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Biochemical Characterization of a Filtered Synaptoneurosome Preparation from Guinea Pig Cerebral Cortex: Cyclic Adenosine 3′:5′-Monophosphate-generating Systems, Receptors, and Enzymes[1]

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Abstract

A particulate preparation was obtained by low speed centrifugation of guinea pig cerebral cortical homogenates prepared with a Krebs-Henseleit buffer. Light microscopic examination, using a reflected light differential interference contrast system, reveals the presence of intact neurons, axonal fragments, glial cells, and erythrocytes along with an abundance of small spherical entities (diameter about 1.1 μm) and snowman-shaped entities (diameter of larger sphere about 1.1 μm, diameter of attached smaller sphere about 0.6 μm). Many unattached smaller spherical entities are also present (diameter about 0.6 μm). Pressure filtration through 5- or 10-μm Millipore filters, followed by low speed centrifugation and resuspension, removes most of the larger entities to afford a suspension composed mainly of the small spherical and snowman-shaped entities. Electron microscopic examination reveals the presence of many synaptosomes with attached resealed postsynaptic entities. It is proposed that these correspond to the snowman-shaped entities to be termed synaptoneurosomes. Accumulations of cyclic AMP elicited by 2-chloroadenosine and histamine, and by combinations of 2-chloroadenosine, histamine, norepinephrine, and forskolin, are lower in filtered than in unfiltered preparations, whereas accumulations elicited by forskolin are unchanged. Levels of adenylate cyclase are reduced by filtration, whereas levels of phosphodiesterase are unchanged. Filtration reduces levels of markers for whole cells and endothelial cells, whereas neuronal markers such as acetylcholinesterase activity and norepinephrine uptake are increased. Levels of S-100 protein, a marker for glial cells, are not significantly decreased. There is no apparent change in the density of many receptors or ion channels. Levels of A_1-adenosine and H_1-histamine receptors are increased, whereas levels of so-called peripheral benzodiazepine-binding sites are decreased.

The sites of formation and physiological function of cyclic AMP in the central nervous system remain obscure more than a decade after the first demonstration of norepinephrine-, histamine-, serotonin-, and adenosine-mediated accumulations of cyclic AMP in brain slices (Kakiuchi and Rall, 1968a, b; Sattin and Rall, 1970). Adenylate cyclases are present at both postsynaptic (dendritic) and presynaptic neuronal loci and on glial and endothelial (capillary) cells (De Robertis et al., 1967; Joo et al., 1975; Weller, 1977; French et al., 1978). All such sites could contribute to generation of cyclic AMP in brain slices. Of particular interest are the synergisms between biogenic amines and adenosine which have been investigated extensively in brain slices with respect to cyclic AMP generation (Daly, 1977). These synergisms are most striking in slices from higher brain regions, namely, cerebral cortex and hippocampus (Daly et al., 1981). Such synergisms do not occur in membrane preparations or in cultured glial or neuronal cells. Recently, however, synergistic interactions of somatostatin and norepinephrine were reported for cultured astrocytes (Rougan et al., 1983). Synergisms between amines and adenosine do occur in a particulate preparation obtained from brain tissue after homogenization in a Krebs-Ringer buffer rather than sucrose (Daly et al., 1980a). These preparations, originally described by Chasin et al. (1974), contain many pinched off and resealed pre- and postsynaptic vesicular entities (Horn and Phillipson, 1976; Daly et al., 1980a). Electron microscopic examination revealed, in addition to various other elements, large numbers of membrane-bound sacs containing mitochondria and small vesicles of various sizes (Daly et al., 1980a). Many of these sacs were attached through a typical synaptic density to an often smaller presynaptic sac clearly identified as a synaptosome by its content of synaptic vesicles. The term synaptoneurosome is suggested for entities in which a presynaptic sac (synaptosome) is attached to a resealed postsynaptic sac (neurosome). It appears possible that the postsynaptic portions of the synaptoneurosomes represent the site of accumulation of cyclic AMP which occurs in these particulate preparations in response to catecholamines, serotonin, adenosine, and vasoactive intestinal peptides (Chasin et al., 1974; Psychoyos, 1978; Daly et al., 1980a, 1982; McNeal et al., 1980; Seamon et al., 1981; Psychoyos et al., 1982a, b). These crude preparations aggregated readily and were, therefore, not amenable to many purification techniques, although a fractional centrifugation has been attempted (Psychoyos et al., 1982a, b). In an effort to size the preparation prior

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to further purification and investigation, homogenates containing the synaptoneurosomes were pressure filtered through 5- or 10-μm Millipore filters. The resulting filtered preparation was found under light microscopic examination to contain a preponderance of small spherical and snowman-shaped entities, the latter thought likely to represent synaptoneurosomes. Cyclic AMP accumulations in response to biogenic amines and adenosine were reduced in the filtered preparation, whereas responses to forskolin, a general activator of adenylate cyclase (Seamon et al., 1981), were not significantly changed. Synergistic responses to combinations of amines, adenosine, and forskolin were reduced. In an effort to determine whether removal of particular cellular entities or a subfraction of synaptoneurosomes might be responsible for the reduction in responsiveness of the cyclic AMP-generating systems, levels of cell marker enzymes, proteins, receptors, and adenylate cyclase and phosphodiesterase were determined.

Materials and Methods

All of the radioactive ligands were from New England Nuclear (Boston, MA). Hydrofluor was from National Diagnostics (Somerville, NJ). *p*-Nitrophenylphosphate and γ-glutamyl-*p*-nitroanilide were from Sigma Chemical Co. (St. Louis, MO). Butyrylthiocholine was from Aldrich Chemical Co., Inc. (Milwaukee, WI). Forskolin was from Calbiochem-Behring Corp. (La Jolla, CA). The 10-μm (LCWP-047) and 5-μm (SMWP-047) filters were from Millipore Corp. (Bedford, MA). All other compounds, salts, and reagents were from standard commercial sources.

Krebs particulates. The particulate preparations were obtained from guinea pig cerebral cortex using a modified Krebs-Henseleit (KRBS) buffer, pH 7.4 (Krebs and Henseleit, 1932), containing adenosine deaminase (10 μg/ml). The buffer consisted of 118.5 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.18 mM KH_2PO_4, 24.9 mM $NaHCO_3$, and 10 mM glucose and was aerated with O_2:CO_2 (95:5) to adjust the pH to 7.4. Brains from one or two male Hartley guinea pigs (250 to 300 gm) were removed immediately following decapitation and placed on an ice-cooled Petri dish, and slices of gray matter were cut manually with a cooled razor blade from the cerebral cortex. The slices (1 gm/brain) were then homogenized by hand (five strokes) in 7 ml of KRBS buffer using a glass-glass homogenizer. The homogenate was diluted with 35 ml of KRBS buffer. For pressure filtration the homogenate was passed through a prefilter consisting of three layers of a nylon material (100 mesh) using a Millipore filter holder and then filtered through a Millipore 10-μm filter (LCWP-047) or a 5-μm filter (SMWP-047). Filtered and unfiltered solutions were centrifuged at 1000 × *g* for 15 min. The supernatant fractions were decanted and the pellets resuspended in 10 ml of KRBS buffer. For certain assays the pellet was resuspended in an appropriate medium as described (see below). Protein was determined by the method of Lowry et al. (1951).

Morphological characterization. The filtered and unfiltered particulate preparations from guinea pig control cerebral cortex were examined by electron microscopy as described previously (Daly et al., 1980a) and by a modification of the reflected light differential interference contrast system first described by Nomarski and Weill (1954) coupled to a video image recorder (Inoue, 1981; Allen and Allen, 1982). The microscope used was a Zeiss photomicroscope II set up for incident light differential interference contrast. The reflective surface was an aluminized coverslip coated by high vacuum sublimation with silicon monoxide and was attached to a standard microscope slide (Hass, 1950) (Evaporated Metal Films Inc., Ithica, NY). The coverslip was siliconized before the specimen was applied. The optics used were a Zeiss Epiplan 100/1.23 objective with a Nomarski prism and a Bausch and Lomb high ultraplane eyepiece in a vertical photo tube. A Sony AVC-3450 black and white video camera was independently supported on a bracket above the microscope. The whole apparatus was isolated from vibration on a pneumatically suspended Ealing optical bench. A Sony AV-8400 receiver recorded the images. Still photographs of images were made with a Canon AE-1 camera held on a rigid mount using 24F Kodak Technical Pan Film. True diameter calibration of images was obtained using photographs of the triple boundary grid lines of a hemacytometer (AO improved Newbauer Hemacytometer, Buffalo, NY). The distance from the inside left grid border to outside right grid border measured directly with a calibrated lens was 5.0 μm and agreed with the manufacturer's specifications. The three intermediate projection lenses, 1.25, 1.60, and 2.0, yielded magnifications of × 4400, 5300, and 6900. Measurements of images from the brain preparation were compared to those of isolated human platelets (Weiss, 1977) and isolated human granulocytes prepared according to the method of Dooley and Takahashi (1981). The measured diameters of these cell types were in complete agreement with literature values (Weiss and Greep, 1977).

Cyclic adenosine monophosphate accumulation in particulate preparations. Pellets from filtered and unfiltered particulate preparations (50 mg of protein) were resuspended in 10 ml of KRBS buffer containing adenosine deaminase (10 μg/ml) and incubated under O_2:CO_2 (95:5) with [^{3}H]adenine (0.1 ml; specific activity, 2.3 to 4.0 Ci/mmol) for 15 min at 37°C followed by one wash in cold KRBS buffer and final resuspension in 25 ml of KRBS buffer. After 30 min postincubation with O_2:CO_2 the preparation was transferred in 1-ml aliquots to scintillation vials. The preparation was allowed to incubate in the vials for 10 min after addition of the various agents. The contents were then transferred to Microfuge tubes and centrifuged for 15 sec in a Brinkman centrifuge. The supernatant was decanted and the pellet was resuspended in 1.2 ml of 6% trichloroacetic acid containing 0.15 μM cyclic AMP. The percentage conversion to [^{3}H]cyclic AMP of the [^{3}H]adenine-labeled nucleotides of the particulate preparation was determined as described by Daly et al. (1980a). This prelabeling technique afforded results comparable to those obtained by measurement of endogenous cyclic AMP. [^{3}H]Cyclic AMP was isolated as described (Salomon et al., 1974).

In certain experiments the total accumulation of radioactive cyclic AMP in the medium and the tissue was measured as follows. Membrane vesicular preparations were prelabeled with [^{3}H]adenine as described above. Aliquots of the membrane suspension (1 ml) were added to scintillation vials and incubated at 37°C for 10 min. Agonists (0.075 ml) were added and the assay was stopped 10 min later with 0.125 ml of 50% trichloroacetic acid containing cyclic AMP (1 mM). Total [^{3}H]cyclic AMP accumulation was then measured as described by Daly et al. (1980a). Parallel with these experiments, cyclic AMP accumulation was measured in the pelleted tissue as described above.

Alkaline phosphatase. Pellets were resuspended in KRBS buffer (10 to 100 μg of protein/200 μl), and alkaline phosphatase activity was assayed as described (Bergmeyer et al., 1974).

γ-Glutamyltranspeptidase. The pellets (~15 mg) were resuspended in 3 ml of a 10 mM Tris buffer (pH 8.6) containing 0.1% Triton X. This tissue was incubated at 0°C for 30 min and then centrifuged at 20,000 × *g* for 45 min (Djuričić and Mršulja, 1977). Solubilized γ-glutamyltranspeptidase activity was assayed as described (Szasz, 1974).

Adenylate cyclase. Pellets (4 to 20 mg of protein) were homogenized in a Dounce homogenizer (7 to 10 strokes) using 5 ml of 50 mM Tris buffer (pH 7.5) containing 0.1 mM $CaCl_2$. The homogenate was centrifuged at 20,000 × *g* for 10 min, then washed once with an additional 5 ml of buffer and recentrifuged. Assay of adenylate cyclase activity was according to the method of Seamon et al. (1981), except that EGTA in the incubation medium was replaced with 1 mM dithiothreitol.

The amount of adenylate cyclase activity that was accessible to exogenous [^{32}P]ATP in the particulate preparation was assayed before and after hypotonic lysis. Adenylate cyclase activity was also measured using the same procedure as described above, except that the assay was performed in a KRBS buffer with the unfiltered and filtered particulate preparations. In another portion, the vesicles were first lysed in 50 mM Tris buffer (pH 7.5) containing 0.1 mM $CaCl_2$, and then enzyme activity was measured. The increase on lysis represented the amount of adenylate cyclase that was present in vesicular entities and was responsible for accumulations of [^{3}H] cyclic AMP in adenine-labeled preparations. The activity prior to lysis represented enzyme in membrane fragments and perhaps inside-out resealed membrane fragments.

Phosphodiesterase. The pellets (2 to 6 mg of protein) were homogenized in 1 ml of 20 mM Tris buffer (pH 7.5) containing 1 mM $MgCl_2$, 3 mM 2-mercaptoethanol, and 0.1 mM EGTA. The homogenates were then centrifuged for 60 min at 100,000 × *g*. The supernatant was decanted and the pellet was resuspended in 1 ml of the above buffer. Assay of phosphodiesterase activity in supernatant and pellet fractions was carried out in 0.1 ml of 50 mM Tris buffer (pH 7.5) containing 5 mM $MgCl_2$ and 50 μM $CaCl_2$, at 37°C for 10 min. Aliquots (10 μl) of supernatant diluted 10-fold and of the resuspended undiluted pellet were assayed for low and high Km enzyme activity. The low Km enzyme reaction mixture contained 1 μM cyclic AMP and 0.56 μCi of [^{3}H]cyclic cAMP. The high Km reaction mixture contained 50 μM cyclic AMP and 1.13 μCi of [^{3}H]cyclic AMP. Blank values were obtained with no addition of enzyme or with boiled enzyme. The reaction was terminated by heating the incubation tubes in a 95°C bath for 3 min. All assays were carried out in triplicate or quadruplicate. The unreacted cyclic AMP was separated from the 5′-AMP product by using a polyacrylamide-boronate affinity gel, Affigel 601 (Bio-Rad Laboratories, Richmond, CA) in a modification of the method of Davis and Daly (1979). Econocolumns (Bio-Rad Laboratories) were prepared with a 0.7-ml bed volume of gel in distilled water. Carrier 5′-AMP (0.2 μmol) was added to each assay tube in 0.4 ml of

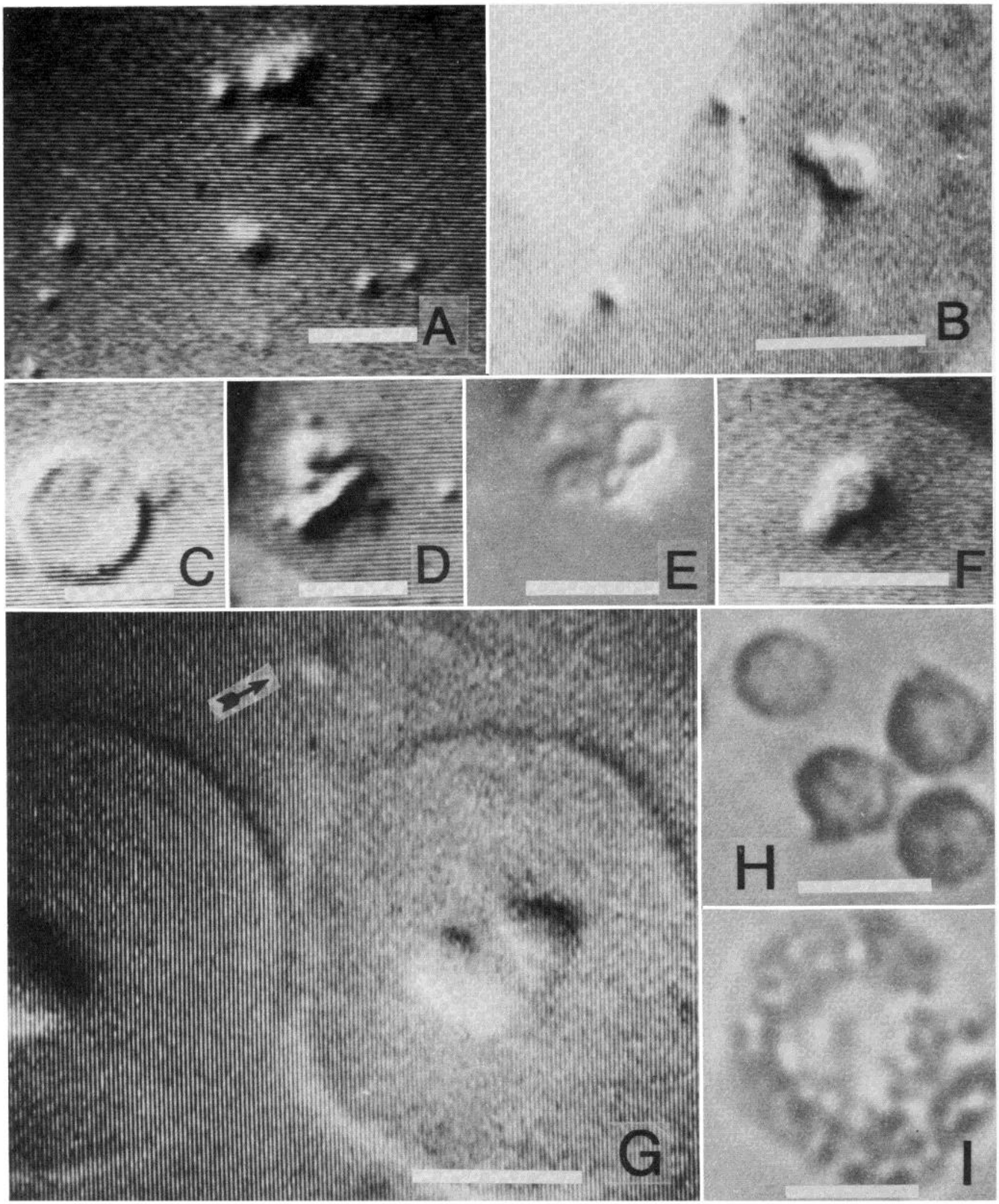

Figure 1. Light microscopic observations of the filtered particulate preparation (*A* to *G*) and reference cells (*H* and *I*). *A*, Appearance of vesicles illustrating the two major populations of vesicles: the 0.6-μm-diameter vesicles and the larger 1.1-μm vesicles. Magnification × 4400. *B*, An example of a vesicle in composite or "snowman" form (synaptoneurosome) and two examples of the smaller vesicles. Occasionally, as seen here, the smaller vesicle has an attached plume, presumably of membranous origin. Magnification × 6900. *C*, Erythrocyte with an adjacent 1.1-μm vesicle. Magnification × 4400. *D*, A typical multivesicular cluster. Magnification × 4400. *E*, A "snowman" figure illustrating the apparent structural unity of two vesicles, phase contrast Nomarksi optics. Magnification × 5300. *F*, A "snowman" figure without the filamental plume (see Fig. 1*B*). Magnification × 6900. *G*, Large, intact cells which may be oligodendrocytes with an adjacent 1-μm vesicle (*arrow*). Magnification × 5300. *H*, Platelets. Magnification × 4400. *I*, Granulocyte. Magnification × 4400. *Bars*, 5 μm.

TABLE I

Dimensions of neurosomes, synaptosomes, and reference cells

Component	Diameter[a] (μM)	Volume[b] (μM^3)	Surface Area[b] (μM^2)	Surface/Volume
Neurosome	1.10 ± 0.5	0.60 ± 0.04	3.4 ± 0.8	5.8
Synaptosome	0.56 ± 0.15	0.30 ± 0.04	2.0 ± 0.1	6.7
Platelets[c]	3.6 ± 0.2	24.8 ± 0.02	41.2 ± 0.1	1.7
Erythrocyte	7.2 ± 0.8			
Granulocyte	12.8 ± 2.0	1096 ± 4	514 ± 13	0.5
Oligodendrocyte[d]	12.6 ± 0.9	1047 ± 4	499 ± 3	0.5
Neuron[e]	14.3; 21.7	3032	1014	0.3

[a] Values are the mean ± SEM for 30 measurements.

[b] The volume and surface areas were estimated from geometric formulas for spheres or elipsoids.

[c] Platelets appeared to be spheres; no semiaxis difference could be distinguished.

[d] Cell type entitled "oligodendrocyte" is illustrated in Figure 1*E*.

[e] The short and long axes of a typical oval-shaped neuron (see Fig. 1) are presented rather than the diameter.

0.1 M HEPES-0.1 M NaCl buffer (pH 8.5). Samples were applied to the columns followed by a wash with 9 ml of HEPES-NaCl buffer (pH 8.5) to elute unreacted cyclic AMP; then 7 ml of 0.05 M sodium acetate buffer (pH 4.8) was applied to the columns to elute 5′-AMP. Recoveries were 50 to 70% and were determined by measuring absorbance at 258 nm. Radioactivity in aliquots (2 ml) of the sodium acetate eluate was measured in 13 ml of Hydrofluor to determine conversion of [^{3}H]cyclic AMP to [^{3}H]-5′-AMP.

Acetylcholinesterase assay. Pellets were homogenized in sucrose (0.04 M) using a Dounce homogenizer to give a homogenate with 3 mg of protein/ml. The pH was adjusted to 7.4 and acetylcholinesterase activity was assayed as described (Siakotos et al., 1969).

Pseudocholinesterase assay. Pellets were homogenized in 0.1 M phosphate buffer (pH 8.0) to give a homogenate with 0.5 to 1.0 mg of protein/ml. Pseudocholinesterase was assayed as described (Ellman et al., 1961).

Receptor-, enzyme-, and channel-binding assays. Pellets were suspended in appropriate medium. Binding assays with ^{3}H-ligands were performed using a filtration technique according to the methods cited for each assay.

α_1-Receptor binding. [^{3}H]WB 4101 (8 nM, 24.7 Ci/mmol) was used with phentolamine (10 μM) to determine nonspecific binding (modification of Greenburg et al., 1976). The incubation was in 50 mM Tris (pH 7.7) at 25°C for 15 min.

α_2-Receptor binding. [^{3}H]Clonidine (5 nM, 20.4 Ci/mmol) was used with phentolamine (10 μM) to determine nonspecific binding (Atlas et al., 1982). The incubation was in 50 mM Tris (pH 7.5) with 2 mM magnesium acetate at 25°C for 40 min.

β-Receptor binding. [^{3}H]Dihydroalprenolol (12 nM, 35.6 Ci/mmol) was used with propranolol (400 μM) to determine nonspecific binding (Skolnick

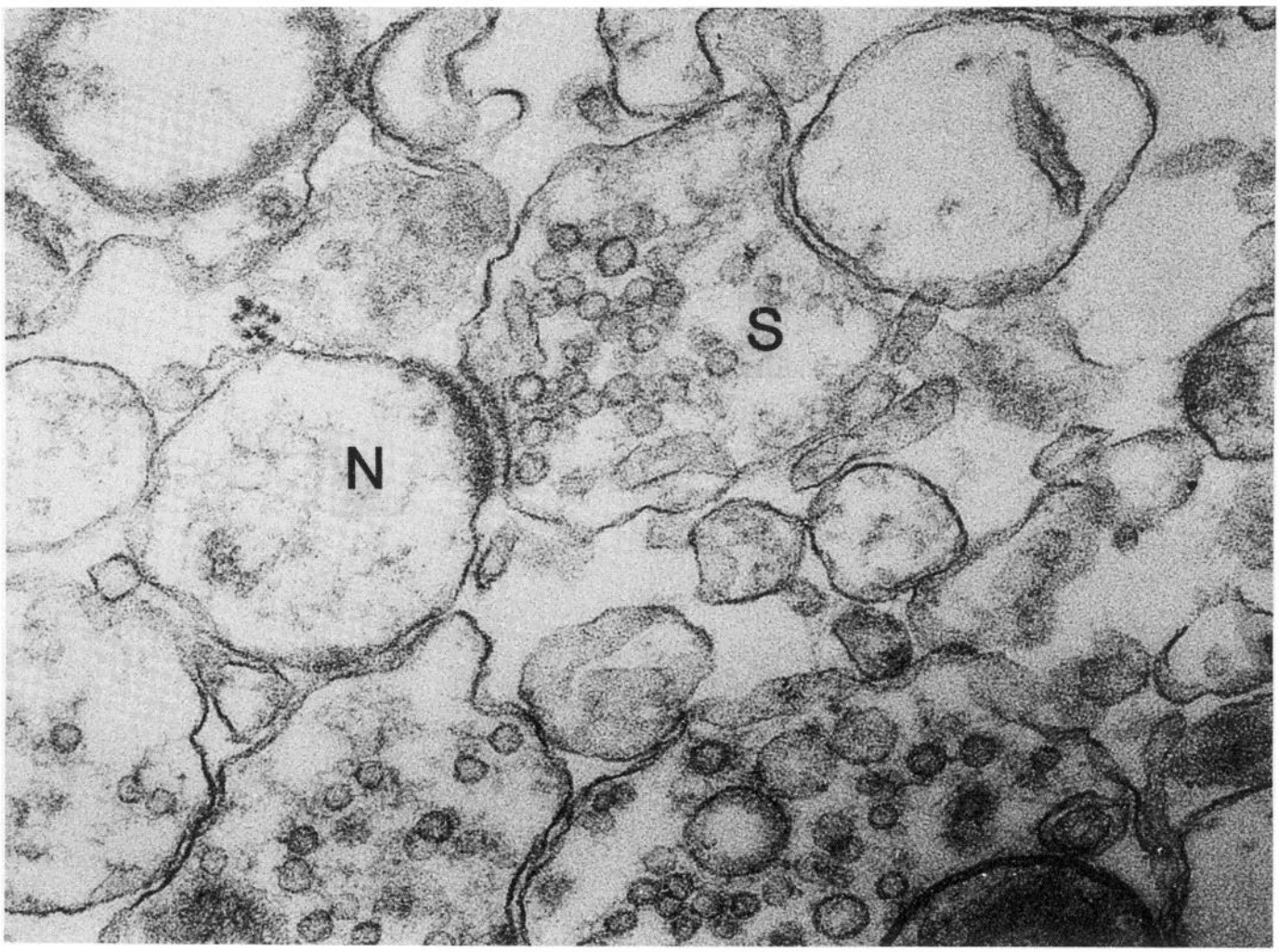

Figure 2. Electron micrograph of the filtered particulate preparation from homogenates of slices of cerebral cortex from guinea pig (see "Materials and Methods"). This section illustrates a typical profile of a synaptoneurosome. The presynaptic profile (*S*) with characteristic presynaptic vesicles is clearly attached at the postsynaptic density to a postsynaptic structure, a neurosome (*N*). Magnification × 96,500. The synaptosome and neurosome portions often appear similar in size, as in this example. In some cases (see Daly et al., 1980a) the neurosome portion appears smaller. This apparent discrepancy with light microscopic analysis could be due to selective shrinkage of the neurosome portion during fixation. The relative size is also dependent on the plane through which the synaptosome and neurosome are cut.

TABLE II

Distribution of visible elements in a filtered preparation[a]

Component	Approximate Diameter (μm)	Number/100 Fields
Synaptosomes	0.6	252
Neurosomes	1.0	67
Synaptoneurosomes		
Simple forms	1.6	106
Multiple forms	>1.6	35
Large Vesicles	>2	10
Oligodendrocytes	12	7
Erythrocytes	7	7
Unidentified		6

[a] One hundred frames were examined from a suspension of a filtered preparation containing 1 mg of protein/ml of KRBS buffer.

TABLE III

Accumulations of [^{3}H]cyclic AMP elicited by various agents in unfiltered adenine-labeled particulate preparations: Effects of preparation and incubation in two different Krebs buffers

Agent (μM)	[^{3}H]Cyclic AMP (% conversion)	
	A Krebs-Ringer	*B* Krebs-Henseleit
None	0.25 ± 0.02	0.41 ± 0.06
Histamine (100)	2.30 ± 0.92	4.06 ± 0.36
2-Chloroadenosine (100)	3.05 ± 0.64	6.00 ± 0.63
plus norepinephrine (100)	5.72 ± 1.13	9.54 ± 0.44
plus histamine (100)	5.75 ± 1.01	14.36 ± 3.03
Norepinephrine (100)	0.30 ± 0.03	0.60 ± 0.04
plus histamine (100)	2.94 ± 0.73	6.25 ± 0.45

[^{3}H]Adenine-labeled particulate preparations were isolated and incubated in (*A*) a Krebs-Ringer bicarbonate glucose buffer (pH 7.4) prepared according to the method of Daly et al. (1980a), or (*B*) a Krebs-Henseleit bicarbonate glucose buffer (pH 7.4) prepared according to the method of Psychoyos et al. (1982a) containing 4.7 mM potassium and 2.5 mM calcium ions. Incubations with agents were for 10 min, after which [^{3}H]cyclic AMP was determined as described under "Materials and Methods." Values are ±SEM for three or four experiments.

et al., 1977). Incubations were in 50 mM Tris (pH 7.5) containing 0.3 M NaCl at 37°C for 30 min.

H_1-Histamine receptor binding. [^{3}H]Mepyramine (6 nM, 20 Ci/mmol) was used with chlorpheniramine (10 μM) to determine nonspecific binding (Hill et al., 1978). Incubations were in 50 mM Na^+/K^+ phosphate buffer (pH 7.5) at 30°C for 60 min.

H_2-Histamine receptor binding. [^{3}H]Tiotidine (2 nM, 79.5 Ci/mmol) was used with cimetidine (10 μM) to determine nonspecific binding (modification of Gajtkowski et al., 1983). Incubations were in 50 mM Na^+/K^+ phosphate buffer (pH 7.5) at 30°C for 30 min.

A_1-Adenosine receptor. [^{3}H]Cyclohexyladenosine (1 nM, 11.4 to 25.0 Ci/mmol) was used with 2-chloroadenosine (10 μM) to determine nonspecific binding (Bruns et al., 1980). Incubations were in 50 mM Tris (pH 7.7) at 25°C for 60 min.

Serotonin uptake site. [^{3}H]Imipramine (5 nM, 75 Ci/mmol) was used with buffer lacking sodium ion to determine nonspecific binding (Rehavi et al., 1983). Incubations were in 50 mM Tris (pH 7.4) containing 5 mM KCl with and without 120 mM NaCl at 0°C for 60 min.

Central benzodiazepine site. [^{3}H]Diazepam (2 nM, 85.3 Ci/mmol) was used with clonazepam (1 μM) to determine nonspecific binding (Squires and Braestrup, 1977). Incubations were in 50 mM Tris (pH 7.4) at 0°C for 45 min.

Peripheral benzodiazepine site. [^{3}H]Ro 5-4864 (5 nM, 76.9 Ci/mmol) was used with Ro 5-4864 (1 μM) to determine nonspecific binding (Schoemaker et al., 1981). Incubations were in 50 mM Tris (pH 7.4) at 0°C for 90 min.

Opiate-binding site. [^{3}H]Naloxone (5 nM, 34.3 Ci/mmol) was used with morphine (1 μM) to determine nonspecific binding (modification of Nagamatsu et al., 1982). Incubations were in 50 mM Tris-HCl (pH 7.4) at 25°C for 30 min.

Na^+/K^+ ATPase site. [^{3}H]Ouabain (1 μM, 18 Ci/mmol) was used with buffer lacking $MgCl_2$ and ATP to determine nonspecific binding (Swann et al., 1981). Incubations were in 50 mM Tris-HCl (pH 7.5) with and without 2 mM ATP and 2 mM $MgCl_2$ at 37°C for 60 min.

Calcium channel site. [^{3}H]Nitrendipine (1 nM, 70 Ci/mmol) was used with nifedipine (1 μM) to determine nonspecific binding (modification of Marangos et al., 1982). Incubations were in 50 mM Tris (pH 7.4) at 25°C for 30 min.

Voltage-dependent sodium channel site. [^{3}H]Batrachotoxinin-B benzoate (50 to 200 nM, 14 Ci/mmol) was used with 300 μM veratridine to determine nonspecific binding (Creveling et al., 1982). Incubations were in 50 mM HEPES buffer (pH 7.4) containing 130 mM choline chloride, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 5.4 mM KCl at 37°C for 30 min.

Norepinephrine uptake. The active uptake of [^{3}H]norepinephrine was determined by the following procedure. Aliquots of resuspended pellets (0.3 ml of KRBS buffer) containing 0.2 to 0.8 mg of protein were preincubated at 37°C or 0°C for 3 min in 12 × 75 mm glass test tubes. Uptake was initiated by the addition of [^{3}H]norepinephrine (0.01 ml) to give a final concentration of 0.1 μM. Uptake was stopped after 5 min by diluting this reaction mixture with ice-cold phosphate-buffered saline (pH 7.4) (PBS) followed by rapid, vacuum-assisted filtration through a glass microfiber filter (Whatman GF/C, 2.4 cm), and then by three rapid washes with cold phosphate-buffered saline (3 ml). Filters were removed and shaken in counting vials containing scintillation fluid (8 ml, Hydrofluor), and the tritium content was determined with a counting efficiency of 37%. Active uptake was defined by subtracting the tritium accumulated at 0°C from the accumulation at 37°C. The accumulation at 0°C was 12.2 ± 2.8% (SEM) of the accumulation at 37°C. The accumulations of [^{3}H]norepinephrine at 0°C and 37°C were linear from 1 to 9 min and at protein concentrations from 0.2 to 0.8 mg. In practice the reaction mixtures contained 1.5 μCi of isotope of which less than 1% was retained by the glass filter. Uptake values are expressed as picomoles per minute per milligram of protein ± SEM for four to six determinations.

S-100 radioimmunoassay. S-100 protein was iodinated using $^{125}I_2$ (>350 mCi/ml). The reaction mixture contained 0.1 M sodium phosphate (pH 7.1, 50 μl), $^{125}I_2$ (20 μl, >350 mCi/ml), S-100 protein (1 mg/ml of H_2O, 5 μl), and chloramine T (1 mg/ml of H_2O, 10 μl). The reaction was stopped 1 min later using sodium metabisulfite (1 mg/ml, 30 μl) and KI (0.1 M, 100 μl). The mixture was desalted on a PD 10 column (Pharmacia Fine Chemicals, Uppsala, Sweden) which had been pre-equilibrated with PBS containing 0.05% Triton X.

Pellets were homogenized in 5 mM Tris buffer and centrifuged at 40,000 × *g*. The solubilized S-100 protein in the supernatant was then assayed. The assay mixture consisted of S-100 (1 mg/ml of H_2O or 50 μl of the supernatant), rabbit anti-S-100 serum (200 μl of a 1:800 dilution in PBS), 50 μl of [^{125}I]S-100 (~70,000 cpm). After incubation for 24 hr at 4°C the antigen/antibody complexes were separated from free antigen by the double antibody technique using goat anti-rabbit-γ-globulin serum. All samples were run in duplicate. Controls containing 250 μl of PBS plus [^{125}I]S-100 were also included (Uozumi and Ryan, 1973). A radioimmunoassay standard curve was determined with the S-100 protein and used to calculate the amount of S-100 in the brain preparations. The bovine brain S-100 and antiserum against bovine brain S-100 were generously provided by Dr. Blake Moore (Washington University, St. Louis, MO).

DNA assay. Pellets were homogenized in water with a Dounce homogenizer to yield 11 mg of protein/ml. DNA was measured according to the method of Dische (1955).

Results

Morphological characterization. The availability of a light microscopic system permitting real-time magnification of × 4400 to × 6900 prompted a re-examination of the components present in the unfiltered 1000 × *g* particulate preparation: the preparation contains intact neurons, axonal fragments, dendritic processes, glial cells, erythrocytes, cell nuclei, unidentified whole cells, large empty spherical sacs, myelin fragments, and many large clumps of unidentifiable "debris." The presence of these components in this low speed sediment of a crude homogenate is not surprising and is in agreement with earlier descriptions (see Chasin et al., 1974; Horn and Phillipson, 1976; Daly et al., 1980a; Psychoyos et al., 1982a). The presence of intact cells in the preparation was not clearly apparent in our previous studies (Daly et al., 1980a) and those of others due primarily to the sampling limitations inherent in electron microscopy.

A significant observation resulting from the real-time visualization of unfixed preparations is the detection of an abundance of small, spherical bodies, to which in many cases a second, smaller spherical body is attached, giving a snowman appearance (Fig. 1, *B*, *E*, and *F*). Since the preparation was observed while suspended in buffer, these smaller bodies are in constant motion and thus can be

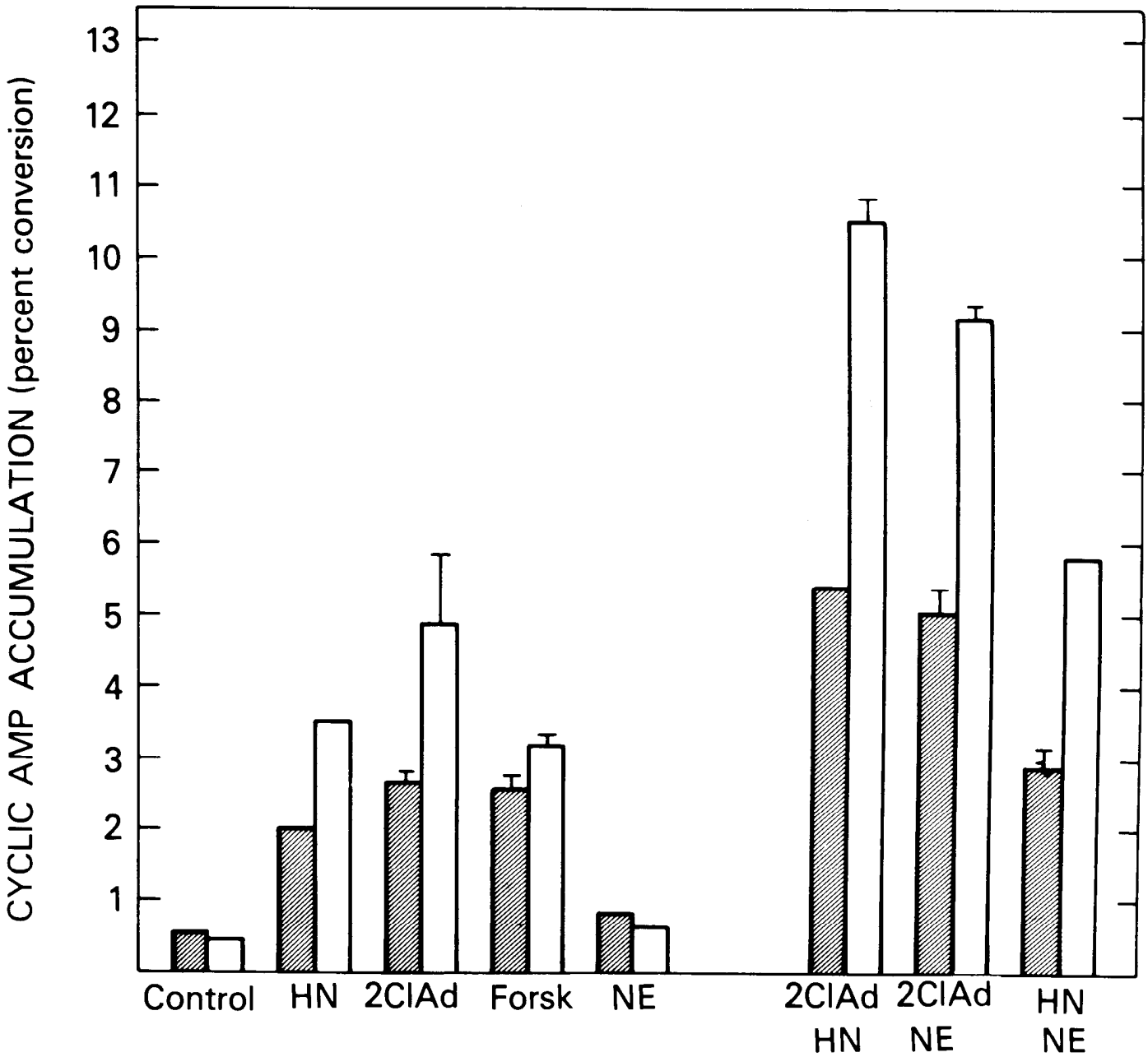

Figure 3. [^{3}H]Cyclic AMP accumulation (percentage of conversion) in filtered (*hatched bar*) and unfiltered (*open bar*) particulate preparations in the presence of various agents (100 μM). histamine (*HN*), 2-chloroadenosine (*2ClAd*), norepinephrine (*NE*), and forskolin (*Forsk*). Accumulations of [^{3}H]cyclic AMP were measured as described under "Materials and Methods." Values are means ± SEM (n = 3) of a typical experiment. *Error bars* are not shown when too small to depict.

observed on all sides as they pass through the visual field. They are clearly spherical and seem to consist of two distinct populations: the more numerous spheres, with a mean diameter of 0.6 μm, and the second group, having an apparent mean diameter of 1.1 μm (Table I). Measurements made from photographs of the video tape confirmed our initial impression that these two groups consist of sacs which are very nearly uniform in size. Although the snowman configuration, illustrated in Figure 1, *B*, *E*, and *F*, is the most numerous of the various complex forms of such small entities, many of the 1-μm sacs had attachments of two, three, and sometimes more of the smaller sacs (see Fig. 1, *A* and *D*). These various forms are firmly attached to one another, and the large and small parts cannot be separated by physically disturbing the suspension or by increasing the ionic strength of the suspension to approximately 400 mM by adding NaCl or KCl solutions. Some of the larger spherical vesicles (1.1 μm) do not have smaller vesicles attached to them. The relatively small and uniform size of the vesicular entities immediately prompted attempts to purify the preparation by filtration. Diluted suspensions of the crude 1000 × g × 15 min sediment were passed through various nylon mesh cloth and controlled pore filters ranging from 3 to 10 μm pore sizes. The most satisfactory procedure consisted of a preliminary filtration of homogenates or resuspended particulate preparations through a nylon mesh (100 mesh) and then through 5- or 10-μm Millipore filters (see "Materials and Methods"). Following this filtration step the visible components of the preparation consist almost entirely of small spherical and snowman-shaped bodies (Fig. 2*B*). Only occasional erythrocytes and what appear to be intact oligodendrocytes are observed (Fig. 1, *C* and *G*). Apparent dimensions of the various entities and of reference cells are in Table I. The relative abundance of simple (spherical) and multiple (snowman-shaped) forms and other elements are given in Table II.

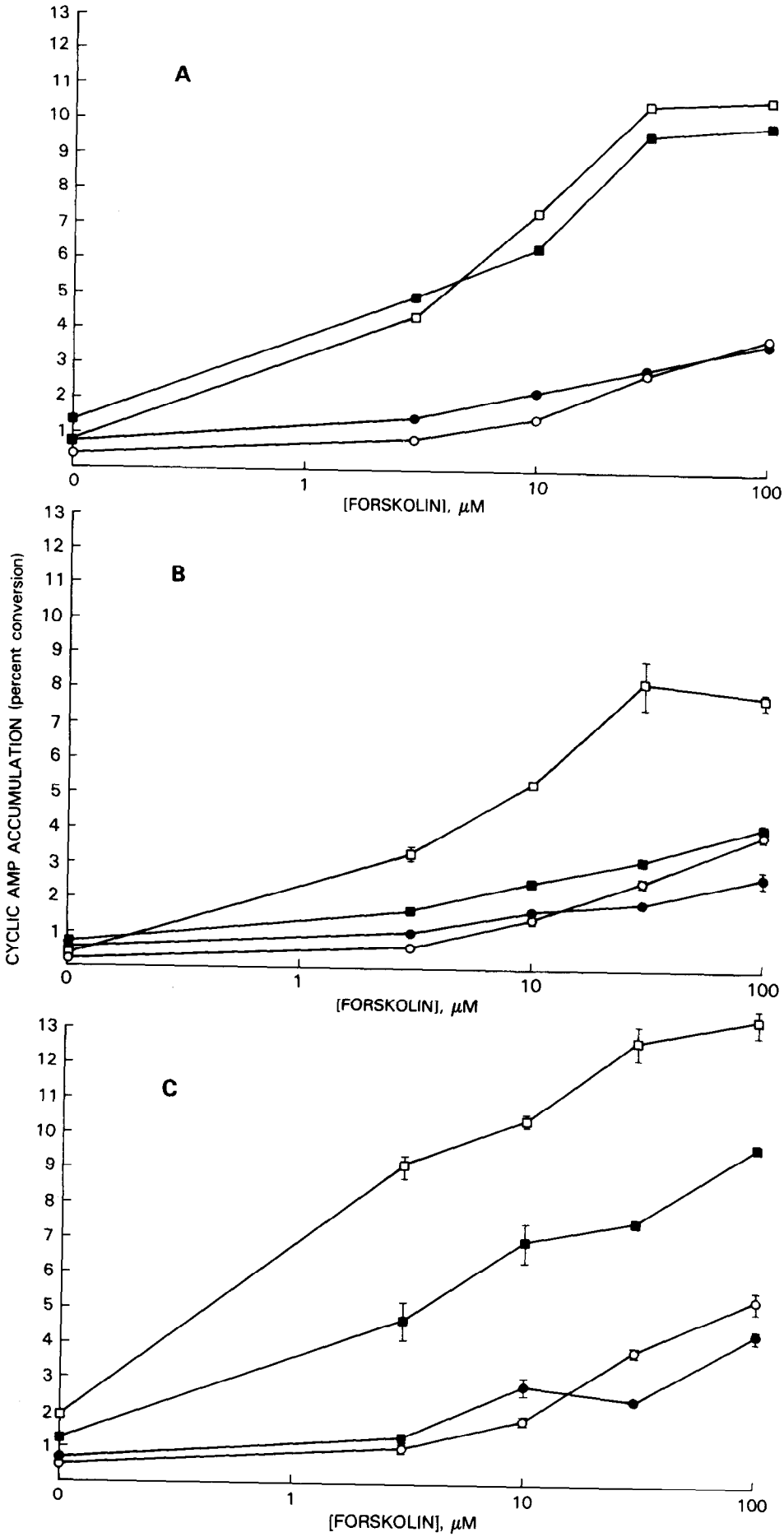

Figure 4. Dose-response relationships for agonist-induced [^{3}H]cyclic AMP accumulations in [^{3}H]adenine-labeled particulate preparations from guinea pig cerebral cortex. The figure shows the effect of forskolin (0.1 to 100 μM) alone in the filtered preparation (●) and in the unfiltered preparation (○) and in the presence of the following: *A*, histamine (2 μM), filtered (■) and unfiltered (□); *B*, 2-chloroadenosine (2 μM), filtered (■) and unfiltered (□); *C*, histamine (2 μM) plus 2-chloroadenosine (2 μM), filtered (■) and unfiltered (□). Results are means ± SEM of a typical experiment. *Error bars* are not shown where they are smaller than the *symbol*.

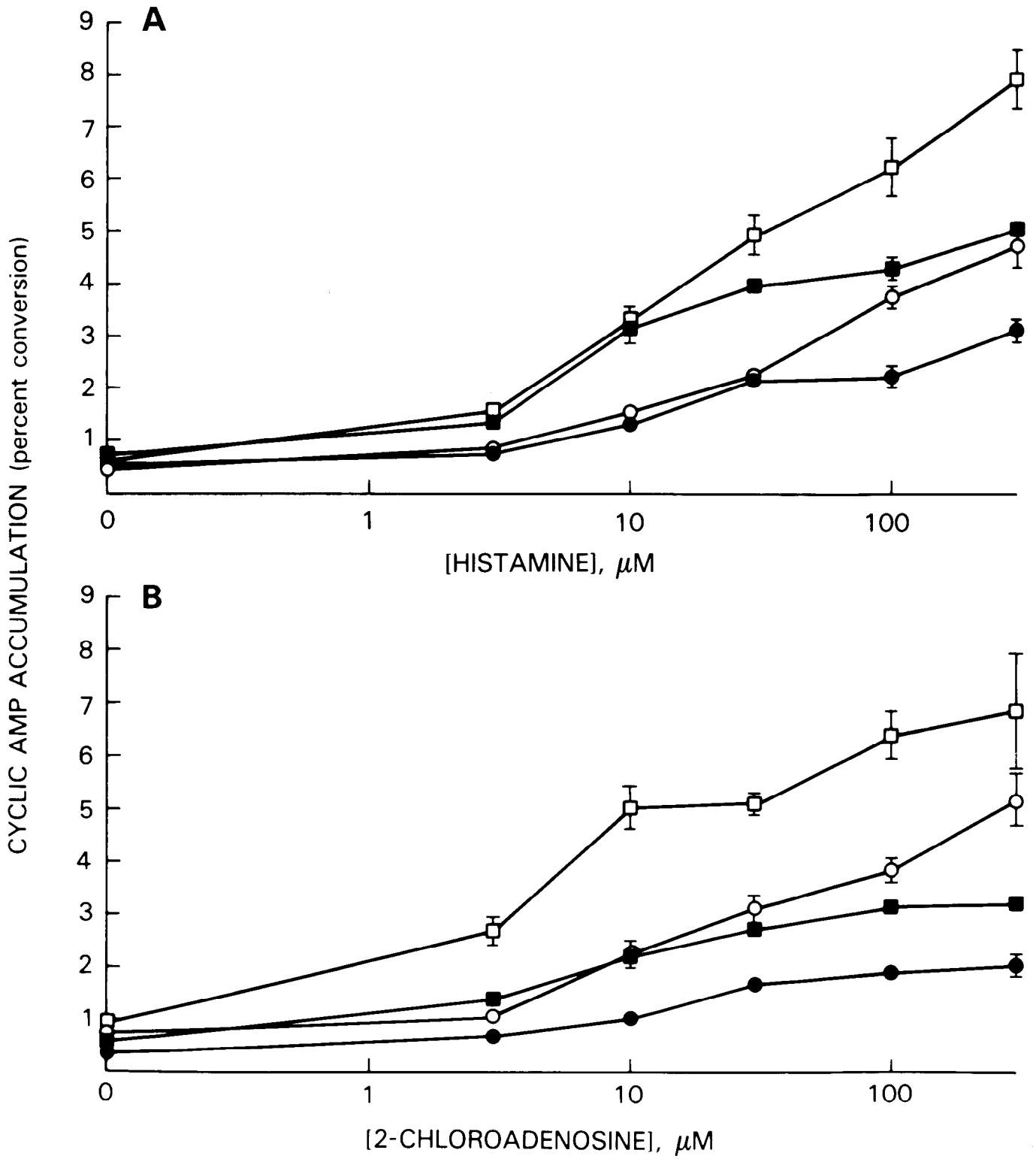

Figure 5. Dose-response relationships for histamine- and 2-chloroadenosine-elicited accumulations of [^{3}H]cyclic AMP in [^{3}H]adenine-labeled particulate preparations from guinea pig cerebral cortex in the presence or absence of forskolin (1 μM). Histamine (*A*) or 2-chloroadenosine (*B*) results are shown for filtered (●) and unfiltered (○) conditions. Histamine (*A*) plus forskolin or 2-chloroadenosine (*B*) plus forskolin are shown for filtered (■) and unfiltered (□) conditions. Results are expressed as means ± SEM of a typical experiment. *Error bars* are not shown where they are smaller than the *symbol*.

Electron microscopic examination of filtered preparations reveals the presence of free mitochondria, granular endoplasmic reticulum, small clusters of ribosomes, small sacs of unknown nature, many presynaptic sacs (synaptosomes), and the presynaptic sacs attached to postsynaptic membrane-bound sacs (see Fig. 2). The latter appear to correspond to those previously observed by electron microscopy in the unfiltered preparation (Daly et al., 1980a). It would seem appropriate to call the postsynaptic membrane-bound sac a neurosome since it is derived from the postsynaptic neuron. The composite entities, consisting of a neurosome with one or more presynaptic bodies (synaptosomes) attached to it, would then be termed a synaptoneurosome.

TABLE IV

Comparison of ^{3}H-ligand binding in membranes from guinea pig cerebral cortex particulate preparations

Binding studies were performed as described under "Materials and Methods." All experiments were performed in triplicate or quadriplicate and repeated three to six times on different preparations.

Binding Site or Receptor	Ligand	Concentration	Unfiltered Preparation	Filtered Preparation
		nM	*fmol/mg of protein*	
Adenosine (A_1)	[^{3}H]Cyclohexyladenosine	1	33 ± 4	71 ± 3[a]
α_1-Adrenergic	[^{3}H]WB 4101	8	142 ± 21	132 ± 11
α_2-Adrenergic	[^{3}H]Clonidine	5	78 ± 12	76 ± 11
β-Adrenergic	[^{3}H]Dihydroalprenolol	12	205 ± 24	194 ± 24
Histamine (H_1)	[^{3}H]Mepyramine	6	236 ± 35	332 ± 20[b]
Histamine (H_2)	[^{3}H]Tiotidine	2	26 ± 5	20 ± 1
Serotonin Uptake	[^{3}H]Imipramine	5	42 ± 5	45 ± 5
Central Benzodiazepine	[^{3}H]Diazepam	2	117 ± 38	94 ± 34
Peripheral Benzodiazepine	[^{3}H]Ro 5-4864	5	286 ± 31	174 ± 22[a]
Opiate	[^{3}H]Naloxone	5	92 ± 12	76 ± 12
Na^+/K^+ ATPase	[^{3}H]Ouabain	1000	21,000 ± 3,000	24,000 ± 6,000
Ca^{2+} Channel	[^{3}H]Nitrendipine	1	229 ± 80	224 ± 75
Na^+ Channel	[^{3}H]Batrachotoxin-B benzoate	5–200	3,620 ± 500[c]	3,960 ± 500[c]

[a] $p < 0.05$.
[b] $p < 0.1$.
[c] B_{max} value.

TABLE V

Comparison of endothelial, neuronal, glial, and whole cell marker concentrations in unfiltered versus filtered particulate preparations from guinea pig cerebral cortex

Cell marker concentrations were measured as described under "Materials and Methods" in the unfiltered and filtered preparations. Results are means ± SEM of two to four experiments on different preparations expressed in milligrams of protein.

Cell Type	Marker	Unfiltered	Filtered
Endothelial cells	γ-Glutamyltranspeptidase (nmol/mg/min)	47 ± 12	27 ± 8
	Alkaline Phosphatase (nmol/mg/min)	49 ± 18	11 ± 1[a]
	Pseudocholinesterase (nmol/mg/min)	0.13 ± 0.02	0.15 ± 0.03
Neurons	Acetylcholinesterase (nmol/mg/min)	15 ± 1	20.0 ± 2[a]
	Norepinephrine Uptake (pmol/mg/min)	0.25 ± 0.02	0.43 ± 0.02[b]
Glia	S-100 Protein (ng/mg)	180 ± 33	150 ± 35
Whole cell or nuclei	DNA (mmol/mg)	40 ± 4	20 ± 3[b]

[a] $p < 0.1$.
[b] $p < 0.05$.

Accumulation of cyclic AMP in filtered and unfiltered preparations. Accumulations of [^{3}H]cyclic AMP elicited by adenosine, norepinephrine, and histamine in [^{3}H]adenine-labeled vesicular preparations decreased with time (McNeal et al., 1980). It appeared that low levels of endogenous adenosine resulted in a slow loss of receptor-induced cyclic AMP production and that desensitization could be prevented by the presence of exogenous adenosine deaminase. Adenosine deaminase (10 μg/ml) was therefore used in our present studies to eliminate any effects of endogenous adenosine.

Using a modified Krebs-Henseleit buffer, decreasing the amount of homogenization, assuring adequate mixing, and increasing the ratio of surface to incubation volume was recently reported by Psychoyos et al. (1982a) to result in greatly enhanced accumulations of cyclic AMP in vesicular preparations in response to various agents. A comparison of results obtained with the Krebs-Ringer buffer used in prior studies (Chasin et al., 1974; Daly et al., 1980a) and those with Krebs-Henseleit buffer are given in Table III for the unfiltered preparations. Because of the somewhat greater responsiveness in the latter, Krebs-Henseleit buffer was used in subsequent comparisons of filtered and unfiltered preparations. A comparison of different agonist-induced accumulations of [^{3}H]cyclic AMP in the unfiltered and filtered preparations reveal the expected synergisms with norepinephrine plus histamine, and norepinephrine plus 2-chloroadenosine (Fig. 3). The filtered preparations are not as responsive as the unfiltered preparations, and synergisms are reduced but still present.

TABLE VI

Adenylate cyclase activity in unfiltered and filtered particulate preparations from guinea pig cerebral cortex

The particulate preparations were obtained as described under "Materials and Methods" and adenylate cyclase activity was assayed with 0.025 mM ATP containing 0.5 μCi of [α-^{32}P]ATP at 30°C for 10 min in the presence of various concentrations of forskolin. The EC_{50} value in the unfiltered preparation for forskolin is 0.45 μM and in the filtered preparation is 0.52 μM. Results are expressed as mean ± SEM for three experiments.

	Adenylate Cyclase Activity (pmol/mg/min)	
	Unfiltered	Filtered
Basal	117.2 ± 9.7	79.2 ± 4.5[a]
Forskolin		
0.1 μM	153.1 ± 31.2	95.8 ± 10.9
1.0 μM	189.9 ± 19.8	115.6 ± 5.2[a]
10 μM	230.8 ± 26.3	135.2 ± 5.9[a]
100 μM	232.4 ± 22.6	139.9 ± 4.5[a]

[a] $p < 0.025$.

Forskolin, a direct activator for the catalytic subunit of adenylate cyclase (Seamon and Daly, 1981) stimulates radioactive cyclic AMP accumulation in a dose-responsive manner in both the unfiltered and filtered preparations (Figs. 4 and 5). Combinations of a low concentration of histamine (2 μM) with varying concentrations of forskolin result in a greater than additive response in both filtered

TABLE VII

Comparison of filtered and unfiltered particulate preparations with a P_2 synaptosomal fraction from guinea pig cerebral cortex

Particulate preparations were obtained as described under "Materials and Methods." The P_2 synaptosomal pellet was prepared in 0.32 M sucrose (0.3 gm/4 ml) by homogenizing the cerebral cortical tissue using a Teflon pestle and then centrifuging the homogenate at 1,000 × *g*. The supernatant was then centrifuged at 25,000 × *g* and the resulting pellet was assayed. The assays are described under "Materials and Methods." The results are expressed as means ± SEM of three determinations in a single experiment except those designated with a superscript *a*, which are means ± SEM of two to three separate experiments.

	Brain Preparation		
	Unfiltered Preparation	Filtered Preparation	P_2 Fraction
[^{3}H]Cyclic AMP Accumulation (multiples of basal)			
Agonist			
2-Chloroadenosine	13.5	5.4	1.7
Histamine	9.1	4.3	1.5
Norepinephrine	1.4	1.5	0.8
2-Chloroadenosine plus Histamine	27.0	10.7	1.6
2-Chloroadenosine plus Norepinephrine	21.0	7.9	1.9
Histamine plus Norepinephrine	11.7	4.0	1.3
Enzymes and Receptors			
Acetylcholinesterase (nmol/min/mg of protein)	15.7		19.5
Pseudocholinesterase (nmol/min/mg of protein)	0.13 ± 0.02[a]	0.15 ± 0.03[a]	0.07 ± 0.01[a]
β-Adrenergic Receptor (fmol/mg of protein)	208 ± 16		136 ± 4
Histamine (H_2) Receptor (fmol/mg of protein)	26 ± 5[a]	20 ± 0.9[a]	18.5 ± 0.2[a]
Adenosine (A_1) Receptor (fmol/mg protein)	43 ± 1	59 ± 3	47 ± 1

and unfiltered preparations (Fig. 4*A*). Combinations of a low concentration of 2-chloroadenosine (2 μM) with varying concentrations of forskolin also result in more than additive responses (Fig. 4*B*). At 100 μM forskolin, the presence of 2 μM 2-chloroadenosine increases the accumulation of cyclic AMP above basal by 30-fold in the unfiltered preparation, whereas in the filtered preparation the presence of 2 μM 2-chloroadenosine produces only an 8-fold increase. Combinations of both 2 μM 2-chloroadenosine and 2 μM histamine with varying concentrations of forskolin result in a slightly greater than additive response (Fig. 4*C*).

Histamine causes a dose-dependent increase in cyclic AMP in both unfiltered and filtered preparations (Fig. 5*A*). Histamine in the range of 3 to 30 μM is equally potent and efficacious in stimulating cyclic AMP accumulation in the unfiltered and filtered preparations. At higher concentrations (100, 300 μM), the response to histamine is greater in the unfiltered preparation. Forskolin at low concentration (1 μM) augments the response to 300 μM histamine by about 1.7-fold in both the unfiltered and filtered preparations. 2-Chloroadenosine (10 to 300 μM) elicits a lower response in the filtered preparation both alone and when augmented by a low concentration of forskolin (Fig. 5*B*).

The decrease in the accumulation of cyclic AMP evoked by certain agents in the filtered preparation does not appear to be a time-related effect (data not shown). Steady-state levels are reached in both preparations at 10 min. The unfiltered preparation at all time points produces larger responses than does the filtered preparation.

The decrease in cyclic AMP accumulations is not due to extrusion or loss of cyclic AMP from vesicular entities in the filtered preparation. In filtered tissue, 2-chloroadenosine evokes a 3.0 ± 0.4% conversion of the labeled nucleotides to [^{3}H]cyclic AMP when the [^{3}H]cyclic AMP content of the pellet is measured. When total [^{3}H]cyclic AMP accumulation is measured (medium plus tissue), the percentage of conversion is 3.4 ± 0.1. The values for pellet and total for histamine stimulation are 1.9 ± 0.3 and 1.6 ± 0.1, respectively, while values for the combination of 2-chloroadenosine and histamine are 5.0 ± 0.2 and 5.2 ± 0.2, respectively.

Apparent densities of receptors, uptake sites, channels, enzymes, and proteins. Filtration does not markedly alter density for many of the central nervous system receptors (Table IV). The density of H_1-histamine receptors, measured with [^{3}H]mepyramine, increases slightly in the filtered preparation (Hill et al., 1978). The density of A_1-adenosine receptors, measured with [^{3}H]cyclohexyladenosine (Bruns et al., 1980), increases nearly 2-fold in the filtered preparation. The so-called "peripheral" benzodiazepine-binding sites were measured using [^{3}H]Ro 5-4864, a specific agonist (Schoemaker et al., 1981): the apparent density decreases about 50% after filtration. The B_{max} values, obtained from a Scatchard analysis of the binding sites for [^{3}H]batrachotoxinin-B benzoate, an Na^+-channel marker (Creveling et al., 1982), are similar in the unfiltered and filtered preparations. The apparent K_d values for filtered and unfiltered tissue are 164 ± 14 nM and 137 ± 13 nM, respectively. It has been assumed in this study that filtration will not alter the K_d values of the other ligands for their binding sites and, therefore, that binding at a single ligand concentration will provide an estimate of receptor density.

Endothelial enzyme markers such as alkaline phosphatase and γ-glutamyltranspeptidase (Spatz, 1982) decrease in activity after filtration (Table V). However, activity of another purported endothelial enzyme marker, pseudocholinesterase (Mršulja and Djuričić, 1980), remains constant. Acetylcholinesterase, a neuronal marker enzyme (Mršulja and Djuričić, 1980), increases in activity after being filtered by approximately 25%. There is only a 20% decrease in the amount of soluble S-100 protein, a purported glial marker (Moore, 1972), after filtration (Table V).

The DNA concentration in the unfiltered and filtered preparations was assayed as a marker of whole cell or nuclear contamination (Table V). There is a decrease of 50% in the filtered preparation when compared to the unfiltered preparation. Light microscopy of the unfiltered preparation had shown significant numbers of whole cells (see above). The decrease in DNA content upon filtration is consonant with removal of many of these cells. The remaining DNA detected in the filtered preparation may be free or adsorbed DNA or nuclei, since light microscopic examination indicates that nearly all of the whole cells are removed during filtration (Table II).

Basal levels of adenylate cyclase in the filtered preparation are lower than the unfiltered preparation (Table VI). Forskolin (10 μM) increases the enzyme activity by 1.7-fold in the filtered preparation and by 1.9-fold in the unfiltered preparation. The relative amounts of intravesicular adenylate cyclase and of membrane or inside-out vesicular adenylate cyclase appear similar in filtered and unfiltered particulate preparations (data not shown): there was a 60% increase in adenylate cyclase after lysis in both preparations. The intravesicular adenylate cyclase, which is responsible for accumulations of [^{3}H]cyclic AMP in [^{3}H]adenine-labeled preparations, was equated with the increase in activity obtained upon lysis of the vesicular entities. Phosphodiesterase activity, both soluble and membrane bound, measured at low and high concentrations of cyclic AMP were similar in filtered and unfiltered preparations. In both cases the low K_m activity was about 500 pmol/mg of protein/min in the particulate and about 2,000 in the supernatant, whereas high K_m activity was about 5,000 in the particulate and about 25,000 to 30,000 in the supernatant (data not shown).

Comparisons were made with the filtered and unfiltered particulate preparations and the "classical" P_2-synaptosomal fraction (Table VII). The accumulation of cyclic AMP elicited by the different agents is dramatically lower in the P_2 pellet, and the synergisms produced by

combinations of the agonists are nonexistent. Thus, it would appear that the P_2-synaptosome fraction contains much lower levels of the vesicular entities in which various agents elicit accumulations of cyclic AMP. Acetylcholinesterase activity is somewhat higher in the P_2 pellet compared to the unfiltered tissue, whereas pseudocholinesterase activity is slightly lower. β-Adrenergic and H_1-histamine receptor densities are lower in the P_2 fraction, but A_1-adenosine receptor densities are about the same in the P_2 fraction and the unfiltered particulate tissue.

Discussion

Morphological characterization. Previous descriptions of the components present in a low speed sediment fraction of brain tissue homogenized in Krebs buffer indicate that a major component of this sediment consists of membrane fragments resealed to form a variety of pleomorphic vesicles (Chasin et al., 1974; Horn and Phillipson, 1976; Daly et al., 1980a, b; Psychoyos et al., 1982a). These vesicles include synaptosomes and synaptosomes attached to postsynaptic membranes which have resealed to form composite vesicles or clusters of vesicles. Our present observations suggest that the small vesicles, those which pass through a 5- to 10-μm filter, represent two distinct populations with diameters of approximately 1 and 0.6 μm (Fig. 1, Tables I and II). These vesicles may be the same as the small vesicles, 0.3 to 0.6 μm, observed by scanning electron microscopy (Psychoyos et al., 1982a) and the 0.1- to 0.8-μm vesicles observed by transmission electron microscopy (Horn and Phillipson, 1976). The smaller size may result from the effects of fixation, dehydration, and embedding and, in the case of transmission electron microscopy, the fact that a given section will not always pass through the maximum diameter of a vesicle. Transmission electron microscopy of the present unfiltered and filtered particulate preparations not only reveals classical synaptosomes but synaptosomes which are clearly attached at sites of postsynaptic densities to another resealed vesicle (Fig. 2). This composite entity is probably identical with the many "snowman"-shaped vesicles observed by the light microscopic system (Fig. 1). By light microscopy, the larger postsynaptic vesicles are observed alone or with one or more attached smaller vesicles, probably synaptosomes. An appropriate term for the combined pre- and postsynaptic vesicles would be synaptoneurosomes (see above). The vesicles of postsynaptic origin with apparent diameters of 1 μm without an attached synaptosome would then be termed neurosomes, recognizing that these vesicles are a part of a heterogeneous population including many empty membrane ghosts. The preparation also would appear to contain large numbers of free synaptosomes, i.e., the spherical vesicles with diameters of about 0.6 μm.

The present light microscopic observations, while lacking the ultrastructural definition afforded by electron microscopy, permits both real-time observation of *unfixed* vesicles and a much larger sampling of the components of the preparation. The correspondence of the observed diameters of various cell types (Table I) with literature values supports the present estimations of the size of the vesicular entities suggested to be synaptoneurosomes and neurosomes. Since measurements of vesicular diameters have been made in physiological medium and without the distorting effects of fixation, it is probable that these estimations are closer to the true size of the vesicular entities. That a substantial fraction of the vesicles present in a filtered preparation have uniform diameters suggests that vesicular diameter is controlled by some physical parameter of brain membranes, the cell type of origin, the media, and/or the technique of homogenization. The uniform size and spherical configuration allows the estimation of both the volume and surface area of the vesicles (Table I).

Cyclic AMP accumulations. Cyclic AMP accumulations in the particulate preparations must occur in functional entities containing receptors linked to adenylate cyclase and a regenerating source of ATP which can be labeled through incubation with radioactive adenine. Undoubtably, steady-state levels of cyclic AMP reflect, as they do in brain slices, the sum of formation by adenylate cyclases, degradation by phosphodiesterases, and regenerative formation of ATP by energy-consuming reactions (loss of phosphocreatine and/or oxidative phosphorylation). The responses of the particulate preparations appear to reflect those of major compartments of brain slices, and such particulate preparations appear to provide the starting point for isolation and identification of the compartments associated with the major cyclic AMP-generating loci of the central nervous system. Synaptosomal preparations including the P_2 fraction exhibit much less responsive cyclic AMP-generating systems (see Table VII and Daly et al., 1980a).

Whereas filtration yields a more homogeneous preparation which is enriched in synaptoneurosomes and small vesicles, responses of the cyclic AMP-generating systems to certain agents are decreased in this preparation. Histamine, 2-chloroadenosine, forskolin, and combinations of these agents with each other and with norepinephrine elicit accumulations of cyclic AMP in both filtered and unfiltered preparations (Figs. 3 to 5). The response to forskolin is not decreased by filtration, whereas the responses to 2-chloroadenosine and histamine or combinations of 2-chloroadenosine or histamine with other agents are attenuated. Various explanations for the reduction in responses are possible. One possibility is that some minor cellular entity, lost upon filtration, is involved with the larger accumulations of cyclic AMP in the unfiltered preparation. Indeed, larger vesicular entities, which would be partially excluded or possibly fractured by filtration, may provide greater responses than the smaller entities in the filtered preparation. Such an explanation is attractive since, in the smaller synaptoneurosomes, regeneration of ATP might be more of a determining factor than in larger entities. Alternatively, filtration might remove or fracture entities which support or potentiate responses in synaptoneurosomes. Finally, filtration might release proteases or other factors which could decrease receptors or coupling of receptors to adenylate cyclase in synaptoneurosomes. A variety of experiments were carried out to explore these various possibilities.

Adenylate cyclase and phosphodiesterase activity were assessed to determine whether alterations in these enzymes would correlate with the reduced responsiveness of the filtered preparation. Adenylate cyclase activity (basal values) is less in the filtered preparation (Table VII). The reason for the slight reduction in total adenylate cyclase upon filtration is unknown. Adenylate cyclase activity is lower in white matter than in gray matter (Daly, 1977), while basal activity of the enzyme is higher in brain capillaries compared to whole brain homogenate (Karnushina et al., 1980). The activity of vesicular adenylate cyclase represents about 60% of total adenylate cyclase in the filtered and unfiltered preparation. Therefore, the filtration procedure is not selectively concentrating membrane fragments at the expense of vesicular entities that incorporate [^{3}H]adenine and in which radioactive adenine nucleotides can be converted to [^{3}H]cyclic AMP. Phosphodiesterase activity, both soluble and membrane bound, is similar in filtered and unfiltered preparations. Thus, an activation of phosphodiesterases appears unlikely to account for reduced responses in the filtered preparation.

Histamine responses. Histamine elicits accumulations of cyclic AMP in guinea pig cortical particulate preparations through both H_1-receptor and H_2-receptor components (Psychoyos, 1978). The H_1-receptor component is, in large part, dependent on the presence of adenosine, whereas the H_2-component is directly responsible for accumulations of cyclic AMP in the presence of adenosine deaminase (present data) and for the synergistic responses seen between histaminergic and adrenergic agents (Daly et al., 1980a, b). The levels of H_2-histamine receptors are unchanged after filtration. Histamine stimulates cyclic AMP accumulation in capillary-rich preparations from brain (Karnushina et al., 1980), and partial removal of endothelial entities (see below) might be responsible in part for the reduction in histamine responses after filtration.

Adenosine responses. Adenosine elicits accumulations of cyclic AMP in brain slices through a relatively low affinity A_2-adenosine receptor, the effects of which on adenylate cyclase are not detect-

able in brain membranes (Daly et al., 1983). The A_2-receptor-mediated response to 2-chloroadenosine is markedly decreased by filtration. Rat cerebral microvesicle preparations (Huang and Rorstad, 1983) and primary cultures of glioblasts (Sturgill et al., 1975) have adenylate cyclases which are stimulated by 2-chloroadenosine. The reduction in 2-chloroadenosine responses upon filtration might, therefore, be due to partial removal of endothelial and glia elements, but this remains to be proven. There is no satisfactory ligand for the A_2-adenosine receptor in cerebral cortical preparations (see Daly, 1984).

Synergistic responses. Biogenic amines, such as histamine, norepinephrine, and serotonin, markedly increase the magnitude of accumulations of cyclic AMP elicited by adenosine (Daly, 1977; Daly et al., 1980b). The mechanism remains unclear although the receptors, namely, α_1-adrenergic and H_1-histaminergic, are usually not coupled to adenylate cyclase. The receptor binding studies reveal no change in the density of α_1-, α_2-, or β-adrenergic receptors upon filtration (Table IV). Therefore, the decrease in the response to norepinephrine in filtered preparations cannot be accounted for by a decrease in receptor density. The potentiation of adenosine responses by histamine in brain preparations occurs via an H_1-receptor (Daly, 1977). Since the density of H_1-receptors increases after filtration (Table IV), other factors must be responsible for the reduction in synergism between the histamine and 2-chloroadenosine after filtration. It appears likely that the reduction in augmentation of the 2-chloroadenosine response by histamine or by norepinephrine is related to the marked loss in responsiveness of the A_2-adenosine receptor-controlled systems after filtration.

Forskolin responses. Forskolin causes marked accumulations of cyclic AMP in brain slices and markedly potentiates receptor-mediated responses. Conversely, receptor agonists can augment forskolin responses (Seamon and Daly, 1981; Seamon et al., 1981; Daly et al., 1982). The direct effects of forskolin are not markedly changed upon filtration, but the augmentation of a forskolin response produced by low concentration of 2-chloroadenosine is greatly reduced upon filtration. This again probably reflects attenuation of responses mediated via A_2-adenosine receptors. A low dose of histamine markedly augments the forskolin-induced response in both unfiltered and filtered preparations.

Receptor density. The densities (femtomoles per milligram of protein) of all of the receptors tested remain relatively constant upon filtration except for the density of the inhibitory A_1-adenosine receptor, the "peripheral" benzodiazepine-binding sites, and the H_1-histamine receptor. The binding of [^{3}H]cyclohexyladenosine, a specific A_1-adenosine receptor agonist (Bruns et al., 1980), is increased 2-fold upon filtration. This adenosine receptor subclass is responsible for inhibition by adenosine of adenylate cyclase in brain preparations (Cooper et al., 1980). The A_1-adenosine receptor appears localized to presynaptic terminals of excitatory neurons in the cerebellum (Goodman and Snyder, 1982; Goodman et al., 1983). It appears that the filtered preparation is concentrated in elements which contain A_1-receptors, probably presynaptic entities.

The density of "peripheral" benzodiazepine-binding sites measured by [^{3}H]Ro 5-4864 binding decreases by one-half upon filtration, suggesting that these sites may be localized on microvessels, glial, or neuronal elements not isolated in this system. Density of "peripheral"-type benzodiazepine receptors increases markedly in rat striatum after kainic acid lesions (Schoemaker et al., 1982). This increase occurs during profound gliosis, suggesting glial localization. Density of "central" benzodiazepine-binding sites does not change upon filtration (Table IV). Such sites are probably associated with neuronal elements in the central nervous system (Mohler and Okada, 1977; Gallagher et al., 1981).

There is no change in density of binding sites for [^{3}H]imipramine, a specific marker for serotonin uptake sites (Rehavi et al., 1983), upon filtration. These serotonin uptake sites are located both on glia (Haber et al., 1977) and on neurons (Rehavi et al., 1983).

The density of opioid receptors measured with [^{3}H]naloxone does not change with filtration (Table IV). Localization of binding of [^{3}H]naloxone to synaptic elements has been reported (Pert and Snyder, 1973).

Na^+/K^+ ATPase measured by [^{3}H]ouabain binding is unaltered upon filtration (Table IV). Na^+/K^+ ATPase activity is equal in the brain parenchyma and brain capillaries (Spatz, 1982). Denervation decreases its levels and hyperinnervation increases its levels in brain, suggestive of a high density in neuronal entities (Swann et al., 1982).

Densities of binding sites for the Ca^{2+} channel marker [^{3}H]nitrendipine (Marangos et al., 1982) are similar in filtered and unfiltered preparations (Table IV). [^{3}H]Nitrendipine-binding sites in brain appear to be largely on neuronal entities (Murphy et al., 1982) rather than on glia or endothelial cells.

The density of the binding sites for [^{3}H]batrachotoxinin-B benzoate on voltage-sensitive sodium channels is similar in unfiltered and filtered preparations (Table IV). [^{3}H]Batrachotoxinin-B benzoate binding occurs in voltage-dependent sodium channels which are probably present at *high* densities only in neuronal elements and not in glial or endothelial cells (see Creveling et al., 1982). However, the density of voltage-sensitive Na^+ channels is not uniform in neuronal cells—being, for example, very high in nodes of Ranvier—and, thus, a loss of neuronal entities (axons) with higher densities of Na^+ channels might offset the effect of loss of glial and endothelial elements with their low density of voltage-dependent Na^+ channels.

Enzyme and protein markers. Endothelial and glial cell enzyme markers were measured to estimate the levels of such entities in unfiltered and filtered preparations. Alkaline phosphatase, γ-glutamyltranspeptidase, and pseudocholinesterase are in higher concentrations in brain capillary membranes than in the parenchyma (Mršulja and Djuričić, 1980). The ratio of the activities of these enzymes in capillaries to the activities in the parenchyma is 3.9 for alkaline phosphatase, 5.3 for γ-glutamyltranspeptidase, and 8.0 for pseudocholinesterase. Filtration results in a 1.7-fold decrease in γ-glutamyltranspeptidase activity compared to the activity in the unfiltered tissue (Table V). There is a 4.9-fold decrease in alkaline phosphatase activity, whereas there is no change in pseudocholinesterase activity. The decrease in two of these endothelial cell markers suggests that the amount of endothelial entities has been reduced upon filtration, but the lack of effect on pseudocholinesterase is inexplicable.

Acetylcholinesterase activity, a marker for neuronal cells (Mršulja and Djuričić, 1980), increases by about 40% upon filtration, which suggests that neuronal tissue is being concentrated by the filtration step.

Norepinephrine uptake sites are localized in brain, primarily on the presynaptic terminals of neurons. Norepinephrine uptake increases by 1.7-fold in the filtered preparation, indicating an enrichment in neuronal presynaptic entities upon filtration.

In the central nervous system the S-100 protein is found in higher concentration in the white matter than in the gray matter (Moore, 1972). Most of the evidence, obtained by degeneration (Moore, 1973) as well as with immunohistofluorescence or electron microscopic procedures utilizing peroxidase-labeled antibody to S-100 protein (Matus and Mughal, 1975; Ludwin et al., 1976), indicates that S-100 protein in the central nervous system is located primarily in the glial cytoplasm. This protein is present in both astrocytes and oligodendrocytes. A radioimmunoassay of the soluble S-100 protein in the filtered and unfiltered preparations (Table V) reveals that approximately 20% of the soluble S-100 is lost on filtration, suggesting only a slight decrease in oligodendroglia or gliosomes derived from astrocytes in these preparations.

Comparison to crude synaptosome preparations. Certain characteristics of the particulate preparations were compared to a P_2-synaptosomal fraction (Table VII). Cyclic AMP accumulations elicited by various agents are dramatically lower in the P_2 fraction than in the particulate preparations. Synergistic responses are not observed in the P_2 fraction. Thus, the P_2 fraction is inappropriate for use in studying synergistic accumulations of cyclic AMP. The neuronal marker, acetylcholinesterase, is higher in the P_2 fraction, and the

endothelial enzyme marker, pseudocholinesterase, is lower in the P_2 fraction than in the particulate preparation. Receptor densities in the P_2 fraction also differ from those in the particulate preparations. The β-adrenergic receptor density is lower in the P_2 fraction. A_1-Adenosine receptor density is somewhat lower, whereas the H_2 receptor density is about the same. The major difference between the particulate preparations and "classical" synaptosome preparations and indeed the importance of the particulate preparations is their retention of the major cyclic AMP-accumulating entities present in brain.

Conclusions. The present filtration technique reduces whole cell and endothelial contamination of a Krebs-Henseleit particulate preparation from guinea pig brain, yielding a preparation markedly enriched, based on light microscopic examination, in small, spherical, and "snowman"-shaped entities. The latter may consist of a resealed presynaptic sac or sacs (synaptosomes) fused to a larger resealed postsynaptic sac (neurosome), and the term synaptoneurosome has been suggested. At present there is certainly significant contamination of the preparation, perhaps consisting mainly of resealed astrocytic processes (gliosomes). The reductions in cyclic AMP accumulations elicited by 2-chloroadenosine and combinations of 2-chloroadenosine with other agents upon filtration are as yet not understood. It is possible that larger entities which are selected against by filtration are responsible for greater accumulations of cyclic AMP in response to 2-chloroadenosine in the unfiltered preparations. The filtered preparation contains neuronal markers and receptors at levels similar to those for classical P_2 fractions and appears to be providing a starting preparation for further fractionation and purification of entities responsible for synergistic effects of biogenic amines and adenosine on cyclic AMP generation in the central nervous system. It is the most homogeneous cell-free preparation to date for study of synergistic interactions between amines and adenosine with respect to cyclic AMP generation, and its ease of preparation makes it useful for studies on ion fluxes (Schwartz et al., 1984), membrane potential (Creveling et al., 1980), neurotransmitter release (Ebstein et al., 1982; Ebstein and Daly, 1982a, b), and binding of certain radioligands (Creveling et al., 1982).

References

Allen, R. D., and N. S. Allen (1982) Video-enhanced microscopy with a computor frame memory. J. Microscopy *129:* 3–17.

Atlas, D., Y. Plotek, and I. Miskin (1982) An affinity label for α_2-adrenergic receptors in rat brain. Eur. J. Biochem. *126:* 537–541.

Bergmeyer, H. U., K. Gawehn, and M. Grassl (1974) Enzymes as biochemical reagents. In *Methods in Enzymatic Analysis*, H. U. Bergmeyer, ed., Vol. I, pp. 496–497, Academic Press, Inc., New York.

Bruns, R. F., J. W. Daly, and S. H. Snyder (1980) Adenosine receptors in brain membranes: Binding of N^6-cyclohexyl[^{3}H]adenosine and 1,3-diethyl-8-[^{3}H]phenylxanthine. Proc. Natl. Acad. Sci. U. S. A. *77:* 5547–5551.

Chasin, M., F. Mamrak, and S. G. Samaniego (1974) Preparation and properties of a cell-free hormonally responsive adenylate cyclase from guinea pig brain. J. Neurochem. *22:* 1031–1038.

Cooper, D. M., C. Londos, and M. Rodbell (1980) Adenosine receptor-mediated inhibition of rat cerebral cortical adenylate cyclase by a GTP-dependent process. Mol. Pharmacol. *18:* 598–601.

Creveling, C. R., E. T. McNeal, D. H. McCulloh, and J. W. Daly (1980) Membrane potentials in cell-free preparations from guinea pig cerebral cortex: Effect of depolarizing agents and cyclic nucleotides. J. Neurochem. *35:* 922–932.

Creveling, C. R., E. T. McNeal, J. W. Daly, and G. B. Brown (1982) Batrachotoxin-induced depolarization and [^{3}H]batrachotoxinin-A 20-α-benzoate binding in a vesicular preparation from guinea pig cerebral cortex. Mol. Pharmacol. *23:* 350–358.

Daly, J. W. (1977) *Cyclic Nucleotides in the Central Nervous System*, Plenum Press, New York.

Daly, J. W. (1984) Adenosine receptors. Adv. Cyclic Nucleotide Res. *19:* 29–46.

Daly, J. W., E. McNeal, C. Partington, M. Neuwirth, and C. R. Creveling (1980a) Accumulations of cyclic AMP in adenine-labeled cell-free preparations from guinea pig cerebral cortex: Role of α-adrenergic and H_1-histaminergic receptors. J. Neurochem. *35:* 326–347.

Daly, J. W., W. Padgett, Y. Nimitkitpaisan, C. R. Creveling, D. Cantacuzene, and K. L. Kirk (1980b) Fluoronorepinephrines: Specific agonists for the activation of alpha and beta adrenergic-sensitive cyclic AMP-generating systems in brain slices. J. Pharmacol. Exp. Ther. *212:* 382–389.

Daly, J. W., W. Padgett, C. R. Creveling, D. Cantacuzene, and K. L. Kirk (1981) Cyclic AMP-generating systems: Regional differences in activation by adrenergic receptors in rat brain. J. Neurosci. *1:* 49–59.

Daly, J. W., W. Padgett, and K. B. Seamon (1982) Activation of cyclic AMP-generating systems in brain membranes and slices by the diterpene forskolin: Augmentation of receptor-mediated responses. J. Neurochem. *38:* 532–544.

Daly, J. W., P. Butts-Lamb, and W. Padgett (1983) Subclasses of adenosine receptors in the central nervous system: Interactions with caffeine and related methylxanthines. Cell. Mol. Neurobiol. *3:* 69–80.

Davis, C. W., and J. W. Daly (1979) A simple direct assay of 3′,5′-cyclic nucleotide phosphodiesterase activity based on the use of polyacrylamide-boronate affinity gel chromatography. J. Cyclic Nucleotide Res. *5:* 65–74.

De Robertis, E., G. R. de Lorez, G. R. Arnaiz, M. Alberici, R. W. Butcher, and E. W. Sutherland (1967) Subcellular distribution of adenyl cyclase and cyclic phosphodiesterase in rat brain cortex. J. Biol. Chem. *242:* 3487–3493.

Dische, Z. (1955) Color reaction of nucleic components. In *The Nucleic Acids*, E. Chargoff, and J. N. Davidson, eds., Vol. I, pp. 285–306, Academic Press, Inc., New York.

Djurićić, B. M., and B. B. Mršulja (1977) Enzymatic activity of the brain: Microvessels vs total forebrain homogenate. Brain Res. *138:* 561–564.

Dooley, C., and T. Takahashi (1981) The effect of osmotic stress on the function of the human granulocyte. Exp. Hematol. *9:* 731–741.

Ebstein, R. P., and J. W. Daly (1982a) Release of norepinephrine and dopamine from brain vesicular preparations: Effects of adenosine analogues. Cell. Mol. Neurobiol. *2:* 193–204.

Ebstein, R. P., and J. W. Daly (1982b) Release of norepinephrine and dopamine from brain vesicular preparations: Effects of calcium antagonists. Cell. Mol. Neurobiol. *2:* 205–213.

Ebstein, R. P., K. Seamon, C. R. Creveling, and J. W. Daly (1982) Release of norepinephrine from brain vesicular preparations: Effects of an adenylate cyclase activator, forskolin, and a phosphodiesterase inhibitor. Cell. Mol. Neurobiol. *2:* 179–192.

Ellman, G. L., K. D. Courtney, V. Andres, Jr., and R. M. Featherstone (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. *7:* 88–95.

French, S. W., D. S. Palmer, and M. Calwell (1978) Cytochemical localization of adenylate cyclase in broken cell preparations of cerebral cortex. J. Can. Sci. Neurol. *5:* 33–40.

Gajtkowski, G. A., D. B. Norris, T. J. Rising, and T. P. Wood (1983) Specific binding of ^{3}H-tiotidine to histamine H_2 receptors in guinea pig cerebral cortex. Nature *304:* 65–67.

Gallagher, D. W., P. Mallorga, W. Oertel, R. Henneberry, and J. Tallman (1981) [^{3}H]Diazepam binding in mammalian central nervous system: A pharmacological characterization. J. Neurosci. *1:* 218–225.

Goodman, R. R., and S. H. Snyder (1982) Autoradiographic localization of adenosine receptors in rat brain using [^{3}H]cyclohexyladenosine. J. Neurosci. *2:* 1230–1241.

Goodman, R. R., M. J. Kuhar, L. Hester, and S. H. Snyder (1983) Adenosine receptors: Autoradiographic evidence for their location on axon terminals of excitatory neurons. Science *220:* 967–968.

Greenburg, D. A., D. C. U'Prichard, and S. H. Snyder (1976) Alpha-noradrenergic receptor binding in mammalian brain: Differential labeling of agonist and antagonist states. Life Sci. *19:* 69–76.

Haber, B., R. L. Suddith, M. P. Pacheco, A. Gonzalez, T. L. Ritchie, and S. Glusman (1977) Model systems for the glial transport of biogenic amines and GABA: Clonal cell lines and the filum terminal of the frog spinal cord. In *Dynamic Properties of Glia Cells*, E. Schjoffeniels, G. French, L. Hertz, and D. B. Tower, eds., pp. 193–206, Pergamon Press, New York.

Hass, G. (1950) Preparation, structure, and applications of thin films of silicon monoxide and titanium dioxide. J. Am. Ceramic Soc. *33:* 353–360.

Hill, S. J., P. C. Emson, and J. M. Young (1978) The binding of [^{3}H] mepyramine to histamine H_1 receptors in guinea pig brain. J. Neurochem. *31:* 997–1004.

Hollingsworth, E. B., C. R. Creveling, E. T. McNeal, and J. W. Daly (1984) A filtered neurosome enriched preparation from brain: Morphology, receptors, enzymes, and cyclic AMP responses. Fed. Proc. *43:* 1093.

Horn, A. S., and O. T. Phillipson (1976) A noradrenaline sensitive adenylate cyclase in rat limbic forebrain: Preparation, properties and the effects of agonists, adrenolytics and neuroleptic drugs. Eur. J. Pharmacol. *37:* 1–11.

Huang, M., and O. P. Rorstad (1983) Effects of vasoactive intestinal polypeptide, monoamines, prostaglandins, and 2Cl-adenosine on adenylate cyclase in rat cerebral microvessels. J. Neurochem. *40:* 719–726.

Inoue, S. (1981) Video image processing greatly enhances contrast, quality and speed in polarization-base microscopy. J. Cell Biol. *89:* 346–356.

Joo, F., I. Toth, and G. Jancso (1975) Brain adenylate cyclase: Its common occurrence in the capillaries and astrocytes. Naturwissenschaften *62:* 397–398.

Kakiuchi, S., and T. W. Rall (1968a) The influence of chemical agents on the accumulation of adenosine 3′,5′-phosphate in slices of rabbit cerebellum. Mol. Pharmacol. *4:* 367–378.

Kakiuchi, S., and T. W. Rall (1968b) Studies on adenosine 3′,5′-phosphate in rabbit cerebral cortex. Mol. Pharmacol. *4:* 379–388.

Karnushina, I. L., J. M. Palacios, G. Barbin, E. Deux, F. Joo, and J. C. Schwartz (1980) Studies on a capillary-rich fraction isolated from brain histaminergic components and characterization of the histamine receptors linked to adenylate cyclase. J. Neurochem. *34:* 1201–1208.

Krebs, H. A., and K. Henseleit (1932) Untersuchungen über die Harnstoffbildung im Tierkörper. Hoppe-Seylers Z. Physiol. Chem. *210:* 33–66.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193:* 265–275.

Ludwin, S. K., J. C. Kosek, and L. F. Eng (1976) The topographical distribution of S_{100} and GFA proteins in the adult brain: An immunohistochemical study using horseradish peroxidase labeled antibodies. J. Comp. Neurol. *165:* 197–207.

Marangos, P. J., J. Patel, C. Muller, and A. M. Martino (1982) Specific calcium antagonist binding sites in brain. Life Sci. *31:* 1575–1585.

Matus, A., and S. Mughal (1975) Immunohistochemical localisation of S_{100} protein in brain. Nature *258:* 746–748.

McNeal, E. T., C. R. Creveling, and J. W. Daly (1980) Cyclic AMP-generating systems in cell free preparations from guinea pig cerebral cortex: Loss of adenosine and amine responsiveness due to low levels of endogenous adenosine. J. Neurochem. *35:* 338–342.

Mohler, H., and T. Okada (1977) Benzodiazepine receptor: Demonstration in the central nervous system. Science *198:* 849–851.

Moore, B. W. (1972) Chemistry and biology of two proteins, S_{100} and 14-3-2 specific to the nervous system. Int. Rev. Neurobiol. *15:* 215–225.

Moore, B. W. (1973) Brain specific protein. In *Proteins of the Nervous System*, J. D. Schneider, ed., pp. 1–12, Raven Press, New York.

Mršulja, B. B., and B. M. Djurićić (1980) Biochemical characteristics of cerebral capillaries. In *The Cerebral Microvasculature*, H. M. Eisenberg, and R. L. Suddith, eds., pp. 29–43, Academic Press, Inc., New York.

Murphy, K. M., R. J. Gould, and S. H. Snyder (1982) Autoradiographic visualization of [^{3}H]nitrendipine binding sites in rat brain: Localization to synaptic zones. Eur. J. Pharmacol. *81:* 517–519.

Nagamatsu, K., Y. Kido, F. Ferao, T. Ishida, and S. Toki (1982) Effect of morphine on opiate receptor binding and morphine-elicited analgesia. Life Sci. *31:* 1451–1457.

Nomarski, G., and A. R. Weill (1954) Reflected differential interference contrast. Bull. Soc. France Minera. Crist. *77:* 840.

Pert, C. B., and S. H. Snyder (1973) Properties of opiate-receptor binding in rat brain. Proc. Natl. Acad. Sci. U. S. A. *70:* 2243–2247.

Psychoyos, S. (1978) H_1- and H_2-histamine receptors linked to adenylate cyclase in cell-free preparations of guinea pig cerebral cortex. Life Sci. *23:* 2155–2162.

Psychoyos, S., J. Dove, B. Strowbridge, and I. Nusynowitz (1982a) Highly activatable adenylate cyclase in [2·^{3}H]adenine-prelabeled vesicles prepared from guinea pig cerebral cortex by a simplified procedure. J. Neurochem. *38:* 1437–1445.

Psychoyos, S., C. J. Ford, and M. A. Phillips (1982b) Inhibition by etazolate (SQ 20009) and cartazolate (SQ 65396) of adenosine-stimulated [^{3}H]cAMP formation in [2-^{3}H]adenine-prelabeled vesicles prepared from guinea pig cerebral cortex. Biochem. Pharmacol. *31:* 1441–1442.

Rehavi, M., P. Skolnick, and S. M. Paul (1983) Subcellular distribution of high affinity [^{3}H]imipramine binding and [^{3}H]serotonin uptake in rat brain. Eur. J. Pharmacol. *87:* 335–339.

Rougan, G., M. Nobel, and A. W. Mudge (1983) Neuropeptides modulate the β-adrenergic response of purified astrocytes *in vitro*. Nature *305:* 715–717.

Salomon, Y., C. Londos, and M. Rodbell (1974) A highly sensitive adenylate cyclase assay. Anal. Biochem. *58:* 541–548.

Sattin, A., and T. W. Rall (1970) The effect of adenosine and adenine nucleotides on the cyclic adenosine 3′,5′-monophosphate content of guinea pig cerebral cortex slices. Mol. Pharmacol. *6:* 13–23.

Schoemaker, H., M. Morelli, P. D. Deshmukh, and H. I. Yamamura (1982) [^{3}H]Ro 5-4864 benzodiazepine binding in the kainate lesioned striatum and Huntington's diseased basal ganglia. Brain Res. *248:* 396–401.

Schoemaker, H. M., B. S. Yamamura, and H. Yamamura (1981) High affinity benzodiazepine binding sites for [^{3}H]Ro 5-4864 in the rat cerebral cortex. Eur. J. Pharmacol. *71:* 173–175.

Schwartz, R. D., P. Skolnick, E. B. Hollingsworth, and S. M. Paul (1984) Barbiturate and picrotoxin-sensitive chloride efflux in rat cerebral cortical synaptoneurosomes. FEBS Lett. *175:* 193–196.

Seamon, K. B., and J. W. Daly (1981) Forskolin: A unique diterpene activator for cyclic AMP generating systems. J. Cyclic Nucleotide Res. *7:* 201–224.

Seamon, K. B., W. Padgett, and J. W. Daly (1981) Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc. Natl. Acad. Sci. U. S. A. *78:* 3363–3367.

Siakotos, A. N., M. Filbert, and R. Hisker (1969) A specific radioisotopic assay for acetylcholinesterase and pseudocholinesterase in brain and plasma. Biochem. Med. *3:* 1–12.

Skolnick, P., L. P. Stalvey, and J. W. Daly (1977) Blockade of central β-adrenergic receptors by tazolol (1-isopropylamino-3-(2-thiazolozy)-7-propranolol). Life Sci. *21:* 1655–1664.

Spatz, M. (1982) Progress in cerebral microvascular slices related to the function of the blood-brain barrier. Adv. Cell. Neurobiol. *3:* 311–337.

Squires, R. F., and C. Braestrup (1977) Benzodiazepine receptors in rat brain. Nature *266:* 732–734.

Sturgill, T. W., M. B. K. Schrier, and A. G. Gilman (1975) Stimulation of cyclic AMP accumulation by 2-chloroadenosine: Lack of incorporation of nucleoside into cyclic nucleotides. J. Cyclic Nucleotide Res. *1:* 21–30.

Swann, A. C., S. J. Grant, D. Jablons, and J. W. Mass (1981) Increased ouabain binding after repeated noradrenergic stimulation. Brain Res. *213:* 481–485.

Swann, A. C., S. J. Grant, and J. W. Maas (1982) Brain (Na^+,K^+)-ATPase and noradrenergic activity: Effects of hyperenervation and denervation on high-affinity ouabain binding. J. Neurochem. *38:* 836–839.

Szasz, G. (1974) γ-Glutamyltranspeptidase. In *Methods in Enzymatic Analysis*, H. U. Bergmeyer, ed., Vol. II, pp. 715–720, Academic Press, Inc., New York.

Uozumi, T., and R. J. Ryan (1973) Isolation, amino acid composition and radioimmunoassay of human brain S100 protein. Mayo Clin. Proc. *48:* 50–56.

Weiss, L. (1977) Blood. In *Histology*, Ed. 4, L. Weiss and R. O. Greep, eds, pp 433–457, McGraw Hill Inc., New York.

Weller, M. (1977) Evidence for the presynaptic location of adenylate cyclase and the cyclic AMP-stimulated protein kinase which is bound to synaptic membranes. Biochim. Biophys. Acta *469:* 350–354.