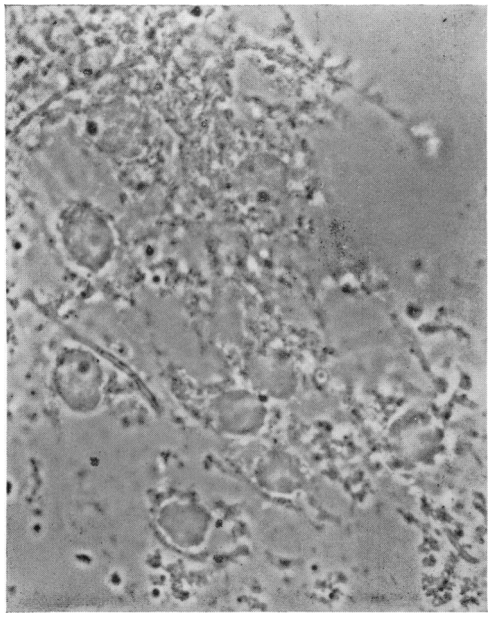


(C)

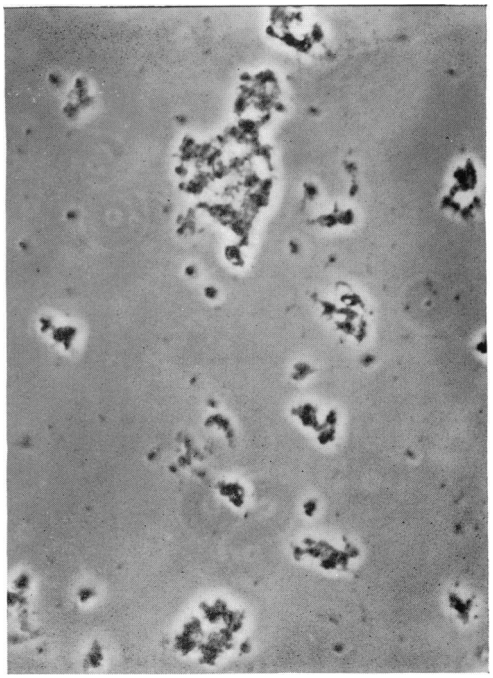


(D)

Plate 1. Phase-contrast photographs of neurons harvested from fraction C after dispersion through nylon bolting and Ficoll gradient centrifugation as described in the text. (A) (×1750) A single cell, presumably shorn of its processes during disruption of the tissue but retaining an axon stump. (B) (×715) A small neuron with two well-defined processes. (C) (×715) and (D) (×640) These are apparently intact, with many processes visible. Focus on the processes has left the cell bodies slightly blurred.



(A)



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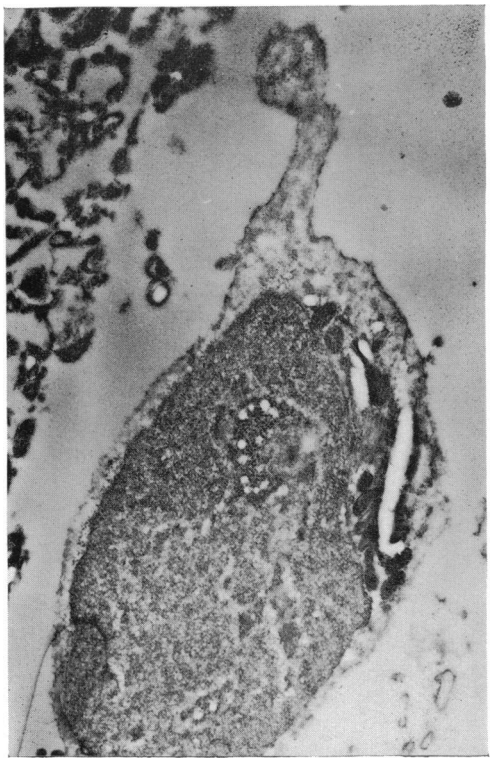

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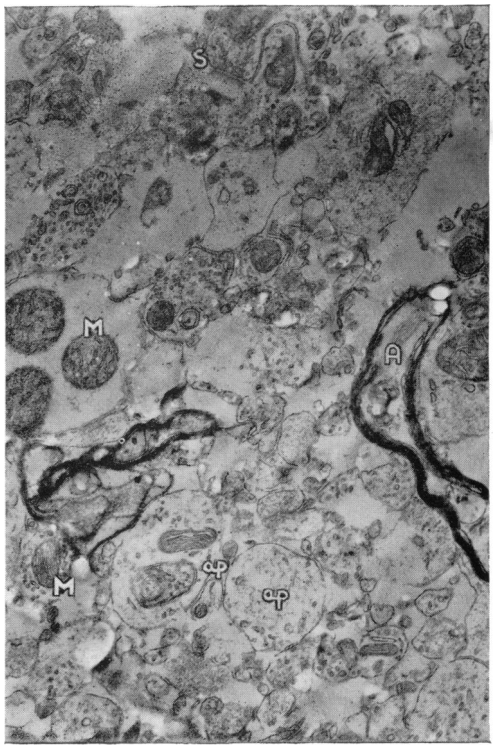

(D)

Plate 2. Phase-contrast photographs of: (A) (× 715) Clump or 'network' of neurons, many of them apparently intact, isolated in fraction C; such groups of cells, apparently held together by their processes, were frequently seen. Glial cells, trapped amongst them, are also visible. (B) (× 610) Glia from fraction B. (C) Electron micrographs of single neuron from fraction C (osmium/methacrylate; × 13960). (D) Electron micrograph of single neuron from fraction B (osmium/Epon; × 13960). Astrocytic processes (ap), mitochondria (M), a synapse (S) and a portion of axon (A) are visible.

Taking the mean of Cragg's figures, and based on a cortical weight of 600 mg., the recovery of neurons in the preparation described here was thus some 6%. If the assumption is made that 70% of the material in the neuronal layer was intact neurons (see Table 3) the average neuronal mass (dry wt.) would be 2300 $\mu\mu$g., a figure within the range quoted by Hydén (1960) for cortical neurons.

Visually, the condition of the neurons recovered in fraction C varied widely. Phase-contrast photography is not suitable for the demonstration of large fields containing many cells, because of problems of depth of focus, but a representative sample of cells at higher magnification is shown in Plate 1 (A–D). Some cells appeared to have lost virtually all their processes, retaining little more than an axon stump or a 'fringe' of cytoplasm. Others had a substantial number of processes and a considerable length of axon. Typically, the perikarya ranged in size from 8 to 25 μ diam. Another common feature, hard to photograph, but visible in Plate 2(A), was groups of neurons apparently held together by a network of processes and frequently containing a number of glial cells trapped between them. Most of the glial contamination of fraction C probably occurred in this way. These networks sometimes survived metabolic experiments without separating into their individual components.

Electron-microscopic observation of fraction C confirmed these observations. Cells ranged from badly damaged, consisting of little more than a nucleus with a surround of adherent cytoplasmic material, to those in which cytoplasmic organization was well retained and portions of external cell membrane could be observed (Plate 2C). Mitochondria and other internal constituents within such cells appeared well preserved.

Glia. Fraction B contained about 18% of the protein recovered from the gradient, although this figure was subject to considerable variation. It consisted of numerous glia, isolated or in 'bundles' similar in appearance to those described by Hamberger (1963) (Plate 2B). The glial nuclei could be observed clearly under phase contrast or after heavy staining with methylene blue. Contaminants present in fraction B included a variety of separated processes, axonal and particulate material from disrupted cells, as demonstrated more clearly by electron microscopy, when sections through axons, neuronal dendrites, nerve-ending particles and mitochondria could be identified as well as glial (astrocytic) processes (Plate 2D). No intact neurons, however, were ever observed in this layer.

Other fractions. Layer A, comprising some 68% of the total protein, was composed largely of clumps of undisrupted tissue, of myelin and of relatively few isolated neurons or glial bundles. Pellet D contained capillaries and axonal material, red blood cells and numerous neuronal nuclei from broken cells. Some apparently well-preserved neurons were also present.

Glial contamination of neuronal fraction

Giacobini (1964) has estimated carbonic anhydrase in neurons and glia of Deiter's nucleus, isolated by the micro-dissection method, and has calculated that the enzyme is 100–120-fold more active in the glia than the neurons. Carbonic anhydrase may thus represent a glial marker, and its activity was therefore measured in the separated fractions from the gradient (Tables 4 and 5) in an effort to obtain information about the possible extent of glial contamination. Compared with the original suspension, carbonic anhydrase was concentrated 2·5-fold in the glial fraction and fourfold in pellet D (which contained red cells). The neuronal layer contained only one-fifth as much carbonic anhydrase as the original suspension on a protein basis. Thus the mean ratio of enzyme in B to that in C was nearly 12. On the assumptions, following Giacobini, that (a) virtually all the carbonic anhydrase measured in the neuronal layer was caused by glial contamination, (b) all that in the glial layer represented glial activity and (c) the ratio of glial/neuronal carbonic anhydrase is 100/1, a figure for the maximum glial contamination of the neuronal layer (on a protein basis) was calculated of $11 \pm 3\%$. It must be noted that this is a maximum figure: any contamination of fraction C by red cells, which contain (Giacobini, 1964) 670 times as much carbonic anhydrase as the neurons, would magnify the apparent glial contamination.

Comparative data on fractions

Succinate dehydrogenase and cytochrome oxidase. Two other enzymes which have been claimed to

Table 4. *Carbonic-anhydrase activity in isolated cortex fractions*

Carbonic anhydrase was determined as described in the Methods section on fractions recovered from five separate gradients, each individual determination being made at four different enzyme concentrations. For description of fractions see the text.

Fraction	Specific activity (units/mg. of protein)
S	1260
A	1191
B (glial)	3488
C (neuronal)	259
D	5212

Table 5. *Estimation of glial contamination of neuronal fraction from rat cortex*

Ratios of carbonic-anhydrase activity in different fractions in the five experiments of Table 4. The glial contamination of the neuronal fraction was calculated on the basis of the Giacobini ratio as described in the text.

Expt.	Ratios of activities B/S	C/S	B/C	Max. glial contamination of neuronal fraction (%)
1	1·0	0·24	4·1	20
2	1·5	0·18	7·9	12
3	1·8	0·20	8·3	12
4	6·0	0·27	22·7	4
5	2·3	0·14	16·0	6
Mean	2·5	0·20	11·8	11±3

Table 6. *Succinate-dehydrogenase and cytochrome-oxidase activities in density-gradient fractions from rat cortex*

Enzyme activities are expressed in arbitrary units/mg. of protein and were determined as described in the Methods section, each determination being performed in quadruplicate (succinate dehydrogenase) or at at least two different enzyme concentrations (cytochrome oxidase); for description of fractions see the text.

Fraction	Specific activity (units/mg. of protein) Succinate dehydrogenase	Cytochrome oxidase
S	15·2±1·7 (5)	44·4±4·7 (3)
A	18·8±1·4 (5)	44·4±7·1 (8)
B (glial)	14·9±1·2 (5)	*29·6±5·2 (9)
C (neuronal)	16·7±1·0 (5)	*54·3±6·1 (9)
D	*4·7±0·3 (5)	50·0±5·6 (9)
Recovery (%)	87±4	96±7

* Significantly different from S ($P < 0·01$)

have a differential distribution between neurons and glia, at least in the Deiter's nucleus of the rabbit, are cytochrome oxidase and succinate dehydrogenase. Hamberger (1963), measuring these enzyme activities by a micro-diver technique in micro-dissected neurons and glial bundles, found both about 2·5-fold concentrated in the glia compared with the neurons, although these differences varied as between neuronal and capillary glia and were apparently markedly subject to variations depending upon the amount of prior mechanical stimulation the animal had received (see also, e.g., Hamberger, Hydén & Lange, 1966).

Measurement of the two enzymes in fractions recovered from the gradient showed (Table 6) that succinate dehydrogenase was distributed equally between neuronal and glial fractions whereas cytochrome oxidase was somewhat concentrated in the neuronal layer (neuronal/glial ratio 1·8).

Chemical analysis of the fractions. The phospholipid, phosphoprotein, 'nucleic acid' (largely RNA), 'acid-precipitable phosphate' (largely DNA) and 'residual organic phosphate' (largely phosphatidopeptide) contents of the separated fractions were determined to provide some absolute data on the composition of the cell fractions. Results are shown in Table 7. On a protein basis, there was a concentration of each of these constituents except for phospholipid in the neuronal layer; most strikingly, the nucleic acids and phosphoprotein were concentrated twofold and 3·5-fold respectively in the neuronal fraction. Concentration also occurred in pellet D.

Absolute concentrations of the different constituents in the suspension were similar to those found earlier in cortex slices (Rose, 1965*b*) with the exception of phospholipid, which was increased by 80%. This reflects the fact that the suspension contained a considerable amount of white matter not present in cortex slices.

Metabolic behaviour of neuronal and glial fractions

Oxygen uptake. The metabolic behaviour of the neuronal and glial fractions was examined by following their respiration manometrically for periods up to 2 hr. To check whether respiration was affected by the period of 3 hr. necessary for isolation of the fractions from the initial suspensions, uptake of oxygen was measured in the suspension immediately on preparation, and again after storage in crushed ice for 3 hr. (Table 8). In the absence of added substrate, respiration was linear for the first 30 min., and then began to decline. With added glucose respiration was stimulated, initially by some 60%, and uptake continued linearly for up to 2 hr. Endogenous respiration was almost unaltered by cold storage, but some impairment of the tissue's capacity to metabolize added glucose was apparent, stimulated uptake of oxygen being only 70–80% of that in the fresh preparation.

Table 7. *Chemical composition of isolated cortex fractions*

For preparation and description of fractions see the text. Results are means of six experiments in each case.

	Composition of phosphate compounds in fractions (mμmoles of phosphate/mg. of protein)				
Fraction	Phospholipid	'Nucleic acid'	'Residual organic phosphate'	Phospho-protein	'Acid-precipitable phosphorus'
S	565	5·5	27·6	1·6	8·2
A	472	4·4	19·9*	1·8	6·5*
B (glial)	451*	3·5*	25·9	1·7	3·4*
C (neuronal)	520	11·0	40·2*	5·7*	14·1*
D	545	11·0*	60·4*	4·6*	13·3*
Recovery (%)	99	108	111	121	85

* Significantly different from S ($P<0·01$).

Table 8. *Respiration of cortex suspensions after cold storage*

Cortex suspensions were made in 10% Ficoll as described in the Methods section, and their respiration in Elliott–Henderson medium ± 10 mM-glucose was followed for 2 hr. either immediately or after storage for 3 hr. in crushed ice. Results of incubation with glucose are expressed ± S.E.M. Numbers of experiments are given in parentheses.

	Uptake of oxygen (mμmoles/mg. dry wt.)			
	60 min.		120 min.	
Suspension	Without glucose	With glucose	Without glucose	With glucose
(*a*) Fresh	272 (3)	445 ± 19 (8)	406 (3)	870 ± 26 (8)
(*b*) Stored	283 (3)	340 ± 14 (5)	375 (3)	638 ± 29 (5)
100(*a*/*b*)	101	76	93	72

Table 9. *Respiration of neuronal and glial fractions with various substrates*

Fractions were prepared and incubations carried out in Elliott–Henderson medium as described in the Methods section. All substrate concentrations were 10 mM. Uptake of oxygen is expressed as mμmoles of O_2/mg. dry wt. ± S.E.M. (numbers of experiments are given in parentheses).

	Suspension		Glia		Neurons	
Substrate added	O_2 (mμmoles)	Stimulation (%)	O_2 (mμmoles)	Stimulation (%)	O_2 (mμmoles)	Stimulation (%)
(*a*) 60 min. incubation						
None	234 ± 15 (15)	—	173 ± 13 (15)	—	117 ± 6 (14)	—
Glucose	430 ± 37 (15)	188	378 ± 33 (7)	219	394 ± 14 (8)	335
Pyruvate	330 ± 25 (6)	141	345 ± 26 (6)	200	368 ± 37 (6)	315
Glutamate	250 ± 17 (6)	107	216 ± 12 (8)	125	213 ± 18 (6)	182
(*b*) 120 min. incubation						
None	329 ± 23 (17)	—	249 ± 22 (10)	—	174 ± 8 (14)	—
Glucose	642 ± 45 (11)	195	860 ± 70 (4)	345	823 ± 75 (6)	475
Pyruvate	555 ± 26 (6)	169	596 ± 30 (4)	238	690 ± 83 (6)	395
Glutamate	342 ± 23 (6)	108	345 ± 24 (8)	138	344 ± 25 (6)	199

These data may be compared with those obtained for respiration of brain-cortex slices under equivalent conditions. Thus, in the absence of added substrate, fresh cell suspensions took up 272 mμ-moles of oxygen/mg. dry wt./hr., compared with a figure of 275 found for cortex slices in a similar incubation medium. In the presence of 10 mM-glucose, uptake in the first hour of incubation was

445mμmoles of oxygen/mg., compared with 580 for the cortex slice (S. P. R. Rose, unpublished work).

Respiration of the stored suspension, and of the isolated glial and neuronal fractions, was examined in the absence of added substrate, and in the presence of 10mM-glucose, -pyruvate or -glutamate (Table 9). Endogenous respiration was lowered in the glial fraction, and substantially lowered in the neuronal fraction. Thus, after 1hr., endogenous uptake of oxygen in the glia was 74%, and in the neurons 50%, of that in the stored suspension. Addition of oxidizable substrate, however, raised oxygen uptake in all three fractions to a similar value. Thus, with glucose, uptake after the first hour was approx. 400mμmoles of oxygen/mg. dry wt. in neurons, glia and suspension; with pyruvate, the stimulation in all three fractions was slightly lower than that with glucose, averaging 345mμmoles of oxygen/mg./dry wt. for each fraction. Variations began to become apparent during the second hour of incubation.

The effect of glutamate on the oxygen consumption of the suspension and glial fraction was only slight, whereas in the neuronal fraction it resulted in a nearly twofold stimulation of respiration over the endogenous value. It cannot, however, be ruled out on the basis of these experiments that a similar stimulation in the suspension and glial fraction was masked by an initially higher rate of endogenous respiration. Oxygen uptake over the second hour suggests that this might indeed be the case with the glial fraction.

Comparison with tissue homogenates. In their capacity to respire with added substrate, cells recovered from the gradient thus behaved strikingly similarly to slices, suggesting that they had retained a considerable fraction of their metabolic integrity. This behaviour can be compared with that of tissue homogenates, prepared by grinding cortical material in the 10% Ficoll medium in a Teflon–glass homogenizer at 3000 rev./min. for a period of 1min. Homogenates so prepared distributed themselves quite differently on the Ficoll gradient (Table 10), layer A being much diminished and B and C increased, whereas their respiration with glucose was decreased threefold compared with the cell suspensions (Table 11). An index of the differences between homogenate fractions and the cell suspensions was the ratio of oxygen uptake with glucose compared with that with pyruvate as substrate. In the suspensions, respiration with glucose was greater than or equal to that with pyruvate (as it is with brain slices); in the homogenate fractions, the reverse was the case, indicating a loss of cytoplasmic glycolytic enzymes.

Lactate dehydrogenase. Confirmation of these conclusions with respect to the retained glycolytic capacity of the isolated cells was obtained by measurement of lactate dehydrogenase in the fractions off the gradient (Table 12). These measurements showed that a substantial proportion (62%) of the lactate dehydrogenase could not be sedimented after the initial preparation of the cell suspension, compared with only 20% of the total protein. Nevertheless, all the lactate dehydrogenase that could be sedimented after the initial suspension had been made could also be recovered after collecting the cells off the gradient, washing them and resuspending. Loss of lactate dehydrogenase could therefore be associated with initial cell breakage and further loss did not subsequently occur, implying that the cells were no longer 'leaky' to lactate dehydrogenase.

Table 10. *Comparison of cortex homogenates with cell suspensions: distribution of dry weight through gradient*

For preparation and nomenclature of fractions see the text; numbers of experiments are given in parentheses.

	Percentage dry wt. recovered	
Fraction	Homogenate	Suspension
A	12 (4)	65 (6)
B	42 (4)	17 (6)
C	29 (4)	9 (6)
D	4 (4)	5 (6)
	—	—
Recovery (%)	87	96

Table 11. *Comparison of cortex homogenates and cell suspensions: respiration of isolated fractions*

Oxygen uptake in mμmoles of O_2/mg. dry wt./hr. in Elliott–Henderson medium; substrate concentration 10mM. Numbers of experiments are given in parentheses: for preparation and nomenclature of fractions see the text.

	Homogenate			Suspension		
Fraction	Glucose (*a*)	Pyruvate (*b*)	100(*a*/*b*)	Glucose (*a*)	Pyruvate (*b*)	100(*a*/*b*)
H or S	152±8 (8)	200±10 (6)	76	430±37 (15)	330±25 (6)	130
B	153±8 (6)	183±9 (6)	83	378±33 (7)	345±26 (6)	109
C	129±25 (6)	185±9 (6)	69	349±14 (8)	368±37 (6)	107

Table 12. *Lactate dehydrogenase in isolated cortex fractions*

Lactate dehydrogenase was measured as described in the Methods section in (*a*) original suspension and (*b*) original suspension and isolated fractions after washing once, removing supernatant and resuspending pellet in 10% Ficoll medium. For fractions and nomenclature see the text. Specific activity in Wróblewski units (Bergmeyer *et al.* 1963). Mean of four experiments, each assay performed at three different enzyme concentrations.

Fraction	Percentage recovery: Lactate dehydrogenase	Percentage recovery: Protein	$10^{-3}\times$ Specific activity (units/mg. of protein)
(*a*) Original suspension			
S	100	100	22·3±3·6
S-pellet	38	80	
(*b*) After washing			
S-pellet	100	100	9·4±1·8
A-pellet	35	38	9·2±0·9
B-pellet	41	41	11·1±2·3
C-pellet	18	21	7·1±0·8
D	2	14	2·2±0·6
Recovery (% of S-pellet)	96	114	

DISCUSSION

The simple method that has been developed provides a means of obtaining relatively large numbers of neurons, in the sense that this word is used by Hydén and his co-workers (Hydén, 1960; Hamberger, 1963), comparatively free of glia and other contamination, by a procedure based on gentle disruption of the cortex followed by gradient centrifugation. Within 3 hr., up to $1{\cdot}5\times10^{7}$ neurons can be collected from 12 rat cortices. At the same time a second fraction enriched in glia but free of neurons can be obtained, the properties of which may be used as a control for the behaviour of the neuronal fraction. Sufficient quantities of material can be obtained by this method to make possible a comparison of the properties of neurons and glia by conventional biochemical techniques such as manometry. Such an approach is not a substitute for the micro-methods developed by Hydén and his co-workers (Hydén, 1960); it does, however, offer a new system for the examination of neuronal–glial interrelations.

The validity of the method must depend on two indices, the relative freedom of the fractions from cross-contamination, and the integrity of the cells. Viewed under phase contrast, a small amount of glial contamination of the neuronal fraction was noticeable. On the criterion of dry weight, or of the ratio of intact neurons to broken cells, a six- to eight-fold purification over the starting material has been achieved (Tables 1–3) and the purity of the fraction could be improved, with some diminution in yield, by lowering the concentration of the supporting sucrose layer of the gradient on which the cells were collected. The glial layer, on the other hand, was virtually free of neurons or neuronal nuclei, though contaminated by myelin, axonal material, mitochondria and nerve-ending particles. A higher degree of purity could probably be achieved by refractionating the harvested cells, but as this would have added substantially to the time necessary to obtain separation, and the emphasis in these experiments has been on obtaining metabolically active preparations, a further purification cycle was not introduced. The morphology of the separated fractions will be discussed in more detail elsewhere.

Apart from the morphological criteria, it was also possible to make a direct comparison between the composition of the neuronal layer and that of neurons prepared by micro-dissection techniques for three variables. Calculation of dry wt./cell and of RNA content/cell, made on the simplifying assumption that 70–75% of the material in the neuronal fraction was, in fact, intact neurons, fell satisfactorily within the range of values quoted by Hydén (1960) based on direct measurements in single cells. Use of the 'Giacobini ratio' (Giacobini, 1964) for the relative concentration of carbonic anhydrase in isolated neurons and glia also enabled an estimate of the extent of glial contamination of the neuronal fraction to be made, and on this basis a maximum figure of some 11%, on a protein basis, could be assigned to glial contamination.

The data reported here refer only circumstantially to the issue of the integrity of the outer membranes of the isolated cells. Thus use of the neuronal/nuclear ratio during the development of the separation method indicated that the integrity

of the neurons depended partly on the choice of Ficoll rather than sucrose as a suspending medium. As measured by this ratio, the fragility of the neurons in all media other than Ficoll was very marked. The other operative factor was the method chosen for disruption of the cortex. In general, any more drastic method than gentle teasing through bolting cloth, whether physical or enzymic, had a deleterious effect on the preparations, even though the proportion of undisrupted tissue was decreased.

Any method of disruption, though, must result in a certain amount of cell damage; axons and processes will be ripped off and cell membranes punctured. The neurons shown in Plate 1 (A–D) show the range of damage produced. Electron microscopy also indicated that fraction C contained a range of cells in varying degrees of integrity. The cell membrane was only sometimes visible in such osmium-fixed preparations, although it is uncertain how far this represents an artifact of the fixation process (M. Kidd & S. P. R. Rose, unpublished work). The integrity of the neuronal cell membrane in isolated neurons has been discussed by Johnston & Roots (1965).

From this point of view, the metabolic experiments reported in Tables 8 and 9 are revealing. The neuronal and glial fractions were concentrated by resuspension and further centrifugation after collection from the gradient, and finally resuspended once more; they must therefore have been largely free of contamination by soluble enzymes leaking out of broken cells. Yet they were capable of actively metabolizing glucose, pyruvate and glutamate for periods of several hours, as well as possessing a substantial endogenous respiratory activity. Indeed, in terms of oxygen uptake, their metabolism in the presence of glucose was only 20–30% lower than that of intact cortex slices. Whereas the enzymes of pyruvate and glutamate oxidation are largely particulate, most of those of glucose metabolism are soluble, with the possible exception of those concerned with the first stages. Thus the glycolytic enzymes might have been expected to leak out of damaged cells during centrifugation, as was the case for example when cell homogenates were treated in a similar way to the cell suspension (Table 11). In this case, respiration was reduced to one-third of that in the suspensions, and pyruvate respiration proceeded rather more rapidly than glucose respiration.

In the neuronal and glial suspensions, on the other hand, as in the intact cortex slice, glucose oxidation proceeded at a rate equal to or slightly higher than pyruvate oxidation. These inferences were confirmed when the distribution of one soluble glycolytic enzyme, lactate dehydrogenase, was examined (Table 12). Whilst considerable loss of lactate dehydrogenase occurred during the initial disruption of the tissue, subsequent leakage during centrifugation, washing and resuspension of the cells was slight.

This observation may be contrasted with the fate of glycolytic enzymes during the preparation of liver cells suspensions in sucrose or polyvinylpyrrolidone media by Branster & Morton (1957) and by Berry (1962*a*,*b*). After centrifugation, Berry could find almost no glycolytic activity in his preparation, whereas oxidation of tricarboxylic acid-cycle intermediates was relatively unimpaired. He could subsequently recover the glycolytic enzymes from the supernatant left after the cells had been harvested.

A more direct demonstration of the integrity of the cell membranes, however, must depend on observations of the active accumulation of ions, of stimulation effects associated with intact membranes, and on the existence of resting potentials in the isolated neurons. On these criteria preliminary evidence suggests that the neuronal fraction contains a significant proportion of at least partially damaged cells. So far, it has not proved possible to demonstrate the existence of resting membrane potential in the the isolated neurons. Although increased respiration in the presence of raised potassium concentrations and some accumulation of amino acids have been observed, such effects are decreased compared with those in the slice (H. F. Bradford & S. P. R. Rose, unpublished work).

The results reported here indicate certain differences between the neuronal and glial fractions. Thus the chemical analyses showed a higher concentration of nucleic acids and of phosphoprotein in the neuronal fraction than in either the initial suspension or the glial fraction (Table 7). Enzymic differences, too, were apparent between the fractions. Although it was not possible to confirm with these cortical cells Hamberger's (1963) observation on the relative concentration of cytochrome oxidase and succinate dehydrogenase in the cells from the Deiter's nucleus, nonetheless, significant differences in the distribution of cytochrome oxidase were found (Table 6). Differences in glutamate metabolism between the fractions were also suggested by the data on oxygen uptake of Table 9 and have been confirmed by studies with ^{14}C-labelled glutamate. Preliminary experiments indicate marked differences between the fractions with respect to the metabolic fate of glutamate, and these are now being extended as part of a general investigation of the metabolic behaviour of the isolated cells under varying conditions.

Note added in proof. Since the submission of this paper, three further procedures for separation of neurons and glia from cortex have been drawn to my

attention. G. Campbell (personal communication) utilizes free-flow electrophoresis for isolating cells initially separated by systemic perfusion of the whole animal with a mixture of trypsin and hyaluronidase, and claims an almost quantitative yield of cells. Rappaport (1966, and personal communication) separates the cells by stirring mouse cortex at 38° in a medium containing sodium tetraphenylboron, but does not further isolate the neurons and glia. Satake & Abe's (1966) procedure depends on acetone extraction, filtration and gradient centrifugation. A neuronal perikaryon preparation, in about 6% yield, is obtained, but no corresponding glial fraction. The average cell mass is apparently only some 5% of that quoted in this paper, presumably partly owing to loss of protein during the extraction procedure. For none of these three procedures is there yet any information on the metabolic state of the isolated cells.

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