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TOPOGRAPHIC STUDIES OF GLYCOPROTEINS OF INTACT SYNAPTOSOMES FROM RAT BRAIN CORTEX

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Summary

Glycoproteins in the external surface of intact synaptosomes from rat brain cortex have been studied by oxidation of exposed galactose and galactosamine groups by galactose oxidase followed by reduction with labeled sodium borohydride. Purified synaptosomes were labeled, disrupted by osmotic shock, and the particulate components were fractionated on diatrizoate to give four synaptosomal membrane fractions (A to D) and a mitochondrial pellet (E). Fractions A and B represent highly purified synaptosomal plasma membranes. After separation of their polypeptides by electrophoresis, 4/5 of the label was present in two bands: one about 72 000 and the other between 7800 and 3200 daltons. Seven other bands were labeled to various degrees: 160 000, 96 000, 53 000, 39 000, 34 000, 23 000 and 16 000 daltons. With isolated membranes (which incorporate 5—6 times more label) 4/5 of label was present in polypeptides in three ranges: 160 000—96 000, 70 000—40 000 and 7800—3200. The number of polypeptides that can be labeled by treatment of isolated membranes is very large. In comparison, glycoproteins whose topographical distribution permits interaction with large molecules at the synaptic surface are very limited. It is further suggested that the external synaptosome membrane involves a relatively tight network of interacting molecules that cannot be readily penetrated by large molecules.

Introduction

Knowledge of the chemical composition and macromolecular organization of the plasma membrane of nerve endings is essential in order to elucidate mechanisms of synaptogenesis, chemical transmitter release and reuptake, and other problems in neurobiology. Studies of axon terminals (synaptosomes) and fractions containing synaptosomal plasma membranes have shown that synaptosomal plasma membranes are enriched in both gangliosides [1—4] and glyco-

proteins [5,6] and these carbohydrate-containing molecules are present on the external surface of neuronal cells [7,8]. Glycoproteins have been localized on the surface of isolated synaptosomes as well as in the synaptic cleft by means of reactions involving lectins [9—11]. Concanavalin A and Ricinus communis binding sites have also been found on the external surface of the post-synaptic membrane facing the synaptic cleft [12] and on the outer surface of attached boutons [13]. The molecules comprising these receptor sites have been indentified by affinity chromatography [14,15] and, recently, by binding of radioactive lectins to glycoproteins following gel electrophoresis [16,17]. The possible role of these carbohydrate-containing molecules in synaptogenesis and in the development of neural pathways has been discussed [18—22].

What is now required is further information on the topographic organization of the membranes of subcellular organelles such as synaptosomes. In this paper we applied the method of Steck [23], namely, sequential application of galactose oxidase and labeled sodium borohydride to intact synaptosomes. By isolation of the membrane components, the label was localized in particular molecules which are present in the exposed surface of the membrane. The results were previously presented in part [24,25].

Materials and Methods

Chemicals. Sodium diatrizoate was purchased from Winthrop Laboratories, New York, N.Y. Tritiated sodium borohydride (approx. 244 mCi/mmol) was purchased from New England Nuclear. Sodium borohydride was obtained from Sigma Chemical Co. Tissue solubilizer (TS-1) was purchased from Research Products International (Elk Grove Village, Ill.).

Enzyme. Galactose oxidase was purchased from Kabi, Stockholm, Sweden. The specific activity of the initial lot was 180 I.U. per mg protein and that of a second lot was 715 I.U. per mg protein. Both preparations were devoid of proteases as tested by sodium dodecyl sulfate (SDS)-gel electrophoresis following digestion of bovine serum albumin and hemoglobin [26]. The enzyme showed a single band on gel electrophoresis in 10% acrylamide-SDS. The identical Coomassie Blue staining patterns obtained for membrane fractions treated with enzyme and without enzyme confirmed the absence of exogenous proteases. Absence of sialidase activity was shown by the identical ganglioside patterns obtained from the membrane fractions treated with and without enzyme.

Preparation of synaptosomes and synaptosomal membranes. Synaptosomes (P_2B fraction) were prepared from brain cortex of male Wistar rats (175—250 g) as described [27]. Synaptosome membranes, prepared by subjecting synaptosomes (labeled or unlabeled) to osmotic shock, were fractionated into 4 membrane fractions (A to D) and a mitochondrial pellet (E) as described [28]. Banding pattern and protein distribution of membrane fractions was similar from both synaptosomes.

Labeling of intact synaptosomes. Synaptosomes (P_2B fraction) were washed 2—3 times either with Krebs Ringer solution (or with 0.32 M sucrose/1 mM phosphate/0.1 mM EDTA) by centrifugation at 27 000 $\times g$ for 15 min to reduce the diatrizoate concentration as well as contaminating membrane fragments. They were then suspended in 5 ml Krebs Ringer solution and divided

into two equal portions. To one, galactose oxidase in 0.2 ml of Krebs Ringer/0.1 mM EDTA was added using 180 I.U. of enzyme for 20—30 mg of synaptosomal protein; to the other, Krebs Ringer/0.1 mM EDTA without enzyme was added. Both portions were shaken gently at 25°C for 3 h, then chilled rapidly to 4°C and centrifuged (15 min, 17 300 × *g*). The 3 h incubation time was used because this was the optimal time for glycoprotein labeling in erythrocyte membranes [29]. Glycolipid labeling may increase up to 24 h [30]. Pellets were resuspended in 2.5. ml of Krebs Ringer/EDTA and 250 μCi of tritium-labeled $NaBH_4$ in 0.4 ml of Krebs Ringer/EDTA was added. After 30 min at 25°C, 4 mg of unlabeled $NaBH_4$ was added and the reaction was continued for 15 min. The reaction mixtures were centrifuged (15 min, 15 300 × *g*). Each pellet was then suspended in 4 ml of 0.32 M sucrose, osmotically shocked and the membranes were then fractionated [28].

Labeling of isolated synaptosome membrane fractions. Synaptosome membrane fractions (A to D) were first isolated and then labeled as described above both with and without pretreatment with $NaBH_4$.

Separation of labeled membranes into lipid and protein fractions. Labeled membranes were shaken with 20 vols. of chloroform/methanol (2 : 1, v/v) and left overnight at 4°C. One-third volume of methanol was added, and after 1 h, insoluble material was collected by centrifugation (9000 × *g*, 10 min, 4°C). The protein pellet was washed with 10 ml of chloroform/methanol (2 : 1, v/v) and set aside for analysis. The organic solvent phases were combined and evaporated to obtain the total lipid fraction. Glycolipids were also extracted by a modification of Folch's procedure [31] involving reextraction of lower and interphase material twice with 'pure solvents upper phase'. Extractions were repeated with 0.88% KCl. Delipidated membrane residue was collected as described above.

Radioactivity measurements. Portions of the labeled fractions were incubated (55°C, 60 min) with 0.2 ml of TS-1 solubilizer and then counted. Counting efficiency was 35—40% as determined with internal standards. Protein was determined as described [27].

Gel electrophoresis. Delipidated proteins were analyzed in 10% polyacrylamide tube gels at pH 8.8 (0.375 M Tris · HCl buffer with 2 mM EDTA) by the procedure of Laemmli [32] as modified by Mahadik et al. [33]. Gels were stained either for glycoprotein with periodic acid-Schiff stain [34] or for total protein with Coomassie Blue. Gels were scanned in a Gilford gel scanner, then sliced (85—100 slices/100 mm gel) and radioactivity was determined for each pair of consecutive slices. Molecular weights in the labeled peaks were determined [35], using standards [33].

Slab gel electrophoresis. Gradient slab gel electrophoresis (0.375 M Tris · HCl buffer, pH 8.8 containing 2 mM EDTA) was carried out [33] using linear gradients of 5.6% to 22% acrylamide for the running gel and 3.5% acrylamide for the stacking gel. Staining patterns of the different runs were highly reproducible.

Results

Labeling of intact synaptosomes. Because of undefined chemical reactions of labeled sodium borohydride, it has been the practice to evaluate the specificity

TABLE I

LABELING OF INTACT SYNAPTOSOMES BY TREATMENT WITH GALACTOSE OXIDASE-BOROHYDRIDE: RADIOACTIVE LABEL INCORPORATED INTO MEMBRANE FRACTIONS

Average of 5 experiments (with same quantity of borohydride of equal specific activity). Results are given in thousands.

Membrane fraction	cpm per mg membrane protein			
	E + BH	BH	Δ (E + BH) − BH	*R* (E + BH)/BH
A	98.1	23.1	65.0 ± 7.9 *	4.2
B	67.5	23.5	44.0 ± 11.2	2.9
C	45.9	25.4	20.5 ± 8.0	1.8
D	27.7	25.5	2.2 ± 2.9	1.1
E	26.1	27.7	−1.6 ± 1.5	0.9

* S.E.

of labeling by means of the ratio of specific activities obtained by treatment of cells or fractions with and without the enzyme ((E + BH)/BH). However, calculation of Δ values [(E + BH) − BH] may also be very instructive. We have studied membrane fractions from synaptosomes after labeling the intact synaptosomes (Table I) and compared the results with those obtained by subjecting the isolated membrane fractions to the same procedure (Table II).

In these experiments the quantity of galactose oxidase used was greatly in excess of that required to oxidize all available galactose and *N*-acetyl galactosamine residues based on the measurements of Churchill et al. [36] and of Margolis et al. [6].

The membrane fractions obtained after labeling of intact synaptosomes showed substantial differences. The highest degree of specific labeling was found in fraction A, less in fractions B and C, and no significant increase over background in fractions D and E (Table I). These differences among the fractions were not altered by washing the synaptosomes three times (under condi-

TABLE II

LABELING OF ISOLATED SYNAPTIC MEMBRANE FRACTIONS WITH GALACTOSE OXIDASE-BOROHYDRIDE

Results are given in thousands.

Membrane fraction	After pretreatment with borohydride (cpm per mg membrane protein)				Without pretreatment by borohydride * (cpm per mg membrane protein)			
	E + BH	BH	Δ (E + BH) − BH	*R* $\frac{(E + BH)}{BH}$	E + BH	BH	Δ (E + BH) − BH	*R* $\frac{(E + BH)}{BH}$
A	426.6	53.8	372.8	7.9	743.5	307.0	436.5 ± 11.2 **	2.42
B	344.7	38.6	306.1	8.9	639.4	275.5	363.9 ± 2.9	2.32
C	167.7	36.5	131.2	4.6	—	—	—	—
D	205.0	50.0	155.0	4.1	—	—	—	—

* Average of 2 experiments.
** S.E.

tions where membrane contaminants such as myelin, vesicles or broken membranes do not sediment) prior to treatment, indicating that a contaminant of the fraction was not responsible for the differences. Without prior enzyme treatment, all the fractions showed similar specific radioactivities resulting from unknown reactions with labeled borohydride.

Labeling of isolated membranes. When isolated membranes were labeled by the galactose oxidase-borohydride method, 5—6 times more label was incorporated than when intact synaptosomes were labeled, and the membrane subfractions did not show a useful degree of discrimination. Labeling of isolated membrane fractions produced a very high background which could be reduced by pretreating these fractions with unlabeled borohydride. This procedure could not be employed with synaptosomes because their intactness did not resist the action of the borohydride. When the background was reduced in this way, the labeling after galactose oxidase treatment was found to be much more specific, showing a value of $R((E + BH)/BH)$ of 8—9 compared with approx. 2.5 for fractions A and B (Table II). Reduction of background by pretreatment with unlabeled borohydride did not appreciably alter the number of groups labeled as judged by the Δ values (373 and 306 vs. 437 and 364) representing groups made susceptible to reduction through enzyme action. Fractions A and B showed a higher degree of labeling than fractions C and D, probably due to their higher concentration of gangliosides and glycoproteins.

Partition of label between proteins and lipids. On partitioning of label between proteins and lipids obtained from the membrane fractions after labeling intact synaptosomes, about 2/3 of the specific label was found in the lipids and about 1/3 was present in the protein from membrane fractions A and B. Although the values of $R((E + BH)/BH)$ were similar, the nonspecific label incorporated in the absence of enzyme was three times higher with lipid than with protein. The Δ values showed a 16—18-fold higher level of labeling of groups appearing in the A and B membrane fractions compared to the D and E fractions.

When isolated membrane fractions were labeled, an even larger proportion of the specific label was found in lipid. The Δ values indicated that over 80% of the specific label was present in the chloroform/methanol-extractable fraction. Our earlier studies [33] showed that the lipid extract contained only negligible amounts of protein.

Characterization of labeled membrane proteins. Because the highest degree of specific labeling was associated with fractions A and B, and evidence presented earlier [28] indicated that these fractions were the purest synaptic membrane fractions, our further studies were restricted to fractions A and B.

Membrane proteins from labeled synaptosomes. Although tube gels have lower resolving capacity than slab gels, they were used initially in order to facilitate detection of glycoproteins with periodic acid-Schiff stain and also to permit instrumental scanning. Staining, scanning, and counting of 10% polyacrylamide gels revealed six labeled bands with molecular weights of 96 000, 72 000, 53 000, 33 000, 25 000 and <17 000 in membrane fraction A obtained after labeling intact synaptosomes (Fig. 1). These bands are designated 1-I to 1-VI, respectively. Band 1-VI (<17 000 daltons) contained the largest amount of label, followed in order by bands 1-II, 1-III, 1-IV, 1-I and 1-V. These

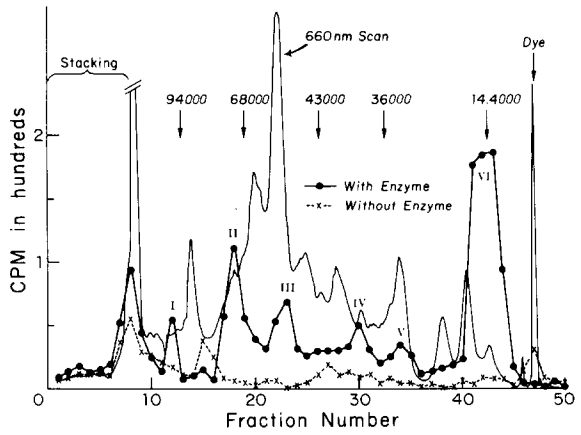


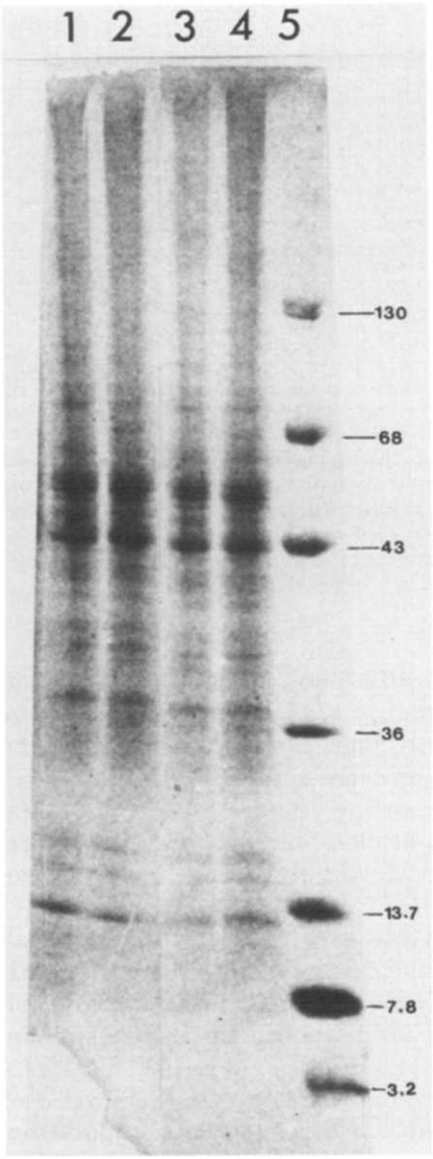
Fig. 1. Polypeptides of synaptic membrane fraction A after surface labeling of intact synaptosomes. Equal amounts of delipidated membrane protein (about 100 μg) from synaptosomes treated with galactose oxidase-[^{3}H]borohydride and with [^{3}H]borohydride alone were subjected to electrophoresis in 10% acrylamide tube gels. Gels were stained with Coomassie Blue, destained and scanned at 660 nm for proteins. Scans from both samples were very similar and, therefore, only one is shown in the figure. Both gels were sliced and radioactivity was counted (as described). Each fraction represents two slices.

bands were all detectable by periodic acid-Schiff stain. A similar labeling pattern was obtained with fraction B. The Coomassie Blue staining patterns were similar for membrane fraction A from synaptosomes treated with enzyme and without enzyme, indicating the absence of protease activity in galactose oxidase. The most intense staining was present in the region of 70 000—40 000 (Fig. 1), and represented over 60% of the total membrane protein. Therefore, most proteins were unlabeled. The intensely stained polypeptides were the predominating bands reported for fraction A [28].

A much higher degree of band resolution was obtained using gradient slab gels. The major polypeptides seen were similar to those previously reported: (A_2, A_4, A_5, C_4, C_7, C_8, C_{11}, C_{13}, D_1 and D_{11}) [28]. The staining patterns with fraction A were similar for material obtained after labeling either intact synaptosomes or isolated membranes (Fig. 2).

When intact synaptosomes were labeled, gradient slab gels (5.6—22%) of fraction A revealed only two polypeptides with relatively high incorporation, one of 70 000 and the other between 7800 and 3200 daltons (Fig. 3). Seven bands with lower incorporation were also seen: 160 000, 96 000, 53 000, 39 000, 34 000, 23 000, and 16 000 daltons. The major labeled peak (70 000 daltons) appears somewhat smaller than the labeled band 1-II (Fig. 1), probably due to calibration problems in gradient slab gels.

Membrane proteins from labeled isolated membrane fractions. When separated in tube gels, the labeled bands had molecular weights of >130 000, 96 000, 80 000, a broad band of 70 000—50 000, 34 000, 27 000 and <17 000 (Fig. 4.). These bands are designated 4-I to 4-VII, respectively. The largest amount of label was contained in band 4-IV followed in order by bands 4-V, 4-VI, 4-I, 4-VII, 4-II and 4-III. These bands were also glycoprotein-positive but the most intense stain was seen in 4-IV (range of 70 000—50 000).



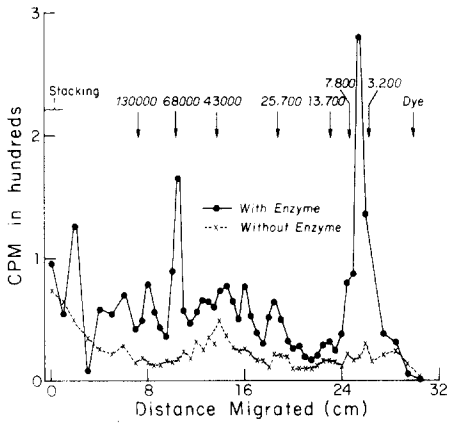


Fig. 2. Polypeptide profile of membrane Fraction A. 1, From synaptosomes treated with borohydride only; 2, from synaptosomes treated with galactose oxidase-borohydride; 3, after treatment of membrane fraction with borohydride; 4, after treatment of membrane fraction with galactose oxidase-borohydride; 5, standards (in thousands of daltons). Gradient slab gel electrophoresis, Coomassie Blue stain, 100 μg membrane protein.

Fig. 3. Gradient slab gel analysis of polypeptides from synaptic membrane fraction A obtained from labeled intact synaptosomes. Equal amounts (about 100 μg) of delipidated Fraction A membrane protein from synaptosomes labeled with galactose oxidase-borohydride and borohydride alone were run. Gels were stained with Coomassie Blue, sliced manually (as indicated), and radioactivity in the slices was determined as described.

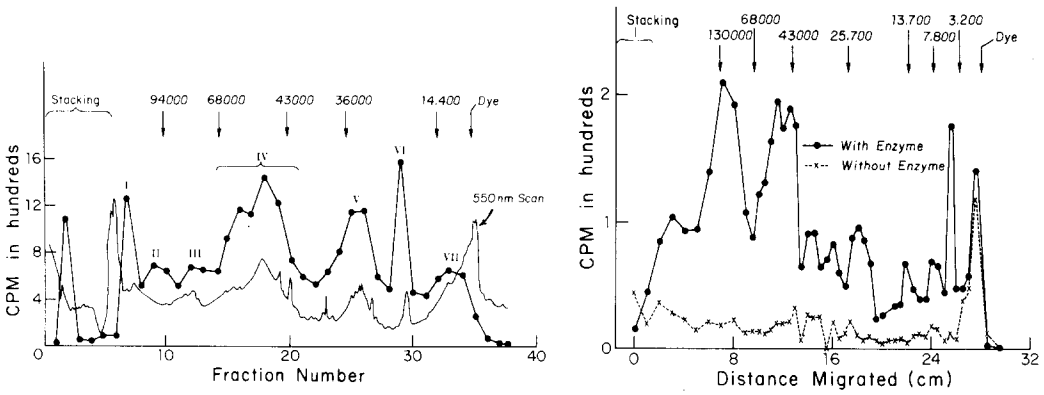


Fig. 4. Polypeptides of synaptic membrane fraction A after labeling of the isolated membrane. Delipidated labeled protein (200 μg) was subjected to electrophoresis in 10% acrylamide tube gel. The gel was stained for glycoproteins with periodic acid-Schiff and scanned at 550 nm. The gel was then sliced and radioactivity counted (as described). Each fraction represents two slices.

Fig. 5. Gradient slab analysis of polypeptides from synaptic membrane fraction A labeled after isolation. Equal amounts (about 100 μg) of delipidated Fraction A membrane protein from isolated membrane labeled with galactose oxidase-borohydride and borohydride alone were run. Gels were stained with Coomassie Blue, sliced manually (as indicated), and radioactivity in the slices was determined as described.

Gradient slab gels (5.6—22%) revealed a high degree of label incorporation into polypeptides in three ranges: 160 000 to 96 000, 70 000—40 000, and 7800—3200 (Fig. 5). A lesser degree of incorporation was seen in four bands: 39 000, 34 000, 23 000, and 16 000. Labeled proteins following pretreatment showed a similar labeling pattern, suggesting that the same groups are detected under both conditions.

Comparison of results. The major glycoproteins accessible and susceptible to galactose oxidase on intact synaptosomes are found predominantly in two bands of 72 000 and 3200—7800 daltons (bands 1-II and 1-VI), whereas the major labeled glycoprotein-positive bands obtained by treatment of the isolated membrane fractions were different (4-IV and 4-V). Most of the polypeptides in the ranges of 160 000—96 000 and 70 000—40 000 only became available for labeling after the membrane of the intact synaptosome was disrupted. The low molecular weight polypeptides (3200—7800) may arise as a result of endogenous proteolytic activity. The greater resolving power of gradient slab gels was necessary to separate polypeptides in the range of 160 000—96 000 (which probably remained at the interface of stacking and running gels in tube gel electrophoresis) and in the range below 7800.

Discussion

Although considerable evidence has now been accumulated on the protein [28,33,37—40] and lipid [1,2,41] composition of synaptic membranes, the organization of these components is still poorly understood.

At least 6 glycoprotein-positive bands were seen when fraction A polypeptides were separated in 10% tube gels. The molecular weights of 96 000, 87 000, a region extending from 70 000 to 45 000, 33 000, 23 000, and

17 000 were consistent with those reported by Morgan et al. [39].

The glycoprotein-positive region from 70 000 to 45 000 daltons corresponds in part to the glycoproteins detected by Kelly and Cotman [17] in synaptic plasma membranes using concanavalin A. They reported extensive labeling with concanavalin A in the region of 52 000—45 000 daltons. Gurd [16] reported the periodic acid-Schiff's positive region to lie between 65 000 and 35 000 daltons.

After galactose oxidase -[^{3}H]borohydride treatment of intact synaptosomes all of these bands incorporate label to some degree. However, the two most prominently labeled bands are 1-VI (17 000 daltons in tube gel; 7800—3200 in gradient slab gel) and 1-II (72 000 daltons in tube gel). It is of special interest that glycoproteins which showed the most incorporation after treatment of the isolated membrane (in the ranges of 160 000—95 000 and 70 000—50 000; and the band at 39 000) were not particularly accessible in the intact synaptosome. Recently we have shown [42] that in the gangliosides of fractions A and B from labeled synaptosomes, about 70% of the label was present in G_{M1} and only small amounts in other gangliosides. With labeled membranes, gangliosides other than G_{M1}, (G_{M2}, G_{D2}, and G_{D1b}) were also labeled appreciably. These studies show that only a fraction of the reactive molecules in synaptosomes were accessible in the intact structure since the specific activity of the polypeptides was almost 5—6-fold higher after labeling isolated membranes than after labeling intact synaptosomes. This was probably not due to incompleteness of reaction since the quantity of enzyme used per mg of protein represented a 10 000-fold excess over that required to oxidize all such groups in synaptosomal membrane proteins [6,36]. No change in the labeling pattern was seen when the enzyme concentration was doubled. Since the protein-staining patterns were similar for material obtained either from labeled synaptosomes or isolated membranes, the 5—6-fold increased labeling cannot be due to contaminants.

The question may be raised as to what kind of structural organization of synaptosomal molecules exists that permits so few of the groups that are present to react when the structure is intact. A reasonable interpretation is that the external synaptosomal membrane involves a relatively tight network of interacting molecules that cannot be readily penetrated by galactose oxidase, and that osmotic lysis causes this network to be disrupted.

Morphological analysis of membrane fractions A and B [29] showed no vesicle contamination. Although some contribution probably comes from the presence of junctional material [17] which may serve to protect external surface molecules from enzyme oxidation, the quantity of such junctional material in synaptosomes is small. This junctional material contains major polypeptides in the range of 70 000—45 000 daltons which are poor in galactosyl residues [17]. Therefore, the large differences (between labeling of synaptosomes and isolated membranes) found in this region are indicative of a structural organization of the synaptic membrane that is not associated with junctional elements. Gurd [16] also has shown by means of lectin binding that glycoproteins in the range of 65 000—34 000 daltons are non-junctional. It is possible that groups present on the internal surface of membranes can be labeled in isolated membranes, but so far there is no satisfactory evidence that carbohydrates are localized at the

cytoplasmic surface. Using galactose oxidase as the membrane-impermeable probe it has been shown that all the reactive glycoprotein and glycolipid is present only at the outer membrane surface of erythrocytes [30]. This model may serve for the plasma membranes of other cells as well.

The degree of labeling of the smaller glycopeptides (7800—3200 daltons) was higher when intact synaptosomes were subjected to this procedure than when isolated membranes were so treated. Although the staining in this region of the gel was weak, these smaller fragments were not glycolipids because membranes delipidated by two extraction procedures gave the same results. They were not free sugars because the gels had been washed well during staining and destaining procedures. The higher labeling may indicate that in the undisrupted synaptic membrane these smaller glycopeptide chains are more accessible to galactose oxidase and become buried or otherwise blocked when the structure is disrupted. Alternatively these smaller molecules may arise through the action during incubation of endogenous proteases that are more active in the intact synaptosome than in the isolated membranes. If this explanation is correct, these proteases must be very specific, since no differences were detected among any of the major polypeptide bands of comparable fraction A membranes based on Coomassie Blue stain. This problem will require further investigation with the application of protease inhibitors.

Following completion of the studies described in this paper, Wang and Mahler [43] reported on the topographic labeling by iodination techniques of rat brain synaptosomes prepared by a different method [40]. Labeled peptides on the external synaptosome surface were identified having molecular weights of 175 000, 97 000, 68 000, 54 000 and 33 000. Although it was suggested that these bands may contain glycoproteins and should thus be susceptible to labeling by the galactose oxidase-borohydride method, we find that only one of these 5 bands, that corresponding to 68 000 daltons, is appreciably labeled in our intact synaptosomes. It is of interest that we also find a very high level of labeling of lipid under our conditions of galactose oxidase-borohydride treatment, whereas the authors of ref. 43 indicate that no lipid is labeled (their experiments involve treatment only of isolated membrane fractions). These discrepancies cannot be explained. It should, however, be noted that very considerable differences exist between the materials and conditions employed by Wang and Mahler and those reported in this paper.

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