RESOLUTION OF THREE DISTINCT POPULATIONS OF NERVE ENDINGS FROM RAT BRAIN HOMOGENATES BY ZONAL ISOPYCNIC CENTRIFUGATION

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ABSTRACT

Conditions have been established for the fractionation of subcellular components of rat forebrain homogenates by zonal isopycnic equilibration in continuous sucrose density gradients using a B-XIV rotor. The fractions were analyzed biochemically and by ultrastructural morphometry. Starting from postnuclear supernates of forebrain homogenates, it has been possible to resolve three distinct populations of nerve endings from one another, as well as from free mitochondria and myelin fragments. The three types of nerve endings differ in their apparent specific gravity, their biochemical properties, and their ability selectively to accumulate exogenous transmitter substances in vitro. These three particle populations are likely to represent, in order of increasing modal equilibrium density, (a) cholinergic nerve endings, characterized by their high content of acetylcholine, (b) γ-amino butyric acid (GABA)-containing nerve endings with high glutamate decarboxylase activity and the ability to accumulate exogenous GABA, (c) adrenergic nerve endings that accumulate exogenous dopamine and noradrenaline and exhibit high monoamine oxidase activity.

INTRODUCTION

Whittaker (1, 2) and de Robertis (3) were the first to isolate nerve ending particles from brain tissue homogenates, and to interpret them as detached, presynaptic boutons sheared off and resealed during homogenization of the tissue. Many other workers have since studied these structures by morphological and biochemical techniques. Under the assumption that the biochemical and biophysical properties of nerve endings reflect the functional characteristics of the synaptic region, and that these properties are largely preserved during homogenization, one would expect to find several distinct populations of nerve endings corresponding to the different systems of neurotransmission. Subfractionation of crude mitochondrial fractions from brain homogenates of different species has indeed provided evidence that nerve endings containing specific transmitters can be resolved from each other (4–7). These results have been obtained mainly by centrifugation through discontinuous density gradients.

The aim of this work was to define nerve ending populations which would be suitable for the study of interactions of psychotropic drugs with synaptic structures. This paper thus reports an analytical procedure for the biochemical, morphological, and functional characterization of nerve endings.

MATERIALS AND METHODS

Fractionation Techniques

Male RAC rats from Tierfarm AG, Sisseln, Switzerland, kept on a standard diet (Nafag pellets,
Biochemical Analyses

The starting material and the fractions obtained were assayed for a number of enzyme activities and for their content of some nonenzymatic constituents. Photometric measurements were performed in a Zeiss PM4 spectrophotometer equipped with a thermostatically controlled automatic cell unit with six cuvettes (Carl Zeiss, Oberkochen, W. Germany), and a transmittance-absorbance converter. For time-course determinations, the absorbance as such, or its logarithm, was recorded on a linear-log W. and W. recorder, model 3011 (W. and W. Electronic Ltd., Basel, Switzerland). Fluorometric assays were measured in an Hitachi-Perkin-Elmer fluorescence spectrometer model 203 (Perkin-Elmer Corp., Electro-Optical Div., Norwalk, Conn.). Automatic analyses were performed in a Carlo Erba automatic chemical analyzer, system 1500 (Carlo Erba, Milan, Italy) consisting of a sample distributor, model SD3, with a cooled sample tray, a proportioning pump, a three-channel flow photometer, model SS27, and Perkin-Elmer recorders, model 159. Radioactivity was measured in a Beckman LS-133 scintillation counter (Beckman Instruments International S.A., Geneva, Switzerland).

Acetylcholine: Acetylcholine was estimated biologically using the dorsal muscle of the leech. A narrow strip of the muscle was suspended under tension of 500 mg in a 10-ml Tyrode bath saturated with 95% O2 plus 5% CO2 at room temperature, and contraction of the strip was recorded axonotomically by means of a strain gauge transducer. To each test sample or acetylcholine standard, eserine (0.1 μg/ml) was added. At this concentration, eserine optimally increased the sensitivity of the muscle and completely inhibited acetylcholinesterase. Before and after each series of determinations, the system was calibrated with increasing concentrations of acetylcholine. Within the range of 1-20 ng of acetylcholine, a steep but reproducible logarithmic dose-response curve was obtained. In fractionation experiments in which the distribution of particulate acetylcholine was measured, all the sucrose solutions used contained 2 × 10^-5 M eserine in order fully to inhibit acetylcholinesterase. Fractions from zonal centrifugations were diluted with twice their volume of 0.32 M sucrose containing eserine, and the particulate material pelleted in an angle rotor, MSE no. 59593, at 3 × 10^6 g-min. The pellets were resuspended in Tyrode’s solution, adjusted to pH 4 by the addition of HCl, and heated in a boiling water bath for 10 min (9). After allowing the samples to cool to room temperature, the precipitate was removed by centrifugation, and the supernate readjusted to pH 7.4 with NaOH and diluted with Tyrode’s solution to a concentration suitable for the assay.

Acetylcholinesterase (EC 3.1.1.7): Acetylcholinesterase was assayed in 50 mM sodium phosphate buffer, pH 7.4, in the presence of 5 mM acetylcholine hydrochloride and 0.01% Triton-X-100. Incubation was carried out at 37°C for 60 min and stopped by the addition of an equal volume of 0.4 M perchloric acid. The precipitate was removed by centrifugation and the supernate was assayed for acetylcholine according to the method of Hestrin.

Percent concentrations are always wt/vol.
cytochrome c was added. The rate of cytochrome c oxidation, which obeys first-order kinetics, was recorded linearly at 350 nm on a linear-log recorder with the help of a transmittance-absorbance converter (see above), according to the principle described by Leighton et al. (8).

GLUTAMATE DECARBOXYLASE (EC 4.1.1.15): Glutamate decarboxylase was measured by determining the amount of $^{14}$CO$_2$ liberated from [1-$^{14}$C]glutamic acid as described by Fonnum (16). Incubation was carried out at 37°C for 60 min in the presence of 2 $\mu$Ci of D-[1-$^{14}$C]glutamic acid (10 mCi per mmol), 25 mM l-glutamic acid, 0.1 mM pyridoxal phosphate, 0.1 M sodium phosphate buffer, pH 6.2, and 0.05% Triton-X-100, using the technique described by Baggilini and Bickel (17). The reaction was stopped by the addition of HCl to a concentration of 0.1 N and incubation was continued for another 30 min in order to ensure complete recovery of $^{14}$CO$_2$. The samples were counted after addition of 10 ml of 0.6% of PBD-scintillator (see Materials), in toluene.

GLUTAMATE DEHYDROGENASE (EC 1.4.1.3): Glutamate dehydrogenase was assayed as described by Beaufay et al. (18), with the following modifications: Glycylglycine, 20 mM, pH 7.7, was used as the buffer, nicotinamide and cyanide were omitted, and the substrate concentration was 0.8 mM. Incubation was carried out at 25°C in 1-ml cuvettes. The reaction was started by the addition of the substrate after a 3-min preincubation which was found necessary to overcome the initial lag phase of the reaction.

LACTIC DEHYDROGENASE (EC 1.1.1.27): Lactic dehydrogenase was assayed at 25°C in 1-ml cuvettes using a medium which contained 50 mM sodium phosphate buffer, pH 7.5, 90 $\mu$M NADH, 0.44 mM sodium pyruvate, and 0.01% Triton-X-100. The reaction was started by the addition of substrate after 1 min of preincubation, and the decrease in absorbance at 340 nm was recorded. Blanks were run without substrate.

MONOAmine OXIDase (EC 1.4.3.4): Monoamine oxidase was determined according to Kraji (19). Incubation was carried out at 37°C for 20 min in 33 mM Tris-Cl buffer, pH 8.5, 51 $\mu$M kynuramine hydrobromide, and 0.01% Triton-X-100. Standard assays contained 3.3 nmol 4-hydroxyquinoline. Blanks were run for each fraction by adding the substrate after the stopping solution.

$\alpha$-NaphthylacetaTE ESTERASE (EC 3.1.1.6): $\alpha$-Naphthylacetate esterase was determined automatically. The liberated $\alpha$-naphthol was measured photometrically after coupling with fast red. Sample (0.45 ml/min) and $\alpha$-naphthylacetate, 0.75 mM in 50 mM sodium citrate buffer, pH 5.8 (1.88 ml/min), were incubated at 37°C for 15 min. Fast red ITR salt, 0.2% in 0.2 M sodium acetate buffer, pH 4.5, containing 1% of Brj (1.36 ml/min),
was then added, and the reaction allowed to proceed at 37°C for 10 min before the mixture entered the flow photometer. Transmittance at 550 nm was recorded on log paper. 20 samples were processed per hour with a 1:2 sampling-to-washing ratio. A standard solution containing 0.1 mM α-naphthol in 20 mM NaOH was used.

5′-NUCLEOTIDASE (EC 3.1.3.5): 5′-Nucleotidase was assayed in 50 mM Tris-HCl buffer, pH 7.7, in the presence of 3 mM AMP, 2 mM magnesium sulfate, and 0.1% Triton-X-100. Incubation was carried out at 37°C for 60 min and stopped by the addition of 3 vol of a solution containing 15% trichloroacetic acid, 3% ascorbic acid, and 0.5% EDTA. Inorganic phosphate was determined as described for ATPase.

PROTEIN: Protein was determined as described by Lowry et al. (8). BSA was used as a standard.

Uptake Studies

The uptake of labeled dopamine, noradrenaline, serotonin, and γ-amino-butyric acid by subcellular particles was studied at 25°C with postnuclear supernates in a modified Krebs-Ringer solution (20). 1 vol of postnuclear supernate obtained from a 10% homogenate was diluted with 3 vol of the medium, preincubated at 25°C for 10 min, and then supplemented with the labeled compound in different concentrations. Uptake was terminated at different time intervals by cooling the reaction mixture in an ice bath. The particulate material was then sedimented at 3 × 10⁶ g·min, rinsed twice with small amounts of isotonic saline, extracted by homogenization in ethanol, and freed from precipitated protein by centrifugation at 10,000 g·min. When the subcellular distribution of the labeled compound was investigated, the 3 × 10⁶ g·min pellets were rinsed with 0.32 M sucrose, resuspended in the same solution, and fractionated as described above. Radioactivity was measured in 0.1-m samples of the ethanol or aqueous fractions after addition of 10 ml of 12% ethanolamine in methanol and 10 ml of 0.6% PBD in toluene. The results are expressed as the particulate-to-medium ratios V/S, where V is the transmitter uptake in millimoles per gram of pellet wet weight (3 × 10⁶ g·min), and S is the initial medium concentration of transmitter in millimoles per milliliter.

Morphological Techniques

The samples were prepared according to the procedure introduced by Baudhuin et al. (21). The fractions were diluted to approximately 0.5 mg/ml of protein, and 0.1 ml portions were mixed with 19 vol of ice-cold 0.1 M cacodylate buffer, pH 7.4, containing 1.5% glutaraldehyde, and adjusted to a toxicity of 340 mmoles by the addition of sucrose. The suspension was filtered through Millipore filter membranes with pore size 0.025 μm (VSWP filters, Millipore AG, Kloten, Switzerland) under nitrogen pressure of 1-2 atm. Filtration time was 20-30 min. The particle pellicle was covered with a second filter of the same type and carried through the subsequent preparation steps. Refixation was performed for 2 h in 1% osmium tetroxide buffered with 50 mM sodium phosphate made isotonic with sucrose. The filters were dehydrated in graded alcohol solutions and released into propylene oxide where the membranes were dissolved within 10–12 hours. The pellicle was embedded in Epon (22), and grey sections were cut at right-angles to the pellicle surface. After staining with uranyl acetate and lead citrate (23) the specimens were examined with a Siemens Elmiskop 101.

The volume densities of nerve endings, and of free and intraterminal mitochondria were determined according to the principles outlined by Weibel (24).

Materials

All solutions were made up in glass-distilled water. Special attention was devoted to the sucrose solutions used for the homogenization of the tissue and for preparing the density gradients. Sucrose “for density gradient centrifugation” (E. Merck A.G., Darmstadt, W. Germany) was used, and the water was freed of carbon dioxide by boiling just before use. Reagents used in the present work were obtained from the following sources: Fluka A.G., Basel, Switzerland, osmium tetroxide, and 2-(4-tertiary butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole (PBD); KAO Atlas Ltd., Tokyo, Japan, Emaol 4130; Koch Light Laboratories Ltd., Colnbrook, Bucks., England, 4-methylumbelliferonyl-2-acetamido-2-deoxy-β-d-glucopyranoside, and 4-methylumbelliferone; E. Merck A.G., Darmstadt, W. Germany, eserine; Packard Instrument Co., Inc., Downers Grove, Illinois, Triton-X-100; Schuchardt Ltd., Munich, W. Germany, titanium sulfate-titanium oxysulfate, and cholesterol; Siegfried Ltd., Zofingen, Switzerland, ethylenelediamine tetraacetic acid (EDTA, disodium salt); Sigma Chemical Co., St. Louis, Mo., AMP, ATP, cytochrome c (type III from horse heart), fast red ITR salt, 4-hydroxyquinoline, kynuramine dihydrobromide, and NADH; TAAB Laboratories, Reading, Sussex, England, glutaraldehyde; Technicon Instruments Corp., Tarrytown, N. Y., Brij-35; Wander Ltd., Berne, Switzerland, acetylcholine hydrochloride, Lamisil. The following labeled compounds were obtained from New England Nuclear, Boston, Mass., γ-amino-[2-3H]butyric acid, [3,4-3H]dihydroxyphenylethylamine, [m]{[1-14C]glutamic acid, [5-3H]hydroxytryptamine, and m}{[7-14C]nor-
### Table I

**Relative Content of Constituents of Rat Forebrain Homogenates in the Postnuclear Supernate E Used as the Starting Material for the Fractionation Experiments**

| Constituent                        | Percent of total activity recovered in E | Recovery | Number of experiments |
|------------------------------------|-----------------------------------------|----------|----------------------|
| Protein                            | 80.9 ± 8.9                               | 106.3 ± 11.5 | 17                   |
| Cytochrome oxidase                 | 89.8 ± 3.6                               | 95.9 ± 10.3  | 17                   |
| Glutamate dehydrogenase            | 84.9 ± 5.5                               | 95.1 ± 16.3  | 14                   |
| Monoamine oxidase                  | 78.5 ± 5.3                               | 104.3 ± 9.5  | 16                   |
| Glutamate decarboxylase            | 98.0                                     | 97.3       | 2                    |
| α-Naphthylacetate esterase         | 81.7 ± 9.5                               | 104.4 ± 9.6  | 17                   |
| Na⁺/K⁺ + Mg⁺⁺-ATPase               | 74.0 ± 12.5                              | 107.2 ± 16.7 | 8                    |
| 5'-Nucleotidase                    | 82.8                                     | 98.5       | 3                    |
| Acetylcholinesterase               | 90.4                                     | 103.5      | 1                    |
| Lactic dehydrogenase               | 91.1 ± 4.0                               | 102.7 ± 14.9 | 11                   |
| N-acetyl-β-glucosaminidase         | 83.1 ± 9.3                               | 106.1 ± 12.8 | 7                    |
| Catalase                           | 79.9                                     | 95.1       | 2                    |
| Cholesterol                        | 72.4                                     | 106.8      | 2                    |

* Mean values. Standard deviation is given if more than three determinations were performed.

† Because of lack of space in the incubation apparatus (17), the distribution of GAD between postnuclear supernate (E) and nuclear pellet was determined only twice.

### Table II

**Enzymes and Other Constituents Measured. Specific Activities and Contents in the Starting Material, and Average Percent Recoveries upon Fractionation.**

| Constituent                        | Specific activity* (mU/mg protein (mean ± SD)) | Average percent recovery upon fractionation (mean ± SD) | Number of experiments |
|------------------------------------|-----------------------------------------------|-------------------------------------------------------|----------------------|
| Cytochrome oxidase                 | 64.6 ± 22.4                                  | 100.9 ± 23.0                                          | 15                   |
| Glutamate dehydrogenase            | 86.3 ± 14.6                                  | 86.6 ± 10.0                                           | 17                   |
| Monoamine oxidase                  | 1.91 ± 0.26                                  | 88.9 ± 9.2                                            | 18                   |
| Glutamate decarboxylase            | 8.30 ± 0.31                                  | 76.6 ± 8.3                                            | 10                   |
| α-Naphthylacetate esterase         | 67.7 ± 14.9                                  | 88.1 ± 14.9                                           | 14                   |
| Na⁺/K⁺ + Mg⁺⁺-ATPase               | 266.0 ± 60                                  | 89.4 ± 14.9                                           | 15                   |
| 5'-Nucleotidase                    | 22.9 ± 2.4                                   | 96.5 ± 11.9                                           | 4                    |
| Acetylcholinesterase               | 113.6                                       | 51.0                                                  | 1                    |
| Lactic dehydrogenase               | 653.0 ± 60                                  | 114.8 ± 17.5                                          | 15                   |
| N-acetyl-β-glucosaminidase         | 1.24 ± 0.2                                   | 90.8 ± 9.8                                            | 10                   |
| Catalase                           | 1.13                                         | 93.0                                                  | 2                    |
| Acetylcholine (µg/g of brain)      | 0.52 ± 0.36                                  | 104.6 ± 23.0                                          | 3                    |
| Cholesterol (mg/g of brain)        | 4.92                                         | 88.1                                                  | 1                    |

* The unit of cytochrome oxidase activity is defined by Cooperstein and Lazarow (40), and that of catalase by Leighton et al. (8). For all other enzymes tested, one unit of activity corresponds to the amount of enzyme which degrades 1 µmol of substrate per min.

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RESULTS

Starting Material

The homogenization of the brain and the preparation of the postnuclear supernate that was used as the starting material yielded very reproducible results. Cell disruption was highly effective as judged by the presence of more than 90% of the total activity of some of the enzymes in the supernate fraction (see Table I). The specific activities of the enzymes and the concentrations of non-enzymic constituents in the starting material of the fractionation experiments are given in Table II. This table also shows the average recoveries for these constituents upon fractionation.

Fractionation Experiments

The fractionation data presented in this section were all obtained in the shallow, large-volume density gradient described under Materials and Methods. Data obtained under different conditions will be briefly commented upon in a subsequent section.

Figs. 1 and 2 show the results of 2 out of 17 such experiments. In describing these data, it is useful to begin with the distribution profiles of cytochrome oxidase and of glutamate dehydrogenase (GDH), which enable us to identify the equilibrium position of the mitochondria (25). The two profiles, although not identical, have a similar shape, and essentially the same modal equilibrium density of 1.182 ± 0.003, and 1.183 ± 0.006, respectively (see Table III). The main cytochrome oxidase peak is frequently accompanied by a minor shoulder or peak in the middle of the gradient where very little GDH activity is found (see Figs. 2 and 3). Monoamine oxidase (MAO), which is known to be associated with the outer membrane of rat liver mitochondria (26), and which is generally considered to be a mitochondrial marker in nervous tissue as well (27, 28), shows a density equilibration profile that differs considerably from that of the two former enzymes. MAO exhibits in most of the experiments a broad peak with modal density of 1.169 ± 0.004. In some experiments, the MAO band is resolved into two strongly overlapping peaks, one coinciding with, the other adjacent to, the peaks of cytochrome oxidase and GDH. The dissociation of MAO and cytochrome oxidase is further documented in Fig. 3 which shows the distributions of both enzymes in eight different experiments.

Despite a rather high variability of the MAO profiles, these data indicate the presence of two MAO-containing particle populations with different mean equilibrium densities. A minor portion of the total MAO activity is always found at the border between the starting zone and the gradient, at a density of approximately 1.100. In this zone,
Figure 2 Isopycnic equilibration of subcellular components of a postnuclear supernate of rat forebrain homogenate. The data are calculated and presented exactly as in Fig. 1. Percentage recoveries were 108 for protein, 104 for lactic dehydrogenase, 77 for a-naphthylacetate esterase, 125 for catalase, 67 for cytochrome oxidase, 83 for glutamate dehydrogenase, 87 for monoamine oxidase, 89 for glutamate decarboxylase, 100 for 5'-nucleotidase, and 92 for N-acetyl-β-glucosaminidase.

we find very high activities of the sodium-potassium-activated, magnesium-dependent ATPase, 5'-nucleotidase, and a-naphthylacetate esterase. These enzymes all exhibit a second much lower and broader peak in the middle of the gradient. In two experiments, the subcellular distribution of cholesterol was found to be almost identical with that of the 5'-nucleotidase activity. These data suggest that the material which accumulates at the border between the starting zone and the gradient represents mostly fragments of the myelin and of the plasma membranes. The minor portion of MAO activity in this zone could indicate the presence of mitochondrial outer membrane fragments. However, other, nonmembrane-bound enzymes, e.g. catalase and N-acetyl-β-glucosaminidase, are also enriched in this zone. They may belong to particles too small to pass the density step under the adopted experimental conditions, or may be localized in fragments of myelinated axons.

Because of the association of glutamate decarboxylase (GAD) (16, 29) and particulate acetylcholine (5, 30) with nerve endings, we have determined their density distributions in a number of

| Average modal density* | Constituent                                    |
|------------------------|------------------------------------------------|
| 1.101 ± 0.010          | α-Naphthylacetate esterase                      |
| 1.104 ± 0.003          | ATPase                                         |
| 1.104                  | 5'-Nucleotidase; acetylcholinesterase           |
| 1.105                  | Cholesterol                                    |
| 1.137 ± 0.003          | Acetylcholine                                   |
| 1.148                  | 5'-Nucleotidase; acetylcholinesterase           |
| 1.149 ± 0.004          | Glutamate decarboxylase                         |
| 1.150 ± 0.003          | ATPase; α-naphthylacetate esterase              |
| 1.150 ± 0.006          | Lactic dehydrogenase                           |
| 1.156 ± 0.005          | N-acetyl-β-glucosaminidase                     |
| 1.156                  | Cholesterol                                    |
| 1.160 ± 0.007          | Monoamine oxidase†                              |
| 1.182 ± 0.003          | Cytochrome oxidase                             |
| 1.183 ± 0.005          | Glutamate dehydrogenase                        |
| 1.199 ± 0.008          | N-acetyl-β-glucosaminidase                     |

* Standard deviations were calculated only if more than three single values were available. Minor peaks accounting for less than 10% of the total, and peaks extending into the sample zone were disregarded.
† In 4 out of 18 fractionations MAO showed 2 peaks with modal densities 1.181 ± 0.002, and 1.166 ± 0.008, respectively.
FIGURE 3  Distribution of cytochrome oxidase (white histograms) and monoamine oxidase activity (shaded histograms) in eight isopycnic equilibration experiments with rat forebrain homogenate. The data are presented exactly as in Fig. 1. Percentage recoveries (±SD) were 91 ± 25 for cytochrome oxidase and 102 ± 7 for monoamine oxidase.

experiments. About half of the GAD activity of each preparation equilibrates in a relatively sharp, symmetrical peak with average modal density of 1.149 ± 0.004 and is well resolved from the MAO activity profile. The remainder of the GAD is retained in the starting zone, together with the soluble proteins. This bimodal distribution could result from the partial release of enzyme from nerve endings during homogenization, as suggested by Salganikoff et al. (29). These authors found no soluble GAD after homogenization in the presence of calcium, and concluded that calcium prevents nerve ending damage and consequently GAD release. However, Fonnum showed that upon addition of calcium, soluble GAD binds strongly to sedimentable material (16). It seems likely, therefore, that the GAD found in the starting zone represents enzyme originally present in the cytosol of neuronal bodies and/or glial cells. The particulate acetylcholine equilibrates in a narrow band with modal density of 1.137 ± 0.003 which is slightly but clearly dissociated from the heavy peak of GAD. This indicates that the nerve endings containing GAD and the cholinergic nerve endings, which we recognize by their acetylcholine content, are distinct entities, and differ sufficiently in their physical properties to be resolved in a sucrose density gradient.

As expected, lactic dehydrogenase (LDH) is mostly retained in the starting zone. A consistent portion of its activity, however, is distributed throughout the gradient and possibly shows a relative enrichment in the middle of the gradient. In a number of experiments, the distributions of N-acetyl-β-glucosaminidase and catalase, which were selected as lysosomal and peroxisomal markers, respectively, were also determined. N-acetyl-β-glucosaminidase activity is found throughout the gradient. Its distribution profile is characterized by two highly reproducible peaks, one with approximately the same modal density as the light peak of the 5'-nucleotidase (and other membrane markers measured), and the other with a modal density of 1.199 ± 0.008. The first-mentioned peak extends over the sample zone into the beginning of the gradient, as does the corresponding peak of protein. Its activity is presumably due to N-acetyl-β-glucosaminidase released from damaged lysosomes, as well as enzyme bound to structures retained within the membrane zone. The distribution of N-acetyl-β-glucosaminidase activity in the gradient supports the notion that brain lysosomes are very heterogeneous. Catalase exhibits a less complex distribution. About one-third of its activity is retained near the starting zone, together with a similar fraction of the N-acetyl-β-glucosaminidase activity (see above). The remainder is found in a broad, symmetrical peak which clearly indicates that most of the enzyme is particle bound. Catalase could be confined to discrete particles or be present in soluble form in the cytosol of nerve endings which are the main particulate component of the median zones of the gradient. The obtained distribution profiles argue in favor of the first possibility. In
fact, if catalase were soluble as is LDH, a much larger fraction of its activity would remain in the sample zone. The protein distribution is characterized by two peaks, each accounting for approximately half of the total content. The first peak covers the sample zone and the beginning of the gradient, while the second, much broader peak has a modal density of 1.150. The highest relative protein contents are always found at the beginning of the gradient together with high activities of the membrane enzymes. In the outer half of the gradient, the protein content decreases steadily with increasing equilibrium density. Very little protein is found together with the highest activity of cytochrome oxidase.

Fractionations in Different Types of Gradients

The experimental conditions of the fractionations described above were adopted on the basis of the results obtained with various types of density gradients. Although none of these systems showed any apparent advantage over the one that was finally used, the results obtained will be briefly described. Steep sucrose gradients extending linearly between the density of the sample (1.04) and 1.20 g/ml were used initially. The density distribution patterns and the average modal densities of most of the constituents were consistent with those presented in the former section. The resolution, however, was poor. A significant improvement was achieved by the introduction of a steep density increment from 1.04 to 1.10 between sample zone and gradient. In this system, myelin and other membranous material could be trapped at the boundary between the sample zone and the gradient, thus leaving a large portion of the equilibration field for heavier particles. We also used Ficoll density gradients (Pharmacia, Uppsala, Sweden) in isotonic sucrose but found it of little advantage. Because of the high viscosity of the gradient material, centrifugation time had to be exceedingly long to allow for particle equilibration. Incomplete equilibration in such gradients resulted in poor resolution of nerve ending populations.

Morphological Results

Because of the heterogeneity of the starting material and the high degree of variability in size of some of the subcellular structures, the fractionated material was processed for electron microscopy under conditions that allowed a morphometrical evaluation of the main structures present (see Materials and Methods). Figs. 4–7 are micrographs of the fractions that were analyzed by morphometry. They were selected from one fractionation experiment on the basis of the biochemical data. They exhibit, in this order, the highest relative activities of cytochrome oxidase (density 1.185), MAO (1.165), GAD (1.155), and the highest content of particulate acetylcholine (1.137). The results of the morphometrical analysis of micrographs providing three to six complete surveys of the pellicle depth are presented in Table IV.

Fig. 4 has a very homogeneous appearance. In accordance with its high cytochrome oxidase activity, it consists almost exclusively of free mitochondria which occupy 74% of the total volume of the structures present. Figs. 5–7 differ strikingly from the mitochondrial fraction but are similar to each other. The main components are profiles of nerve endings of different size and shape. In the MAO-rich fraction (Fig. 5), nerve endings account for 58% while free mitochondria occupy only 12% of the total particle volume. Nerve endings make up 40% of the particle volume in the fraction with the highest GAD content and 56% in the peak fraction of particulate acetylcholine content (Figs. 6 and 7). In the micrographs of these two last fractions, a number of membrane profiles of various shapes are noted. Myelin membranes, however, seem to be totally absent. A large proportion of the nerve ending volume is accounted for by intraterminal mitochondria. They occupy 22, 23, and 18% of the nerve ending volumes in the fractions represented by Figs. 5, 6, and 7, respectively.

Characterization of Nerve Endings by Transmitter Uptake

The properties of the nerve endings were further investigated by assessing their ability to take up exogenous transmitter substances. Postnuclear supernates were incubated in the presence of radioactively labeled dopamine (DA), γ-amino butyric acid (GABA), serotonin (5-HT), or noradrenaline (NA), and the amount of accumulated radioactivity was determined. The particulate fraction obtained was resuspended in sucrose and fractionated by density equilibration. The particulate-to-medium ratios, which are a measure
of transmitter uptake (see Methods), are shown in Table V.

Fig. 8 shows the density distribution of exogenous DA, NA, GABA, and 5-HT accumulated by particles of the postnuclear supernate. The distribution histograms of protein and cytochrome oxidase are given as references. It should be noted that in this type of experiment the protein distributions are not comparable with those obtained using the standard centrifugation procedure (Figs. 1–3). This is due to the fact that the particulate material of the postnuclear supernate is washed in sucrose (see Materials and Methods) before isopycnic fractionation, and therefore most of the soluble protein is removed.

The profiles of transmitter radioactivity are bimodal in all cases. The first peak is found in the starting zone and is likely to represent transmitter which has not been taken up or which has been released during processing of the starting material. The second peak is found together with the particulate material. Its modal density varies according to the transmitter used. The peaks of particle-bound DA and NA are nearly identical. They are found together with the light half of the MAO activity and exhibit a modal density of 1.164. The peak of particle-bound GABA (modal density 1.148) coincides with the peak of particulate GAD activity. Finally, particle-bound 5-HT, which has a modal density of 1.143, is well resolved from the zone of the other two amines, DA and NA, and does not seem to correspond to any of the biochemical constituents measured.

DISCUSSION

Work from a number of laboratories indicates the presence in brain homogenates of distinct types of nerve endings. Cholinergic nerve endings have been partially resolved from noncholinergic endings in rat brain homogenates (4), and from 5-HT-containing nerve endings in guinea pig brain homogenates (5). Furthermore, GABA-containing nerve endings could be dissociated from adrenergic (6) and from serotonergic nerve endings (7) by density gradient centrifugation of brain homogenates from various species.

In the present study, the fractionation of subcellular structures from rat brain homogenates has been attempted under conditions which differ in many respects from those of the above investigations. By omitting the customary prefractionation by differential centrifugation, we have avoided an arbitrary selection of structures present in the homogenate, and also possible artifacts resulting from damage and aggregation of particles. A very dilute postnuclear supernate from rat forebrain homogenate was used as the starting material, and subjected to zonal isopycnic centrifugation through a large-volume, shallow sucrose density gradient in a B-XIV rotor. The maximum angular velocity was limited to 30,000 rpm in order to avoid particle damage through hydrostatic pressure (31). Particulate acetylcholine, glutamate decarboxylase (GAD) activity, and accumulation of exogenous transmitters by selective uptake were adopted as markers for identifying nerve endings. Fractions from the density gradients characterized by these markers were further analyzed by means of ultrastructural morphometry.

Using this approach, we were able to identify three distinct populations of nerve endings: (a) those containing acetylcholine (cholinergic nerve endings) at a modal equilibrium density of 1.137; (b) nerve endings with high GAD activity and the ability to accumulate exogenous GABA, equilibrating at an average density of 1.149; (c) adrenergic endings characterized by high affinity uptake of exogenous NA and DA at an average density of 1.165. Free mitochondria have a modal equilibrium density of 1.182, and are therefore well resolved from nerve endings. In the interest of clarity, the discussion will be focused on the three nerve ending populations mentioned above.

Cholinergic Nerve Endings

The equilibration profile of particulate acetylcholine has a shape that clearly distinguishes it from all other profiles obtained, including that of acetylcholinesterase activity. The distribution of this enzyme determined in a few experiments either as acetylcholine splitting activity, or as the eserine-sensitive hydrolysis of α-naphthylacetate, was indistinguishable from that of the α-naphthylacetate esterase activity. This supports the interpretation of Whittaker (9) that presynaptic acetylcholinesterase activity is low. High relative concentrations of acetylcholine coincide with comparatively low values of protein and very low relative activities of both cytochrome oxidase and GDH which are markers for the intraterminal mitochondria present. This seems to indicate that, in our starting material, cholinergic endings represent a small particle population.
FIGURES 4-7 Survey electron micrographs of fractions separated by isopycnic equilibration as shown in Figs. 1 and 2. See descriptions in the text. X 30,000.

FIGURE 4 Fraction with highest relative activity of cytochrome oxidase and GDH (d = 1.183).

FIGURE 5 Fraction with high relative activity of MAO (d = 1.185) coinciding with highest concentrations of accumulated DA and NA as determined in other experiments.
**Figure 6** Fraction with highest relative activity of GAD ($d = 1.155$), corresponding to highest concentration of accumulated GABA as determined in other experiments.

**Figure 7** Fraction with highest relative concentration of particulate acetylcholine ($d = 1.137$).
TABLE IV

Relative Volume Densities of Nerve Endings and Mitochondria in the Fractions Represented by Figs. 4-7

| Characteristic Constituent | Density (g/ml) | \( V_{V_N}/V_T \) | \( V_{VML}/V_T \) | \( V_{VMT}/V_T \) | \( V_{VML}/V_{VMT} \) |
|---------------------------|---------------|-------------------|-------------------|-------------------|-------------------|
| Cytochrome oxidase        |               |                   |                   |                   |                   |
| GDH                       | 1.183         | 0.08              | 0.71              |                   |                   |
| MAO                       | 1.165         | 0.59              | 0.12              | 0.13              | 0.22              |
| GAD                       | 1.155         | 0.40              | 0.02              | 0.14              | 0.37              |
| Acetylcholine (particulate) | 1.137        | 0.56              | \( \leq 0.01 \)   | 0.11              | 0.18              |

In the first three columns, the relative volume densities of nerve endings \( (V_{V_N}) \), and of free and intraterminal mitochondria \( (V_{VML} \) and \( V_{VMT} ) \) are given with respect to the volume density of all particles present \( (V_T) \). The last column shows the relative volume density of intraterminal mitochondria with respect to the volume density of nerve endings.

TABLE V

High-Affinity Uptake of Dopamine, Noradrenaline, GABA, and Serotonin by Particles of Postnuclear Supernates of Rat Forebrain Homogenates

| Compound        | Concentration in medium | \( V/S^* \) (mean ± SD) | Number of experiments |
|-----------------|-------------------------|-------------------------|----------------------|
| Dopamine        | \( 1 \times 10^{-7} \)   | 26.1 ± 1.6              | 3                    |
| Noradrenaline   | \( 5 \times 10^{-7} \)   | 5.2 ± 0.3               | 3                    |
| GABA            | \( 1 \times 10^{-7} \)   | 12.3 ± 1.1              | 4                    |
| Serotonin       | \( 1 \times 10^{-7} \)   | 17.1 ± 0.6              | 3                    |

* \( V = \) mmoles of compound per gram of particulate material (\( 3 \times 10^6 g \)-min pellet of reaction mixture); \( S = \) initial concentration of compound in the medium (mmoles per milliliter). The incubation time required to reach maximal ratios was 15 min for the amines and 30 min for GABA.

GABA-Containing Nerve Endings

We have identified a second population of nerve endings, on the basis of the density distribution of GAD activity, which differs from that of particulate acetylcholine. In postnuclear supernates, these particles appear to have the property of selective accumulation of exogenous GABA. Particle-bound GAD activity and the accumulated GABA show very similar peaks. This confirms the data of Neal and Iversen (32) which suggest that GAD, and both exogenous and endogenous GABA, are localized in the same particle.

FIGURE 8 Isopycnic equilibration of dopamine, noradrenaline, GABA, and serotonin (shaded histograms) after high affinity uptake by particles of postnuclear supernates of rat forebrain homogenate. The distribution profile of protein (left-hand graphs) and cytochrome oxidase (right-hand graphs) from the corresponding experiments are given as a reference (white histograms). The data are calculated and presented as in Fig. 1. Percentage recoveries were 101 for dopamine, 116 for noradrenaline, 119 for GABA, and 112 for serotonin.
The median zones of the gradient are further characterized by high relative concentrations of protein and of the majority of the enzyme activities measured. In particular, the membrane markers ATPase, α-naphthylacetate esterase, 5'-nucleotidase, and cholesterol exhibit peaks that closely resemble that of particulate GAD. Furthermore, the cytochrome oxidase profiles frequently show a minor shoulder (or peak) accompanying GAD, which is likely to reflect activity from the numerous intraterminal mitochondria. When free mitochondria and plasma membrane fragments are virtually absent, these enzyme activities are best attributed to nerve endings. This indicates that a large portion of these particles equilibrate in the GAD zones. As it seems unlikely that such great numbers of nerve endings originate from GABA neurons, these results suggest that other kinds of nerve endings, having similar physical properties to those containing GABA, may equilibrate in these zones but remain unrecognized because of the lack of specific biochemical markers. There are indeed indications that this may be the case. Uptake studies have shown that particles which accumulate 5-HT have a similar density distribution to those accumulating GABA. The 5-HT particles could well represent a distinct type of nerve ending which cannot be further characterized by the present selection of biochemical markers.

Adrenergic Nerve Endings

Adrenergic endings have been identified in this work as the particles that accumulate both NA and DA. They represent the nerve ending population with the highest equilibrium density, and can be resolved from the GABA- and 5-HT-accumulating particles, as well as from free mitochondria. The equilibration profiles of accumulated NA and DA cover the light half of the MAO profiles. It is remarkable that the peaks of the amine contents coincide with highest relative activities of MAO, while the profiles of all other enzyme activities measured show a generally pronounced diminution in this zone. It is therefore tempting to assume that high specific activity of MAO may be a characteristic property of adrenergic nerve endings.

The relative activity of MAO is about equal in the equilibration zone of free mitochondria and in the peak zone of adrenergic endings. In the latter, cytochrome oxidase activity is comparatively low and free mitochondria account for only slightly more than 10% of the particle volume. The difference between the MAO and cytochrome oxidase distributions suggests the presence of an MAO-containing particle population distinct from the bulk of the free mitochondria. There is no indication that this may represent a subpopulation of free mitochondria. Differences between neuronal and glial mitochondria have been described in rabbit and bovine brain by Hamberger et al. (33). Both types of mitochondria, however, equilibrate at much higher densities than that of the light MAO zones and exhibit similar MAO:cytochrome oxidase activity ratios. The MAO activity found at lower density could be associated with the mitochondria of the adrenergic nerve endings. These would then represent a special kind of mitochondrial biochemically distinguishable from free brain mitochondria. There is indeed evidence in the literature suggesting that intraterminal mitochondria strongly differ in their enzymatic composition from perikarial mitochondria (34, 35). Finally, there remains the possibility that in adrenergic nerve endings, MAO may be partially localized in structures other than mitochondria; for instance, associated with the nerve ending membranes or storage vesicles. Evidence for a nonmitochondrial localization of MAO has been provided for rat heart, salivary gland, and vas deferens (36), as well as for bovine splenic nerve (37).

Preliminary studies on substrate specificity and susceptibility to inhibitors have disclosed kinetic differences between the MAO of the mitochondrial zone and that of the adrenergic ending zone (U. Bretz, unpublished observations). This suggests that multiple molecular forms of MAO which have been described in brain by others (38, 39) may be present in different proportions in these two particle populations.

We thank Dr. R. B. Bolender for his help and advice at various stages of the morphological work.

Part of this work has been presented at the European Symposium of Zonal Density Gradient Centrifugation, Paris, 1972.

Received for publication 10 October 1973, and in revised form 10 January 1974.

REFERENCES

1. Gray, E. G., and V. P. Whittaker. 1960. J. Physiol. 133:35.
2. Gray, E. G., and V. P. Whittaker. 1962. J. Anat. 96:79.
3. De Robertis, E., A. Pellegrino de Iraldi, G.
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