Biochimica et Biophysica Acta, 448 (1976) 57–72

BBA 77425

CARBOHYDRATE COMPOSITION OF CENTRAL NERVOUS SYSTEM SYNAPSES

ANALYSIS OF ISOLATED SYNAPTIC JUNCTIONAL COMPLEXES AND POSTSYNAPTIC DENSITIES

LYNN CHURCHILL*, CARL COTMAN†, GARY BANKER**, PAUL KELLY and LELAND SHANNON***

Department of Psychobiology, University of California at Irvine, Irvine, Calif. 92717 (U.S.A.)

(Received March 5th, 1976)

SUMMARY

The composition of specialized structures present at synapses within the central nervous system was elucidated by biochemical analysis of fractions enriched in synaptic junctional complexes and postsynaptic densities. The results indicate that the synaptic junctional complex is primarily protein together with some glycoproteins. The synaptic junctional complex proteins are similar in amino acid composition to synaptic membrane proteins; they are not especially rich in basic residues, as previously suggested. The major carbohydrates present in the synaptic junctional complex and postsynaptic density glycoproteins are mannose, galactose, and glucosamine, with lesser amounts of fucose, *N*-acetylneuraminic acid, and galactosamine. Comparison with the synaptic membrane fraction indicates that galactose is more concentrated in the synaptic junctional complex and mannose in the postsynaptic density. Glucose is dramatically enriched in both these fractions. Sucrose binding during isolation may partially account for the glucose enrichment.

INTRODUCTION

In 1897 Foster and Sherrington [1] introduced the term synapse (Gk. connection) to describe the "mode of nexus between neuron and neuron." In the subsequent three-quarters of a century the synapse has formed a central focus for detailed morphological, pharmacological, and electrophysiological studies which have provided

* Present address: Department of Pharmacology, University of Wisconsin Medical Center, Madison, Wisc., 53706, U.S.A.

** Present address: Department of Anatomy, Washington University School of Medicine, St. Louis, Miss., 63110, U.S.A.

*** Department of Biochemistry, University of California at Riverside, Riverside, Calif., 92507, U.S.A.

† To whom reprint requests should be addressed.

many of the detailed properties of synapses. By contrast comparatively little is known about synaptic structure and composition at the biochemical level. So far the biochemical composition of the synapse has been examined only indirectly by cytochemical methods.

Recently new isolation procedures for the synaptic substructures, the synaptic junctional complex [2–4] and its constituent, the postsynaptic density [5], have made it possible to determine directly the biochemical composition of the structures in the synaptic region. Isolated synaptic junctional complexes consist of postsynaptic membranes with a prominent postsynaptic density (an electron dense paramembranous structure) often attached via the synaptic cleft to presynaptic membranes [4]. In isolated postsynaptic densities membranes are essentially absent [5]. Since a synapse with a postsynaptic density is usually an excitatory dendritic synapse [5–8], analyses of the composition of the synaptic junctional complex and postsynaptic density will serve to elucidate the molecular components of excitatory dendritic synapses in the central nervous system. In this paper we determine the proportion of protein, phospholipid and carbohydrate in the synaptic junctional complex fraction in comparison with the synaptic membrane fraction, and we analyze the carbohydrate composition and binding properties of the synaptic membrane, synaptic junctional complex and postsynaptic density fractions.

Cytochemical evidence suggests that the synaptic membrane specializations, such as the postsynaptic density and the material present within the synaptic cleft, are mainly proteins. Treatment of the synaptic junctional complexes by proteolytic enzymes destroys their recognizable structure normally observed by electron microscopy [4, 9, 10]. Enzymes that remove carbohydrates do not destroy the recognizable structure.

Cytochemical evidence also indicates that the proteins of the synaptic junctional complex possess special properties. The postsynaptic density, along with other elements in the synaptic cleft, are selectively stained by ethanolic phosphotungstic acid [10] and bismuth iodide-uranyl lead ions [9, 11]. The staining can be prevented by prior acetylation or by trypsin treatment. Because *in vitro* studies on various proteins and model peptides indicated that these stains selectively interact with basic amino acid residues in proteins, Bloom and Aghajanian [10] and Pfenninger [11] proposed that the synaptic junctional complex contains protein enriched in basic amino acids.

Cytochemical studies also indicate that carbohydrates are apparently present at the synaptic junction, particularly within the synaptic cleft. Carbohydrate stains such as ruthenium red and periodic acid-silver methenamine are associated with intercellular spaces at synaptic junctions [12, 13]. Concanavalin A-ferritin conjugates bind to the external surface of the postsynaptic membrane indicating the presence of α-D-mannosyl or glucopyranosyl residues [14, 15]. Neuraminidase, which removes *N*-acetylneuraminic acid, decreases the uranyl acetate staining of the synaptic cleft [16].

Thus previous cytochemical data indicate that proteins and carbohydrates are most likely elements of the central nervous system synapse. Isolated synaptic junctional complex and postsynaptic density fractions retain their basic morphological form, their distinctive staining properties [4, 5] and their lectin binding sites [14], 15] so that the constituents of central nervous system synapses can be analyzed directly.

MATERIALS AND METHODS

Preparative procedures. Preparation of the synaptic junctional complex and postsynaptic density fractions was carried out as previously described [4, 5]. Forebrains of 60–100-day-old, male Sprague-Dawley rats were excised, homogenized and fractionated to yield a synaptic membrane fraction that contained intact synaptic junctional complexes. The synaptic membrane fraction was treated with Triton X-100 (2 mg/mg membrane protein) at 4 °C and sedimented through 1.0 M sucrose to produce the synaptic junctional complex fraction [4]. To prepare a postsynaptic density fraction, the synaptic membrane fraction was treated with 3.9 % (w/v) sodium *N*-lauroyl sarcosinate at 4 °C and fractionated on a sucrose density gradient [5]. The fraction which pelleted through 1.4 M sucrose was taken as the postsynaptic density fraction.

Quantitative electron microscopy. Representative electron microscopic sampling of the subcellular fractions was achieved as previously described [5]. Samples which contained 60–100 μg of protein were pelleted in a Beem capsule [17], fixed in 2 % glutaraldehyde in Caulfield buffer [18], postfixed with 2 % OsO_4 in Caulfield buffer, and stained in block with uranyl acetate in Kellenberger buffer [19]. The material was then dehydrated in graded steps with ethanol and embedded in Epon-Araldite. Sections were cut on an KLB ultramicrotome and counterstained with lead citrate [20]. Electron micrographs were taken sequentially across the entire thickness of the pellets, which were about 120 μm thick. Squares, equivalent to 2 μm on a side in the pellet, were randomly selected in each of the micrographs. The structures in each of the squares were identified and their areas were computed by measuring length and width with a divider or were measured by superimposition of a grid. The width of plasma membranes was arbitrarily taken as 100 Å.

Analysis of protein, phospholipid, and carbohydrate composition. Samples were washed 3–5 times with glass-distilled water to remove sucrose, lyophilized and weighed. The dried samples were extracted with chloroform/methanol by a modification of the Bligh and Dyer procedure [21] for small volumes. The dried samples were wetted with 80 μl of water and extracted with 300 μl of chloroform/methanol (1 : 2, v/v). After addition of 100 μl of chloroform to adjust the chloroform/methanol concentration to 1 : 1, the samples were centrifuged and the supernatant was removed. The extraction procedure was repeated 3–6 times until the formazan was completely extracted from the insoluble residue. The chloroform/methanol-insoluble residue and extract were dried down and weighed, and the protein and phosphorus content determined. In a previous study, this method completely removed all of the lipids from synaptic membranes [22]. The protein content was also analyzed by the method of Lowry et al. [23] using crystalline bovine serum albumin as a standard. The total phosphorus content was determined by the method of Ames [24]. The formazan content was estimated by measuring the absorbance at 495 nm after extraction into chloroform. The quantity present was calculated based on a comparison with known standards.

Analysis of amino acid composition. Lyophilized and weighed samples of synaptic membrane and synaptic junctional complex fractions were hydrolyzed in 6 M HCl at 110 °C for 70 h and analyzed for amino acid content, according to a previously described procedure [25]. Corrections were made for hydrolytic losses of

threonine (13 %), serine (32 %), half-cysteine (17 %) and tyrosine (6 %).

Analysis of carbohydrates. Carbohydrates were analyzed on washed fractions to measure total carbohydrate and on chloroform/methanol-extracted samples to measure protein-bound carbohydrates. *N*-Acetylneuraminic acid was measured by the method of Warren [26] with modifications to accommodate smaller volumns. Samples were hydrolyzed in 0.05 M H_2SO_4 at 75 °C for 1 h, centrifuged, and portions of the supernatant were assayed for *N*-acetylneuraminic acid using this compound as a standard.

Other carbohydrates were analyzed by gas-liquid chromatography utilizing a combination of the methods of Griggs *et al.* [27] and Yang and Hakomori [28]. Samples were hydrolyzed in 2 ml of 3 M HCl at 100 °C for 1.5 h. In pilot studies these conditions were found to provide the optimal compromise for release and preservation of the neutral and amino sugars present in the various fractions. The conical test tubes holding the samples were flushed with N_2 prior to hydrolysis and sealed with Teflon-lined screw caps. The neutral sugar content was also measured following the mild hydrolysis procedure of Kim *et al.* [29] utilizing 2 ml of 0.25 M H_2SO_4 containing 200 mg Dowex 50 resin rotated in an evacuated sealed tube at 100 °C for 24 h. The two hydrolysis conditions yielded nearly equal quantities of neutral sugars. After hydrolysis the carbohydrates were converted to alditol acetate derivatives by the following procedure. After cooling to room temperature, the pH was adjusted to neutrality, and the water was removed by roto-evaporation at 50 °C. The carbohydrates were reduced with 10 mg of $NaBH_4$ in 1 ml of 1 M NH_4OH at room temperature for 3 h. The reduction was stopped by addition of glacial acetic acid drops, and the methyl borate was removed by repeated roto-evaporation (4–5 times) in methanol at 38 °C. Samples were dried overnight in a vacuum dessicator containing P_2O_5. The carbohydrates were then acetylated with glass-distilled acetic anhydride (0.5 ml) at 100 °C for 2 h. Residual acetic anhydride was removed by roto-evaporation with toluene and the dried residue was dissolved in chloroform/water (1 : 1, v/v). Salts were removed by 3–4 washes with water. The alditol acetate derivatives remained in the chloroform phase. The chloroform was evaporated and the residue redissolved in 10–20 μl of pyridine for injection into the gas chromatograph.

Gas chromatography was performed by a Loenco Model 160 gas chromatograph (Loenco, Inc., Mountain View, Calif.) equipped with a hydrogen flame detector. A glass column, 2 m long by 2.4 mm internal diameter, was packed with ECNSS-M (an organosilicon polyester resulting from the combination of ethylene glycol succinate and a silicone of cyanoethyl type purchased from Applied Science Lab, Inglewood, Ca.) on 100–120 mesh Gas Chrom Q (Applied Science Lab). Neutral and amino sugars were determined in one run beginning under isothermal conditions at 180 °C for 20–30 min, followed by a temperature increase up to 210 °C at 6°/min. The temperature of the injector port was set at 233 °C and the detector at 240 °C. Helium was used as the carrier gas with a flow rate of 85 ml/min. The signal from the gas chromatograph was recorded using the linear mode of a Sargent recorder, Model SRL.

Quantitation of the carbohydrates was achieved by planimetric measurements of the peak areas relative to the area of the internal standard, *m*-inositol. The response factor was determined for each carbohydrate, so that losses during hydrolysis could be partially accounted for. The above procedures allow detection and quantitation at

0.25 μg quantities of fucose, mannose, galactose, glucose, glucosamine and galactosamine (or mannosamine). Since mannosamine has not been detected in glycoproteins, the entire peak area is assigned to galactosamine. The carbohydrate content of ovalbumin was shown to agree with previous data [30] within 11–17 %.

Carbohydrate binding. The isolated fractions were resuspended and pelleted 3–5 times in 25 ml of 0.1 mM EDTA to remove excess sucrose. The pellets were resuspended in a modified Krebs buffer (119 mM NaCl, 47 mM KCl, 1.19 mM KH_2PO_4, 12.5 mM $NaHCO_3$, and 2.53 mM $CaCl_2$, pH 7.6). For the initial binding experiments, a 100 μl aliquot of 0.4–0.6 mg synaptic membrane or synaptic junctional complex protein or 0.1–0.2 mg postsynaptic density protein was incubated with 100 μl of 112–117 μmol of unlabeled carbohydrate and 5–10 μC of ^{14}C-labeled carbohydrates (5–360 Ci/mol) in modified Krebs buffer. For galactose and dextran, 54 μmol of unlabeled carbohydrate/100 μl and a 20 % (w/v) solution, respectively, were prepared. Samples were incubated for 75 min at 4 °C. The sequential binding experiments were conducted at 0–2 μmol of unlabeled carbohydrate with 2 nCi ^{14}C-labeled carbohydrates (360 Ci/mol) so that complete removal of any labeled contaminant could be observed. After the incubation period, samples were diluted with 25 ml of 0.1 mM EDTA and pelleted in a 30 rotor for 78 240 $\times g$ for 20 min. The supernatants were removed and pellets resuspended in 25 ml of 0.1 mM EDTA by dispersion with a glass tool. This procedure was repeated 5–6 times until the supernatants contained less than 50 dpm/ml. Then the pellets were sonicated in 150–250 μl of 0.1 mM EDTA and a 100 μl aliquot was solubilized in 900 μl of 1 % sodium dodecyl sulfate and counted in Triton-toluene fluid [31] in a Nuclear Chicago Liquid Scintillation Counter. Internal standards were used to measure efficiencies which were between 65 and 80 %. The Lowry et al. method [23] adjusted for small volumes was utilized to determine the protein remaining in the pellets after washing. The probabilities to determine whether the synaptic membrane and synaptic junctional complex binding were the same were calculated by the *F*-test and *t*-test.

To determine if the label bound to the synaptic junctional complex fraction would co-migrate with cold sucrose on a thin-layer chromatogram, 2 mg of synaptic junctional complex protein was incubated with 50 μCi of [^{14}C]sucrose in the presence of 0.6 M sucrose for 75 min at 4 °C. The majority of the label bound ($1.3 \cdot 10^3$ dpm/mg protein) was freed by boiling in 0.1 mM EDTA for 10 min. After pelleting in a 40 rotor for 144 880 $\times g$ for 30 min, the supernatant was removed and dried in a boiling water bath. The residue was redissolved in water and 3 μl spotted along with 1 μl of 0.15 % cold sucrose on a thin-layer Kieselguhr G plate buffered with acetate [32]. The thin-layer chromatogram was run in ethyl acetate/propanol/water (67 : 24 : 12 v/v), and stained with atomized ethanol/water/H_2SO_4/anisaldehyde (8.5 : 0.5 : 0.5 : 0.5, v/v). Then the thin-layer chromatogram was divided into equal sections and the silica gel removed to be counted in Triton-toluene fluid [31] in a Nuclear Chicago Liquid Scintillation Counter.

RESULTS

Electron microscopic analysis of the subcellular fractions

A qualitative description and quantitative analysis of the synaptic junctional complex fraction by electron microscopy is presented in comparison with the synaptic

membrane and postsynaptic density fractions. These descriptions and analyses are required in order to evaluate the correlation between biochemical analyses of the fractions and the biochemical composition of the synaptic structures.

The synaptic junctional complex fraction contained structures classified as synaptic junctional complexes (arrows in Fig. 1B). The synaptic junctional complex consisted of a segment of postsynaptic plasma membrane with an attached postsynaptic density joined by a synaptic cleft to a segment of presynaptic plasma membrane. When no presynaptic membrane was attached, a segment of postsynaptic plasma membrane with a postsynaptic density was classified as a postsynaptic specialization (S in Fig. 1B). Membrane vesicles unattached to the synaptic junctional complexes were also present in this fraction. The synaptic membrane fraction which served as the source of the synaptic junctional complex and postsynaptic density fractions contained numerous membranes, a few synaptic junctions (arrows) and other structures classified as contaminants (Fig. 1A). In the postsynaptic density

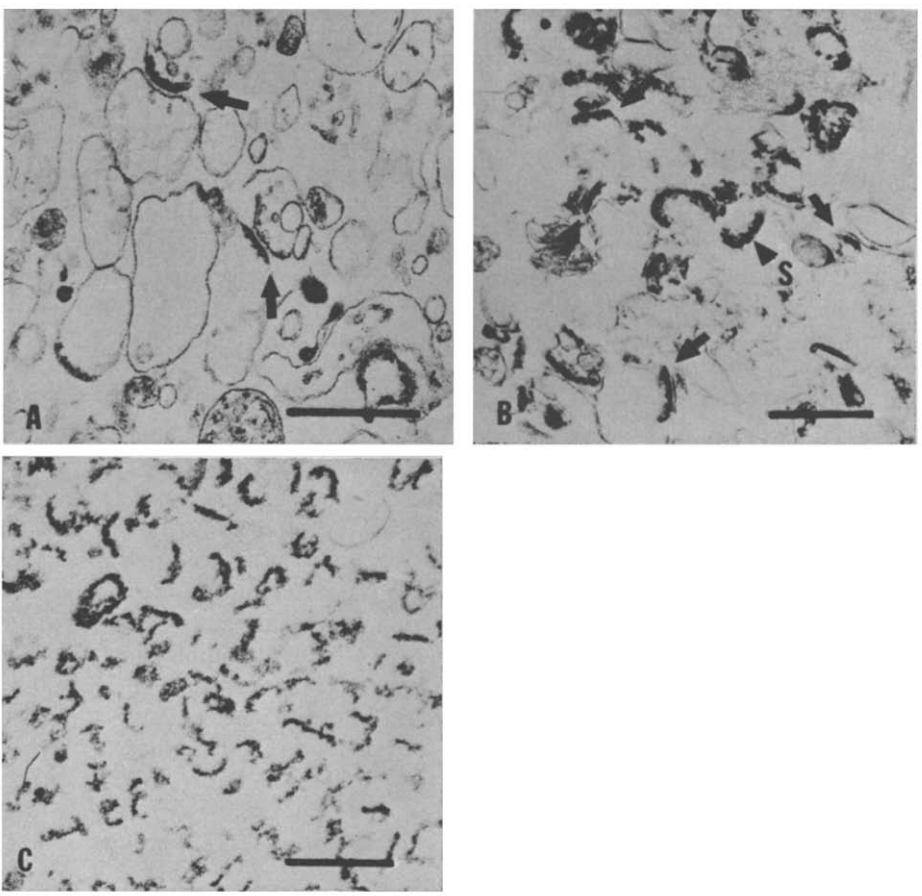

Fig. 1. Electron micrographs of the synaptic plasma membrane (A), synaptic junctional complex (B), and postsynaptic density (C) fractions. The arrows in A and B designate synaptic junctional complexes. The S in B is a postsynaptic specialization. Scale bars represent 0.5 μm.

fraction, the postsynaptic membrane had been removed from the postsynaptic specialization (Fig. 1C). These structures were classified as postsynaptic densities.

Quantitative electron microscopic analysis of the synaptic junctional complex fraction revealed an enrichment in intact synaptic junctional complexes and particularly postsynaptic specializations relative to the synaptic membrane fraction. In the synaptic junctional complex fraction, synaptic junctional complexes and postsynaptic specializations accounted for 40 % of the structures (by number) and occupied 60 % of the cross-sectional area occupied by identifiable structures in the electron micrographs (Table I). Membranes, attached or unattached to synaptic junctions and postsynaptic specializations, made up most of the remaining structures. Most of the attached membrane was presynaptic; postsynaptic membrane beyond the postsynaptic density was usually absent or consisted of a short segment only . For comparison, the synaptic membrane fraction contained primarily membrane vesicles (71 % by number; 49 % by area). Synaptic junctional complexes account for only 4 % of the total area (2 % of the total number) occupied by structures in this fraction. On this basis, the fractionation methods employed result in an approx. 15-fold

TABLE I

QUANTITATIVE ELECTRON MICROSCOPIC ANALYSIS OF SYNAPTIC JUNCTIONAL COMPLEX AND SYNAPTIC MEMBRANE FRACTIONS

The total number of structures counted was 285 and 374 for the synaptic junctional complex fraction. Of the total, 93 and 98 % could be assigned to a specific category. Those unidentifiable could have been either damaged postsynaptic specializations or contaminants.

	Percent of total area of identified structures		
	Synaptic junctional complex fraction		Synaptic membrane fraction*
	Exp. 1	Exp. 2	
Synaptic junctional complexes	16	10	4
Postsynaptic specializations	42	51	
Membrane	30	37	49
(attached to synaptic junctional complex)**	(10)	(15)	
(unattached)	(20)	(22)	
Other contaminants	12	1	46
	Percent of total number of identified structures		
	Synaptic junctional complex fraction		Synaptic membrane fraction*
Synaptic junctional complexes	13	7	2
Postsynaptic specializations	28	35	0
Membrane	57	55	71
(attached to synaptic junctional complex)	(15)	(20)	
(unattached)	(42)	(35)	
Other comtaminants	2	3	27

* Data from Cotman et al. [5].

** The attached membranes include the segments of presynaptic and postsynaptic plasma membranes that are constituents of the synaptic junctional complex.

enrichment in synaptic junctional complexes and postsynaptic specializations in the synaptic junctional complex fraction.

Based on a similar quantitative analysis [5], the postsynaptic density fraction contained 83 % postsynaptic densities which accounted for 90 % of the occupied area in electron micrographs. Small membrane vesicles account for most of the remaining material in this fraction.

The synaptic specializations in the synaptic junctional complex and postsynaptic density fractions differ in several important respects. In the synaptic junctional complex fraction, the synaptic structures were relatively intact, including the postsynaptic density and overlying plasma membrane. A segment of presynaptic membrane is also frequently attached and therefore the material within the cleft is presumably present as well. In the postsynaptic density fraction, the postsynaptic density is present without any attached plasma membrane that can be observed by electron microscopy. Since the postsynaptic density fraction is reasonably pure in postsynaptic densities and the synaptic junctional complex fraction enriched in synaptic junctional complexes and postsynaptic specializations, biochemical analyses of these fractions will provide strong evidence for the biochemical components of the postsynaptic density and implications for the components in the membrane overlying the postsynaptic density and cleft region. It should be pointed out, however, that certain constituents normally native to these structures may be lost during the isolation and cannot be analyzed.

Biochemical composition

The composition of the synaptic junctional complex fraction was examined and compared with that of the synaptic membrane fraction (Table II). The synaptic junctional complex fraction was about three-quarters protein, based on analysis for protein by the Lowry et al. method [23], or on the weight of the chloroform/methanol-insoluble residue. In contrast, the synaptic membrane fraction was about one-half protein.

The synaptic junctional complex fraction contained less lipid than the synaptic membrane fraction based on the dry weight of chloroform/methanol-soluble material. Measurements of phospholipid, based on chloroform/methanol-extracted phosphorus, also showed a lower phospholipid content in the synaptic junctional complex fraction compared with the synaptic membrane fraction (Table II).

Carbohydrates in the synaptic junctional complex fraction accounted for 0.04 mg/mg dry weight, whereas those in the synaptic membrane fraction made up 0.07 mg/mg dry weight. The synaptic junctional complex and synaptic membrane fractions were similar in their content of protein bound carbohydrates (those in the chloroform/methanol-insoluble residue), but the synaptic junctional complex fraction contained less chloroform/methanol-soluble carbohydrate (Table II).

The "histochemical" method used to increase the density of mitochondria during the fractionation procedure generated formazan, which bound to the fractions. Bound formazan was extracted into the chloroform/methanol phase and accounted for part of the dry weight present in the chloroform/methanol extract. It was more abundant in the synaptic junctional complex fraction than in the synaptic membrane fraction.

In both fractions the amount of protein and chloroform/methanol-insoluble

TABLE II

BIOCHEMICAL COMPOSITION OF THE SYNAPTIC MEMBRANE AND THE SYNAPTIC JUNCTIONAL COMPLEX FRACTIONS

The proportions of protein, phospholipid, and carbohydrate are provided for these fractions. The means and S.E. of the experiments are presented with the total number of experiments in parentheses. The results are expressed as mg/mg dry weight.

	Synaptic junctional complex	Synaptic membrane
Chloroform/methanol insoluble	0.74±0.02 (3)*	0.52±0.02 (4)*
Chloroform/methanol soluble	0.26±0.02 (3)*	0.48±0.01 (4)*
Protein	0.77±0.04 (6)	0.49±0.02 (5)
Phospholipid***	0.04±0.003 (6)	0.29±0.02 (5)
Carbohydrate		
Chloroform/methanol insoluble	0.03**	0.04**
Chloroform/methanol soluble	0.01**	0.03**
Formazan	0.07±0.01 (2)	0.01±0.00 (2)

* Recoveries after chloroform/methanol extraction were 98±1 % for the synaptic junctional complex fraction and 102±1 % for the synaptic membrane fraction. In a previous study of synaptic membranes, the procedure for chloroform/methanol extraction of lipids was demonstrated to be complete by fatty acid analysis [22].

** Summation of mean values from gas chromatographic analysis. See Table IV.

*** The phospholipid is determined by measuring the phosphorus content and assuming that 4 μg of phosphorus corresponds to 100 μg of phospholipids.

carbohydrates obtained by analytical methods agreed well with the total chloroform/methanol-insoluble dry weight. For the synaptic membrane fraction, the total lipid content could be approximated from previous experiments which indicate that 75 % of the synaptic membrane lipid is phospholipid [22]. On this basis analytical estimates of lipid and chloroform/methanol-soluble carbohydrates gave reasonable agreement with the total chloroform/methanol-soluble mass. For the synaptic junctional complex fraction, however, only about one-half of the total chloroform/methanol-soluble mass could be accounted for by the constituents analyzed, lipid (assuming 75 % phospholipids), carbohydrate, and formazan. Bound detergent might have accounted for some of the residual chloroform/methanol-soluble material in this fraction. Triton X-100 is bound in large amounts to some lipophilic proteins (up to 20–50 % of their weight) [33].

The data indicate that protein is the major constituent of the synaptic junctional complex fraction. After correction for the formazan content, protein makes up 80 % of the synaptic junctional complex and 50 % of the synaptic membrane fraction. The proportion of protein to lipid in the synaptic junctional complex fraction is roughly what would be expected for a fraction containing equal parts of membrane, consisting of nearly equal amounts of protein and lipid, and postsynaptic densities, which are almost entirely protein [34].

Amino acid composition

Previously it has been suggested that synaptic structures are especially enriched in basic amino acids [10, 11]. To test this possibility, we determined the amino acid

TABLE III

AMINO ACID COMPOSITION OF THE SYNAPTIC JUNCTIONAL COMPLEX AND SYNAPTIC MEMBRANE FRACTIONS

The degree of hydrophobicity in the amino acids of each fraction is summated in the Σf_{f_t} [35]. The polar amino acids are lysine, histidine, arginine, aspartic acid and glutamic acid. The means ± S.E. of three experiments are described.

Amino acid	Synaptic membrane (mol %)	Synaptic junctional complex (mol %)
Lys	6.0 ± 0.1	6.5 ± 0.3
His	1.8 ± 0.1	2.6 ± 0.2
Arg	3.8 ± 0.2	5.0 ± 0.1
Cys (half)	1.7 ± 0.1	0.7 ± 0.4
Asp	9.6 ± 0.03	9.4 ± 0.3
Met	2.7 ± 0.2	1.7 ± 0.6
Thr	6.1 ± 0.1	6.0 ± 0.1
Ser	9.8 ± 0.3	9.3 ± 0.5
Glu	11.0 ± 0.1	12.0 ± 0.1
Pro	4.3 ± 0.3	5.0 ± 0.5
Gly	7.3 ± 0.4	7.7 ± 0.2
Ala	7.7 ± 0.1	7.9 ± 0.2
Val	7.4 ± 0.3	7.1 ± 0.2
Ile	5.2 ± 0.2	4.5 ± 0.1
Leu	8.9 ± 0.3	8.7 ± 0.2
Tyr	2.7 ± 0.1	2.6 ± 0.2
Phe	3.8 ± 0.1	3.3 ± 0.2
Σf_{f_t}	89.1	87.4
Total polar	32.3	35.5

composition of the total protein in the synaptic junctional complex fraction. Our results do not support this contention. The amino acid composition of the synaptic junctional complex fraction did not differ greatly from that of the synaptic membrane fraction. The proportions of arginine, histidine, and glutamic acid were slightly greater in the synaptic junctional complex fraction (about 1 residue/100 residues) (Table III). There were also slightly fewer hydrophobic amino acids in the synaptic junctional complex fraction (about 2 residues/100 residues). These slight differences were also reflected in the overall hydrophobicity which was computed using quantitative estimates of the hydrophobicity of each amino acid [35]. Therefore, although the composition of individual polypeptides present in the two fractions was quite different, the amino acid composition of the combined synaptic junctional complex proteins did not differ dramatically from that of the synaptic membrane fraction. The amino acid composition of the postsynaptic density fraction has also been shown to be similar to the synaptic membrane fraction [34].

Carbohydrate composition

Since cytochemical stains indicate the presence of carbohydrates in the synaptic region [12–15], the carbohydrate composition of the synaptic junctional complex and postsynaptic density fractions was analyzed and compared with that of the synaptic membrane fraction. The carbohydrates, mannose, galactose, glucosamine, fucose, *N*-acetylneuraminic acid, and galactosamine were present in the synaptic membrane,

synaptic junctional complex and postsynaptic density fractions (Table IV). The total amount of protein-bound carbohydrates present in these fractions is roughly similar. The synaptic junctional complex and postsynaptic density fractions contain little carbohydrate associated with lipid, whereas about 40% of synaptic membrane carbohydrate is chloroform/methanol soluble. The concentration of the particular carbohydrate residues in the synaptic junctional complex fraction was similar to that of the synaptic membrane fraction, except that galactose was more concentrated and *N*-acetylneuraminic acid, less concentrated in the synaptic junctional complex fraction. The postsynaptic density fraction had a similar carbohydrate composition to the synaptic junctional complex fraction, except that mannose was present in greater amounts and *N*-acetylneuraminic acid is reduced. The variability in the carbohydrate measurements for the postsynaptic density fractions may be due to variations in the preparation of this material.

Glucose was present in the synaptic junctional complex and postsynaptic density fractions in much greater amounts than in the synaptic membrane fraction. Quantitation of the glucose demonstrated a 5-fold enrichment in the synaptic junctional complex fraction and a 10-fold enrichment in the postsynaptic density fraction relative to the synaptic membrane fraction (Table IV). The detected glucose could be a native constituent of these fractions or could result from the hydrolysis of sucrose which became associated with the material during the isolation procedure. An increase in the number of washes (from 3 to 5) did not reduce the amount of glucose present. Radioactive sucrose placed in the final gradient was almost completely washed out of the pellet by the third wash, although some of the radioactive label persisted within the pellet for another two washes without significant reduction. The possibility that

TABLE IV

CARBOHYDRATE COMPOSITION OF SYNAPTIC MEMBRANE, SYNAPTIC JUNCTIONAL COMPLEX, AND POSTSYNAPTIC DENSITY FRACTIONS

N-Acetylneuraminic acid was measured by the Warren method [26]. All other carbohydrates were measured by gas-liquid chromatography. Carbohydrates not extracted by chloroform/methanol were considered protein bound. Means ±S.E. are for three or more preparations. For each preparation, 3–4 gas chromatographic runs were made. N.D., not determined. The results are repressed as nmol carbohydrate/mg protein.

	Synaptic membrane	Synaptic junctional complex	Postsynaptic density
Total carbohydrates	305	213	286
N-Acetylneuraminic acid	107, 96	35± 1 (4)	12±1 (3)
Glucose	46	228	777
Total protein-bound carbohydrates	207	202	215, 258
N-Acetylneuraminic acid	47±2 (4)	19± 2 (3)	n.d.
Fucose	18, 18	44, 19	12, 21
Mannose	38±2 (3)	43± 4 (3)	154, 106
Galactose	28±1 (3)	44± 4 (3)	46, 16
Glucosamine	63±8 (3)	45±11 (3)	41, 52
Galactosamine	13±4 (3)	20± 1 (3)	5, 19
Glucose	15±1 (6)	101±16 (4)	223, 391

sucrose trapping was responsible for the glucose enrichment seems unreasonable since the synaptic membrane fraction contained the greatest abundance of closed compartments (membrane vesicles, mitochondria, *etc.*) and the least amount of glucose. The postsynaptic density fraction was enriched in glucose by the greatest amount and yet this fraction is nearly devoid of membranes and other potential compartments.

Authentic sucrose, even when hydrolyzed using the mild conditions of Kim *et al.* [29] yielded only an equimolar amount of glucose. The equimolar amount of fructose released during the hydrolysis of sucrose was apparently destroyed by the acid [36]. Authentic fructose, when subjected to reduction and acetylation (acid hydrolysis omitted), yielded as predicted, equal amounts of glucose and mannose at 50 % of the starting fructose concentration. Hence, the acid lability of fructose combined with the epimerization at carbon 2 prohibits the use of fructose as an internal standard to determine the amount of glucose derived from sucrose contamination.

Carbohydrate interactions

To determine if the subcellular fractions bound sucrose or other carbohydrates *in vitro*, experiments were performed utilizing radioactively labeled carbohydrates. Radioactive label from sucrose binds to the synaptic junctional complex and postsynaptic density fractions about five and ten times as much, respectively, as to the synaptic membrane fraction (Table V). Thin-layer chromatography of the label freed from the synaptic junctional complex by boiling (95 % of the total label bound) revealed that 50 % of the label bound co-migrated with sucrose.

Further analyses of the binding properties revealed several unusual characteristics. The majority of the label remained bound after precipitation with trichloroacetic acid indicating a strong bond, yet boiling and sodium dodecyl sulfate solubilization removed the majority of the label. Competition with non-radioactive sucrose reduced the radioactivity 3-fold, yet increased the concentration of non-radioactive sucrose 10-fold (50–560 mM). Since Herrmann [37] previously observed a radioactive contaminant present in some radioactively labeled carbohydrates, sequential binding experiments were performed to determine if a contaminant was present which could be adsorbed from the radioactively labeled carbohydrates. Sequential binding of 2 nCi of [^{14}C]sucrose with the synaptic junctional complex fraction revealed a marked decrease in the amount of label bound with repeated introduction to fresh protein. On the first trial, 2 % of the label bound to 0.05 mg of synaptic junctional complex protein, whereas the second and third trials revealed 0.07 and 0.01 % of the label bound, respectively, to similar amounts of synaptic junctional complex protein. It appeared that at least a part of the radioactive label bound was a contaminant. However, in light of the chromatographic results and the large quantities of glucose present in the synaptic junctional complex fraction after isolation in sucrose, it seems reasonable that a portion of the radioactive label bound in the presence of high concentrations of sucrose is [^{14}C]sucrose.

Some other radioactive carbohydrates also interacted with the synaptic membrane, synaptic junctional complex and postsynaptic density fractions (Table V). Glucose showed a binding pattern similar to sucrose; however, sequential binding experiments indicated that this binding was partly due to a radioactive contaminant. [^{14}C]Fucose also bound selectively to the synaptic junctional complex fraction, but not to the postsynaptic density fraction. Significantly, this radioactive carbohydrate

TABLE V

RADIOACTIVE LABEL BOUND FROM ^{14}C-LABELED CARBOHYDRATES TO THE SYNAPTIC MEMBRANE, SYNAPTIC JUNCTIONAL COMPLEX, AND POST-SYNAPTIC DENSITY FRACTIONS

The ^{14}C-labeled carbohydrates were incubated with the fractions at the specific activity (Ci/mol) noted in the parentheses. The labeled carbohydrates were mixed with unlabeled carbohydrates at final concentrations of 0.6 M for all but galactose, which was 0.3 M and dextran, which was 10 % (w/v). The incubation conditions were 75 min at 4 °C. The means ±S.E. are presented when three experiments were completed. n.d., not determined. The results are expressed as dpm/μg protein.

^{14}C-labeled carbohydrates	Synaptic membrane	Synaptic junctional complex	Postsynaptic density
Sucrose (0.05)	1.5, 4.5	13.2, 16.7	87, 91
Glucose (0.05)	3.0±0.7	5.3± 1.2*	11.1
Fucose (0.05)	0.2±0.1	3.2± 0.9	0.3, 2.1
Galactose (0.12)	2.5, 2.4	4.6± 0.8**	n.d.
Fructose (0.09)	0.9, 0.6	1.0, 1.4**	n.d.
Mannose (0.05)	0.2, 0.4	0.7± 0.2**	n.d.
Dextran (0.3 mC/g)	19.4, 1.7	26.4± 6.7**	n.d.

* The results differed from those for the synaptic membrane fraction with a P value less than 0.07.
** The results differed from those for the synaptic membrane fraction with a P value less than 0.2.

did not contain the radioactive contaminent previously observed, since trichloroacetic acid did not precipitate this label as it did for the radioactive contaminant [37]. The other carbohydrates tested showed little or no selectivity in binding to the synaptic junctional complex fraction as compared with the synaptic membrane fraction.

In conclusion, we demonstrate an unusually large enrichment in glucose in the synaptic junctional complex and postsynaptic density fractions, which is probably due in part to sucrose binding during isolation. Radioactive label from a variety of sugars binds selectively to these fractions.

DISCUSSION

Our data show that the major component of the synaptic junctional complex and its constituent, the postsynaptic density, is protein. Proteins make up 83 % of the synaptic junctional complex fraction and 90 % of the postsynaptic density fraction [34]. In the synaptic junctional complex fraction, and similarly in the postsynaptic density fraction, carbohydrates or lipids constitute the remaining dry weight. Most or all of the lipid in the synaptic junctional complex fraction may be associated with plasma membrane. In this case, the postsynaptic density may contain only protein and small amounts of carbohydrate. Thus, in full agreement with earlier conclusions based on cytochemical results [4, 9, 10], our data show directly that the specialized structures present at synaptic junctions consist predominantly of proteins.

Bloom and Aghajanian [10] and Pfenninger [11] have suggested that the specialized structures present at synaptic junctions are enriched in basic amino acids. They based this suggestion on the affinity of these structures for certain stains (ethanolic phosphotungstic acid and bismuth iodide-uranyl lead) and the loss of

staining after acetylation. Amino acid analysis of the synaptic junctional complex and postsynaptic density fractions which are selectively stained by ethanolic phosphotungstic acid and bismuth iodide-uranyl lead does not reveal any large increase in basic amino acids. Rather the specific staining properties may be due to an increase in the concentration of proteins which have a slightly higher concentration of basic amino acids or the presence of a few minor proteins which are unusually enriched in basic amino acids.

The total carbohydrate concentration in the synaptic junctional complex and postsynaptic density fractions is similar to that in the synaptic membrane fraction, especially after removal of glycolipids. This suggests that the carbohydrates are constituents of the synaptic junctional complex and postsynaptic density rather than of some contaminating structure in these fractions. Further evidence for the presence of carbohydrates in the synaptic junction is provided by electron microscopic analysis of fractions incubated with concanavalin A-ferritin conjugates and Ricin 120-ferritin conjugates [14, 15]. Both of these plant lectins bind selectively to carbohydrate residues on the outer surface of the postsynaptic membrane. Therefore, the presence of carbohydrates at the synaptic junction is indicated by both cytochemical data and direct analysis.

The particular carbohydrates in our synaptic membrane fraction are similar to those reported in fractions of synaptosomal membranes [38, 39]. The concentration of neutral sugars described by Gombos *et al.* [39] is similar, but that of glucosamine is higher than our results indicate. Margolis *et al.* [38] reported a relatively greater proportion of mannose compared to other sugars. The particular carbohydrates in the synaptic junctional complex and postsynaptic density fractions are also the same as in the synaptic membrane fraction; however, there are differences in concentration of protein-bound *N*-acetylneuraminic acid, galactose and mannose. *N*-Acetylneuraminic acid is reduced in both the synaptic junctional complex and postsynaptic density fractions. This reduction correlates with the decrease in membrane present in these fractions and suggests that most of the *N*-acetylneuraminic acid is membrane bound [40, 41]. Breckenridge *et al.* [42] have also observed that Triton-insoluble material is enriched in glycoproteins that do not contain *N*-acetylneuraminic acid. Galactose is present in greater concentration in the synaptic junctional complex fraction and mannose is greater in the postsynaptic density fraction. These differences from the synaptic membrane fraction may reflect the presence of particular glycopeptides in the synaptic cleft region and in the postsynaptic density.

It should be pointed out that the values shown in Table IV for the carbohydrate composition of the various fractions represent average values of fractions which are probably microheterogeneous with respect to the carbohydrate moieties. In addition, the values are not absolute since the optimum hydrolysis conditions used are probably a compromise between release and preservation of the sugar residues.

We discovered the presence of large quantities of glucose in both the synaptic junctional complex and postsynaptic density fractions. Large quantities of glucose have also been reported in other brain subcellular fractions [38, 43]. The glucose in the synaptic junctional complex and postsynaptic density fractions may arise from synaptic glycoproteins (or polysaccharides) which have large amounts of glucose and/or sucrose, present in the isolation media, which binds selectively to synaptic struc-

tures and is hydrolyzed to glucose during analysis. Since some radioactivity from [^{14}C]sucrose binds selectively to the synaptic junctional complex and postsynaptic density fractions, sucrose binding probably accounts for a portion of the glucose present in these fractions. However, since glucose has been detected in glycoproteins in brain [44], and in polymers bound to subcellular fractions [43, 45], the possibility exists that some of the glucose is present as a native constituent of the fractions. From the present experiments it is not possible to determine how much of the detected glucose was present as a native constituent and how much was derived from sucrose. Other radioactive sugars also bound to isolated synaptic junctional complex and postsynaptic density fractions.

The binding of carbohydrates to synaptic junctions and postsynaptic densities indicates that these structures may have "lectin-like" sites for certain sugars. Moreover, this binding may be selective since the radioactive sugars tested do not all bind to the same extent. The binding of radioactive fucose, for example, was enriched in synaptic junctional complex fractions but that of mannose was similar in synaptic membrane and synaptic junctional complex fractions. We would suggest that in the mature nervous system such interactions may play a role in the maintenance, and perhaps in the remodeling, of synaptic connections.

ACKNOWLEDGEMENTS

This work was supported by Grant N.I.N.D.S. NS 08597. L.C. and G.B. were supported by predoctoral fellowships from the National Institutes of Mental Health (MH 51697 and MH 50166, respectively). The authors wish to express appreciation to Dr. Jim Partridge for technical advice in the gas chromatographic analysis and to Dr. Wolfgram for the amino acid analysis. We thank Mr. David Shelton for his technical assistance.

REFERENCES

1 Sherrington, C. (1961) in The Integrative Action of the Nervous System, Yale University Press, New Haven, Conn.
2 De Robertis, E., Azcurra, J. M. and Fiszer, S. (1967) Brain Res. 5, 45–56
3 Davis, G. A. and Bloom, F. E. (1973) Brain Res. 62, 135–153
4 Cotman, C. W. and Taylor, D. (1972) J. Cell Biol. 55, 696–711
5 Cotman, C. W. Banker, G., Churchill, L. and Taylor, D. (1974) J. Cell Biol. 63, 441–455
6 Gray, E. G. (1959) J. Anat. 93, 420–433
7 Gray, E. G. (1969) Prog. Brain Res. 31, 141–155
8 Walberg, F. (1968) in Structure and Function of Inhibitory Neuronal Mechanisms (Van Euler, C., Skoglund, S. and Soderberg, U., eds.), pp. 7–14, Pergamon Press, Oxford
9 Pfenninger, K. H. (1971) J. Ultrastruct. Res. 35, 451–475
10 Bloom, F. W. and Aghajanian, G. K. (1968) J. Ultrastruct. Res. 22, 361–375
11 Pfenninger, K. H. (1971) J. Ultrastruct. Res. 34, 103–122
12 Bondareff, W. (1967) Anat. Rec. 157, 527–536
13 Rambourg, A. and Leblond, C. P. (1969) J. Cell Biol. 32, 27–53
14 Cotman, C. W. and Taylor, D. (1974) J. Cell Biol. 62, 236–242
15 Bittiger, H. and Schnebli, H. P. (1974) Nature, 249, 370–371
16 Bondareff, W. and Sjöstrand, J. (1969) Exp. Neurol. 24, 450–458
17 Cotman, C. W. and Flansburg, D. (1970) Brain Res. 22, 152–156
18 Caulfield, J. B. (1957) J. Biophys. Biochem. Cytol. 3, 827–830

19 Kellenberger, E., Ryter, A. and Sechaud, J. (1958) J. Biophys. Biochem. Cytol. 4, 671–678
20 Venable, J. H. and Coggeshall, R. (1965) J. Cell Biol. 25, 407–408
21 Bligh, E. G. and Dyer, W. (1959) Can. J. Biochem. Physiol. 37, 911–917
22 Cotman, C. W., Blank, M. L., Moehl, A. and Snyder, F. (1969) Biochemistry 8, 4606–4612
23 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
24 Ames, B. (1966) Methods Enzymol. 8, 115–118
25 Wolfgram, F. and Kotorii, K. (1968) J. Neurochem. 15, 1281–1290
26 Warren, L. (1959) J. Biol. Chem. 234, 1971–1975
27 Griggs, L. J., Post, A., White, E. R., Finkelstein, J. A., Moeckel, W. E., Holden, K. G., Zarembo, J. E. and Weisbach, J. A. (1971) Anal. Biochem. 43, 369–381
28 Yang, J. -J. and Hakomori, S. I-. (1971) J. Biol. Chem. 246, 1192–1200
29 Kim, J. H., Shome, B., Liao, T. and Pierce, J. G. (1967) Anal. Biochem. 20, 258–274
30 Fletcher, A. P., Marshall, R. D. and Neuberger, A. (1963) Biochim. Biophys. Acta 74, 311–314
31 Patterson, M. S. and Greene, R. C. (1965) Anal. Chem. 37, 854–857
32 Randerath, K. (1963) in Thin Layer Chromatography, pp. 200–203, Academic Press, New York
33 Helenius, A. and Simons, K. (1972) J. Biol. Chem. 247, 3656–3661
34 Banker, G., Churchill, L. and Cotman, C. W. (1974) J. Cell Biol. 63, 456–465
35 Hjerten, S. and Johansson, K. E. (1972) Biochim. Biophys. Acta 288, 312–325
36 Gottschalk, A. (1972) in Glycoproteins, 2nd edn., pp. 152–154, Elsevier Publishing Co., Amsterdam
37 Herrmann, H. (1974) Anal. Biochem. 59, 293–301
38 Margolis, R. K., Margolis, R. U., Preti, C. and Lai, D. (1975) Biochemistry 14, 4797–4804
39 Gombos, G., Morgan, I. G., Waehneldt, T. V., Vincendon, G. and Breckenridge, W. C. (1972) Adv. Exp. Med. Biol. 25, 101–113
40 Glick, M. C., Comstock, C. A., Cohen, M. A. and Warren, L. (1971) Biochim. Biophys. Acta 233, 247–257
41 Winzler, J. (1970) Int. Rev. Cytol. 29, 77–125
42 Breckenridge, W. C., Breckenridge, J. E. and Morgan, I. G. (1972) in Structural and Functional Proteins of the Nervous System (Davison, A. N., Mandel, P. and Morgan, I. G., eds.), pp. 135–153, Plenum Press, New York
43 Zanetta, J. -P., Morgan, I. G. and Gombos, G. (1975) Brain Res. 83, 337–348
44 Van Nieuw Amerongen, A., Van den Eijnden, D. H., Heijlman, J. and Roukema, P. A. (1972) J. Neurochem. 19, 2195–2205
45 Margolis, R. U., Margolis, R. K. and Atherton, D. M. (1972) J. Neurochem. 19, 2317–2424