Protein Asymmetry in Chick Synaptosomal Plasma Membrane*

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The topographical arrangement of chick synaptosomal plasma membrane polypeptides was examined by lactoperoxidase-catalyzed iodination and trypsin digestion of proteins exposed at the exterior surface. The $^{125}$I-labeling profile on sucrose gradients of lysed, subfractionated synaptosomes generally coincided with the activity of the membrane marker (Na$^+$-K$^+$)-activated ATPase, which suggested that labeling was confined to the synaptic plasma membrane. Two types of experiments confirmed that lactoperoxidase had not crossed the membrane to label the interior of the synaptosome. First, trypsin digestion of labeled synaptosomes altered the Coomassie blue-staining pattern of membrane polypeptides but not the pattern of internal synaptosomal polypeptides. Second, under conditions of extensive membrane labeling, internal polypeptides remained unlabeled. Additional experiments showed that iodination was restricted to the external surface: when synaptic plasma membranes were isolated before lactoperoxidase-catalyzed iodination or incubation with trypsin, all membrane polypeptides were labeled or digested, respectively. However, relatively few polypeptides were labeled or digested when intact synaptosomes served as substrate. This supported the contention that iodination was restricted to the external surface, and, further, suggested that all polypeptides are exposed at one or both membrane surfaces. Eleven polypeptides were labeled to varying degrees by subjecting intact synaptosomes to lactoperoxidase-catalyzed iodination, e.g. lactoperoxidase-catalyzed iodination, and proteolytic digestion were used to investigate these polypeptides. Lactoperoxidase has a molecular weight of about 78,000 and is thought not to cross intact cell membranes. The iodination method depends on a series of reactions of lactoperoxidase with hydrogen peroxide and iodide (13). A lactoperoxidase-iodide complex is formed, and this iodinates primarily surface-exposed tyrosine groups to form monooiodotyrosine. Trypsin is an endopeptidase with a molecular weight of 24,000 which catalyzes the hydrolysis of peptide bonds where the carbonyl group is furnished by a basic residue. We used these enzymes to study the topographical arrangement of synaptic plasma membrane polypeptides. To successfully identify which polypeptides are exposed at the external surface using such techniques one must first demonstrate (a) the inability of the labeling reagents to enter the synaptosomes, and (b) the restriction of the effects of enzymatic modification to the external surface of the synaptic plasma membrane. This report shows that such standards have been achieved.

EXPERIMENTAL PROCEDURES

Preparation of Synaptosomes and Synaptic Plasma Membranes—Synaptosomes and synaptic plasma membranes were prepared from 6- to 8-day-old chicks (male, White Leghorn) as described previously (3). Synaptosomes were obtained from the interface between 7% and 14% Ficoll 400 (Pharmacia) in the Ficoll centrifugation gradient.

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A brain synapse is composed of a presynaptic nerve terminal and a specialization on the postsynaptic neuron which together form a synaptic junction. In the brain, each neuron may have thousands of synapses, and integration of the activity of all of the synapses at any moment largely or entirely determines the behavior of the organism. To study the chemical mechanisms of neuronal interaction, neurochemists have tried to isolate subcellular fractions rich in nerve ending particles or synaptosomes (1, 2). Synaptosomes are nerve terminals which have been sheared off nerve cells by homogenization of brain tissue and have ressealed to form intact presynaptic structures, frequently with postsynaptic material still attached.

The synaptic plasma membrane probably is the most important component involved in the function of synapses. Results on the organization of proteins in this membrane are not currently available because it is difficult to prepare material with little contamination. However, highly purified synaptosomes and synaptic plasma membranes have been isolated recently from chick brain in our laboratory (3).

The topography of polypeptides in the erythrocyte plasma membrane (4-9) and other biological membranes (10-12) lately have been extensively studied. Methods of chemical modification, e.g., lactoperoxidase-catalyzed iodination, and proteolytic digestion were used to investigate these polypeptides. Lactoperoxidase has a molecular weight of about 78,000 and is thought not to cross intact cell membranes. The iodination method depends on a series of reactions of lactoperoxidase with hydrogen peroxide and iodide (13). A lactoperoxidase-iodide complex is formed, and this iodinates primarily surface-exposed tyrosine groups to form monooiodotyrosine. Trypsin is an endopeptidase with a molecular weight of 24,000 which catalyzes the hydrolysis of peptide bonds where the carbonyl group is furnished by a basic residue. We used these enzymes to study the topographical arrangement of synaptic plasma membrane polypeptides. To successfully identify which polypeptides are exposed at the external surface using such techniques one must first demonstrate (a) the inability of the labeling reagents to enter the synaptosomes, and (b) the restriction of the effects of enzymatic modification to the external surface of the synaptic plasma membrane. This report shows that such standards have been achieved.

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Localization of Synaptosomal Membrane Proteins

Synaptic plasma membrane were isolated from the interface between 0.8 and 0.95 M sucrose in the sucrose gradient.

**Iodination of Intact Synaptosomes**—In a typical experiment, washed synaptosomes were suspended in calcium free Krebs-Ringer buffer to a concentration of 1 mg of protein/ml at room temperature. To each milliliter of suspension were added (in order) 140 μl of carrier-free NaI in 7 μl of 0.02 M NaOH (New England Nuclear, Boston, MA.) and 1.2 IU of lactoperoxidase (42 to 57 units/mg, Sigma, St. Louis, Mo.) in 150 μl of Krebs-Ringer buffer. Five 1-μl aliquots of 0.01% H2O2 were added to initiate the reaction, 90 for each addition (total 4.5 μl) to a maximal concentration of 15 μM. The amount of 125I added in all experiments was the same except under the most vigorous labeling condition, in which unlabeled NaI was also added. Other incubation conditions are described in the text and figure legends. After reaction 5 volumes of 6 mm NaI in ice-cold, calcium-free Krebs-Ringer buffer were added and the suspension was centrifuged for 17 min at 17,000 × g, and 4°C. The pellet was washed again with ice-cold Krebs-Ringer buffer and the synaptosomes were lysed to start immunoprecipitation (3).

**Iodination of Purified Synaptic Plasma Membranes**—Synaptic plasma membranes were suspended in calcium-free Krebs-Ringer buffer to a concentration of 1 mg of protein/ml and were labeled under the same conditions as for iodination of synaptosomes. At the end of the incubation, the suspension was diluted with 6 mm NaI in ice-cold, calcium-free Krebs-Ringer buffer and centrifuged (45 min at 100,000 × g, and 4°C). The pellet was resuspended and rewarshed.

**Trypsin Digestion of Labeled Intact Synaptosomes**—Washed, iodinated synaptosomes were suspended in calcium-free Krebs-Ringer buffer (final concentration 1 mg of protein/ml) and trypsin (Sigma) dissolved in the same buffer was added to a final concentration of 0.2 mg/ml. Trypsin was omitted from control experiments. Digestion proceeded for 30 min at room temperature. The reaction was stopped by addition of 0.005 volume of 0.2 M phenylmethylsulfonyl fluoride (Sigma) in ethanol. The suspension was washed twice with 5 volumes of 0.5 N NaOH was added to dissolve the pellet. All incubations were performed to show an absolute requirement for the enzyme in order to show that no other synaptosomal subfraction has a similar radioactivity is incorporated into protein (Fig. 1, Curve d). High levels of incorporation into all gradient fractions were observed. This suggested that under gentle labeling conditions iodination is restricted to the synaptosomal plasma membrane.

The occurrence of low levels of radioactivity in all gradient fractions was due to several factors. Membrane fragments obtained by synaptosomal lysis must vary widely in their size and sedimentation rate, and the sucrose gradient pellet may contain intact or broken synaptosomes. This process all fractions from the sucrose gradient with some synaptic membrane fractions and agrees with the data of Tsai et al. (9). Also, contaminants of the synaptosomal fraction such as microsomes and small pieces of myelin may be labeled during incubation and could release material into all subfractions after lysis. The levels of such contamination are probably small because the labeled bands we observe in our membrane fractions are characteristic of synaptosomal plasma membrane1 (16). Finally, labeled material could be released from the synaptosomal membrane after lysis of the synaptosomes.

**Demonstration of Intactness of Synaptosomes**—Intactness of the synaptosomes is a critical factor in studies of their surface proteins. We showed that the synaptosomal plasma membrane was intact after lactoperoxidase-catalyzed iodination by examining the effects of trypsin on synaptosomal, soluble polypeptides. If the synaptosomes are intact, trypsin should not change the electrophoretic mobility of internal polypeptides. Fig. 2 shows the Coomassie blue-staining patterns of internal, soluble polypeptides from 125I-labeled intact synaptosomes which were trypsin treated, and a pattern from labeled control synaptosomes incubated without trypsin. The

**RESULTS**

**Nature of Protein-labeling Reaction**—At neutral or basic pH the lactoperoxidase-catalyzed iodination of tyrosine is achieved without evidence of I2 formation. Thus, iodination is enzymatic, not chemical (13). The problem of labeling the external surface can be reduced to keeping lactoperoxidase outside the synaptosomes if no labeling occurs without lactoperoxidase. A control experiment without lactoperoxidase was performed to show an absolute requirement for the enzyme as follows. When synaptosomes were incubated in the complete labeling mixture without lactoperoxidase, no radioactivity was incorporated into protein (Fig. 1, Curve a). This showed that iodination was by an enzymatic, rather than a chemical, mechanism. As will be described below, this may not be the case under vigorous labeling conditions.

Many researchers have shown that under proper conditions, lactoperoxidase only iodinates proteins exposed to the outer surface of cells or organelles (4, 6, 9, 11). In our previous study (3), we found the synaptic plasma membrane fraction at the interface of 0.8/0.95 M sucrose in the final centrifugation gradient to be least contaminated based on electron microscopy and enzyme marker data. Membranes isolated from the 0.8/0.95 interface were always labeled to the greatest extent, and the distribution of radioactivity from the sucrose gradient also coincided with the distribution of (Na+/K+)-activated ATPase (a plasma membrane marker) found in the previous study (Fig. 1). The studies of enzyme activity in our previous work show that no other synaptosomal subfraction has a similar distribution. These data suggest that under gentle labeling conditions iodination is restricted to the synaptosomal plasma membrane. When the protein concentration in the incubation was reduced (Fig. 1, Curve d), high levels of incorporation into all gradient fractions were observed. This suggested that under such incubation conditions labeling might not be restricted to the few polypeptides exposed at the synaptosomal external surface.

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mic soluble proteins were precipitated with trichloroacetic acid, fluoride (final concentration 1 mM). After lysis of the synaptosomes washed with distilled water (three times), dissolved in electrophoresis sample buffer, and subjected to electrophoresis on sodium dodecyl sulfate-5.6% polyacrylamide gels which were stained with Coomassie blue. a, trypsin-treated, 0.2 mg of trypsin/mg of synaptosomal protein; b, control, no trypsin; c, synaptoplastic, soluble proteins incubated with trypsin after synaptosomal lysis (0.2 mg of trypsin/mg of soluble protein) and then precipitated with trichloroacetic acid; d, synaptoplastic, soluble proteins from unlabeled, undigested synaptosomes.

Identification of Polyepitides Exposed at One or Both Surfaces of Isolated Synaptic Plasma Membranes—When purified synaptic membranes were iodinated with 125I all major polypeptides were labeled (Fig. 3). There was little difference between the Coomassie blue-stained polyacrylamide gel patterns of polypeptides from the labeling of intact synaptosomes or purified synaptic membranes. In the gel pattern of labeled membranes about 5% of the radioactivity remained at the gel origin. When we varied the iodinating conditions we obtained qualitatively similar results, although the absolute amounts of radioactivity incorporated into polypeptides differed. These data indicate that all polypeptides have some groups capable of being iodinated by lactoperoxidase exposed at the surfaces of purified chick synaptic plasma membranes.

Identification of Polyepitides Exposed at Exterior Surface of Intact Synaptosomes—Tsai et al. (9) found that by varying incubation parameters different results were obtained for lactoperoxidase-catalyzed iodination of erythrocytes and HeLa cells. To optimize labeling conditions we incubated intact synaptosomes with lactoperoxidase under a wide variety of incubation conditions. The results are shown in Figs. 4 to 9.

Fig. 4 describes labeling of intact synaptosomes under vigorous incubation conditions. Using 24 units/ml of lactoperoxidase and 300 μM H2O2 combined with an iodide concentration of 4.2 μM (specific activity 48 μCi/mmol) resulted in high levels of incorporation into synaptic plasma membrane polypeptides. If incorporation into the larger polypeptides was greater in these experiments, the pattern would be quite similar to the labeling pattern of isolated synaptic plasma membranes (compare Figs. 3 and 4). This suggested that labeling was not confined to the external surface of the membrane. We confirmed that assertion by examining the labeling pattern of soluble, synaptoplastic polypeptides obtained after lysis of the iodinated synaptosomes (Fig. 5, Curve a). Probably all of the synaptoplastic polypeptides were labeled under these conditions. Eight picomoles per mg of iodide was incorporated into synaptoplastic total protein. The corresponding patterns are identical. When synaptoplastic proteins were isolated first and then treated with trypsin they were extensively cleaved (Fig. 2). This showed that the lactoperoxidase-labeled synaptosomes are still intact in our system, because trypsin did not digest internal polypeptides.

FIG. 1. Distribution of radioactivity on a sucrose gradient after lysis of synaptosomes labeled by lactoperoxidase-catalyzed iodination. Synaptosomes were iodinated under various conditions as described in the procedure. The concentrations of synaptosomal protein and lactoperoxidase per ml of incubating mixture and the total concentration of H2O2 were: a, 1 mg of protein, zero unit of lactoperoxidase (control), 75 μM H2O2; b, 2 mg of protein, 1.2 units of lactoperoxidase, 75 μM H2O2; c, 2 mg of protein, 6 units of lactoperoxidase, 75 μM H2O2; d, 1 mg of protein, 6 units of lactoperoxidase, 75 μM H2O2. Protein from all fractions of the sucrose gradients was precipitated with 5% trichloroacetic acid, purified, and counted as described in the procedure.

FIG. 2. Effects of trypsin digestion on internal, soluble, synaptoplastic polypeptides. Washed and iodinated intact synaptosomes (labeled as described in the procedure) were digested with trypsin in calcium-free Krebs-Ringer buffer for 30 min at room temperature. The reaction was stopped by addition of phenylmethylsulfonyl fluoride (final concentration 1 mM). After lysis of the synaptosomes and removal of particulate material by centrifugation the cytoplasmic soluble proteins were precipitated with trichloroacetic acid, washed with distilled water (three times), dissolved in electrophoresis sample buffer, and subjected to electrophoresis on sodium dodecyl sulfate-6.8% polyacrylamide gels which were stained with Coomassie blue. a, trypsin-treated, 0.2 mg of trypsin/mg of synaptosomal protein; b, control, no trypsin; c, synaptoplastic, soluble proteins incubated with trypsin after synaptosomal lysis (0.2 mg of trypsin/mg of soluble protein) and then precipitated with trichloroacetic acid; d, synaptoplastic, soluble proteins from unlabeled, undigested synaptosomes.

FIG. 3. Densitometric scan and radioactivity profile after sodium dodecyl sulfate-polyacrylomide gel electrophoresis of polypeptides labeled after lactoperoxidase-catalyzed iodination of purified synaptic plasma membranes. Purified synaptic plasma membranes were iodinated as described in the procedure at a membrane concentration of 1 mg of protein/ml; lactoperoxidase, 1.2 units/ml; and H2O2, 15 μM (total). TD indicates the position of the tracking dye.
We did not attempt to distinguish between these two possibilities by examining the effects of trypsin on the hydrogen peroxide, and I\textsuperscript{251} to 6 units/ml, 75 \mu M of lactoperoxidase, and 300 \mu M of H\textsubscript{2}O\textsubscript{2} (after five additions). After iodination, synaptic plasma membranes were purified and subjected to sodium dodecyl sulfate-tube gel electrophoresis. The gel was sliced into 1-mm slices and radioactivity was determined as described in the procedure.

Coomassie blue-staining pattern of the internal proteins from synaptosomes labeled under these conditions. The results are shown in Fig. 6. Under these conditions, no significant incorporation was found in internal (cytoplasmic, soluble) proteins (Fig. 5, Curve b). It is possible that these proteins occurred originally on the external surface of intact synaptosomes and were solubilized during labeling or isolation of the synaptic plasma membranes. We prefer the more conservative view that under these labeling conditions active iodinating species crossed the synaptosomal membrane to label internal constituents. For this reason we again reduced the levels of lactoperoxidase and hydrogen peroxide in our incubations. We kept the concentration of \textsuperscript{125}I at 0.065 \mu M to maintain sufficient levels of incorporation for unambiguous results.

Fig. 7 shows typical results from such an incubation. A Coomassie blue-staining pattern of membrane polypeptides is included with the incorporation pattern to give an inkling of the specific activities of various labeled membrane polypeptides. Under these conditions, no significant incorporation was found in internal (cytoplasmic, soluble) proteins (Fig. 5, Curve c). This suggests that labeling was restricted to the synaptosomal membrane. Despite the decreased concentrations of lactoperoxidase and hydrogen peroxide utilized in these incubations, the overall labeling pattern of synaptic plasma membrane polypeptides appeared rather similar to that shown in Fig. 6. The major difference is that labeling of the polypeptide with a molecular weight of 146,000 was no longer observed. It may be that steric hindrance prevents iodination of this polypeptide except under vigorous conditions, although the possibility that these proteins occurred originally on the external surface of intact synaptosomes and were solubilized during labeling or isolation of the synaptic plasma membranes. We prefer the more conservative view that under these labeling conditions active iodinating species crossed the synaptosomal membrane to label internal constituents. For this reason we again reduced the levels of lactoperoxidase and hydrogen peroxide in our incubations. We kept the concentration of \textsuperscript{125}I at 0.065 \mu M to maintain sufficient levels of incorporation for unambiguous results.

Not all membrane polypeptides were iodinated by these relatively gentle labeling conditions. This indicates either that lactoperoxidase-catalyzed iodination occurred only on the external surface of the intact synaptosomal membrane or that iodination occurred on both membrane surfaces but that most proteins are completely buried within the membrane and are only exposed after synaptosomal lysis and membrane isola-
Localization of Synaptosomal Membrane Proteins were iodinated as described in the procedure with 140 pCi of $^{141}$I/ml, external labeling of soluble protein. Synaptosomes (1 mg of protein/ml) were iodinated as described in the procedure with 140 pCi of $^{141}$I/ml, 1.2 units/ml of lactoperoxidase, and 15 μM H$_2$O$_2$ (total after five additions). The latter hypothesis seems untenable since literally hundreds of experiments have indicated both that lactoperoxidase can be used as a membrane-impermeable, surface-labeling reagent in a wide variety of cells and subcellular organelles and that no membrane protein has been described with no groups exposed at one or both membrane surfaces. Thus, we feel justified in concluding that lactoperoxidase catalyzes iodination only at the external surface of intact chick synaptosomes under these labeling conditions. At least nine polypeptides were iodinated by the lactoperoxidase-labeling system in this experiment (Fig. 7). They have apparent molecular weights of 130,000, 100,000, 60,000, 42,000, 34,000, 29,000, 26,000, 24,000, and 19,000. The three peaks with the largest molecular weights contained two-thirds of the radioactivity.

We also made the labeling conditions less vigorous by doubling the concentration of synaptosomal protein to 2 mg/ml (Fig. 8). The incorporation of $^{141}$I into membrane proteins decreased and the labeling of polypeptides of lower molecular weight (34,000, 29,000, and 19,000) almost disappeared. The labeling was sufficiently low that the shoulders of labeled peaks at 100,000 and 60,000 in other experiments became labeled peaks of 92,000 and 52,000.

In another set of experiments the concentrations of synaptosomal protein and lactoperoxidase were maintained at 2 mg/ml and 1.2 units/ml, respectively, but the concentration of hydrogen peroxide was increased to 45 μM by adding 15 aliquots over a period of 12 min instead of five aliquots distributed over 5 min. The labeling pattern (Fig. 9) was similar to the pattern shown in Fig. 7 except that the peak at 42,000 was more highly labeled. It appears that no single set of labeling conditions may be considered optimal because labeling under different conditions produced similar results (Figs. 7 and 9), and labeling under more than one set of conditions has provided extra information about surface-exposed polypeptides (Figs. 7 and 8). These results indicate that at least 11 major polypeptides with molecular weights of 130,000, 100,000, 92,000, 60,000, 52,000, 42,000, 34,000, 29,000, 26,000, 24,000, and 19,000 have groups exposed on the external surface of chick synaptosomes. Since all major polypeptides in isolated synaptic plasma membranes were labeled by lactoperoxidase-catalyzed iodination, we conclude that polypeptides other than these 11 either do not have iodinatable groups exposed at the exterior surface or that their groups are not iodinatable by lactoperoxidase unless vigorous labeling conditions are employed, e.g. the polypeptide of molecular weight 146,000.

Proteolytic Effects of Trypsin on Intact Synaptosomes and Isolated Synaptic Plasma Membranes – Hitzemann et al. (17) found that a trypsin concentration of 0.2 mg/ml in a synaptosomal suspension (1 mg of protein/ml in Krebs-Ringer buffer) did not alter the morphology of nerve ending particles after a 45-min incubation at 37°. However, they reported that trypsin treatment did cause a small decrease in the protein content and an appreciable loss of glycoprotein. To further study the disposition of plasma membrane polypeptides intact synaptosomes previously labeled on the outer surface with $^{141}$I were digested with trypsin. Fig. 10 shows that the synaptosomal plasma membrane polypeptide pattern and the radioactivity profile both were altered. Trypsin almost completely digested the three labeled components with molecular weights of 146,000, 130,000, and 100,000 because the Coomassie blue-stained bands and the radioactivity peaks of these components almost disappeared. About half of the radioactivity was removed from the broad, labeled peak with a molecular weight range of 66,000 to 57,000, but the Coomassie blue-stained peak was not greatly changed. We believe that this peak is a group of polypeptides not completely resolved in our gel system. New radioactivity peaks at 72,000 and 67,000 in the trypsin-treated
Localization of Synaptosomal Membrane Proteins

Mahler et al. (18, 19) have reported that polypeptides of similar molecular weights were not labeled when rat brain synaptosomes were subjected to lactoperoxidase-catalyzed iodination. Third, these large polypeptides. It is difficult to imagine what conformation they could assume so as not to be labeled by lactoperoxidase, although primarily exposed at the external surface. On the other hand, if they repeatedly crossed the membrane so that the bulk of the polypeptide chain was within the lipid bilayer it is unlikely that they would be so easily extracted by 0.6 M KCl. Finally, a precedent for such an internal localization exists in the spectrin polypeptides of erythrocyte membranes, which have molecular weights similar to these extrinsic synaptosomal membrane polypeptides. It will be interesting to see how extensive the analogy becomes.

One polypeptide which was not changed by trypsin was that at 109,000. This polypeptide was not labeled when synaptosomes were subjected to lactoperoxidase-catalyzed iodination. We conclude that this polypeptide is localized at the interior surface of the synaptosome or, if it is a transmembrane polypeptide, that a relatively small region is exposed at the exterior surface. The labeled polypeptide at 92,000 was also not modified by trypsin in these experiments. Mahler and co-workers were unable to iodinate a 92,000-dalton polypeptide in their synaptosomal membrane preparation unless the synaptosomes were previously lysed (18, 19). We do not have an explanation for this discrepancy. The data in Fig. 10 suggest that labeled peaks of 52,000 or less were not digested by trypsin. However, migration of labeled products of trypsin digestion of higher molecular weight species in this region precludes certainty about this point.

Performing the trypsin incubation with a more dilute solution of trypsin (0.1 mg/ml) yielded essentially identical results (not shown). Interestingly, there was no difference in the pattern and degree of labeling if synaptosomes were iodinated first and then digested with trypsin or were first digested and then iodinated. This suggests that these levels of trypsin do not uncover "cryptic" sites as has been reported by Morrison and co-workers after trypsin digestion of intact erythrocytes (20).

When isolated synaptic plasma membranes were treated with trypsin (0.2 mg/mg of membrane protein) in calcium-free Krebs-Ringer buffer not all polypeptides were completely digested (Fig. 11a). Large peptides persisted despite our increasing the trypsin concentration to 0.5 mg/mg of membrane protein. This suggested that some polypeptides are not accessible to trypsin and appears to conflict with our previous data (Fig. 3) indicating that all proteins can be labeled by lactoperoxidase-catalyzed iodination under similar incubation conditions. A possible explanation of this apparent contradiction is that some of the synaptic membranes have resealed. If that were so, lactoperoxidase could still label proteins of unsealed membranes and a polyacrylamide gel polypeptide pattern would contain label in every peak. The polypeptide pattern after trypsin digestion would still show high molecular weight peaks from polypeptides located on the internal surface of the resealed synaptic membranes. To test this possibility we incubated synaptic membranes with 0.2 mg of trypsin/mg of membrane protein in a hypotonic medium (1 mM KPO4, 0.1 mM EDTA, pH 8.0) which should decrease the ability of the synaptic membranes to reseal. Under these conditions all polypeptides were digested (Fig. 11b). This suggests that all polypeptides of lysed and nonresealed chick synaptic plasma membranes were accessible to trypsin. This also supports our previous conclusion that all major polypeptides are exposed to at

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**Fig. 10.** Effects of trypsin on intact, iodinated synaptosomes. Synaptosomes were iodinated as described in Fig. 7. They were then suspended in calcium-free Krebs-Ringer buffer (1 mg of protein/ml) and digested with 0.2 mg/ml of trypsin for 30 min at room temperature. The reaction was stopped by addition of phenylmethylsulfonyl fluoride (final concentration 1 mM). Synaptosomes were washed twice and then lysed to prepare synaptic plasma membranes. a, control, incubated without trypsin; b, trypsin-treated. TD indicates the position of the tracking dye. Synaptosomal pattern were undoubtedly digestion products of higher molecular weight material because they did not appear in patterns from undigested membranes. Faster mobility of digestion products probably accounted for the increased size of peaks of radioactivity and Coomassie blue staining at 29,000 and 26,000 observed after surface digestion (Fig. 10).

We were surprised to note that polypeptides with molecular weights of 200,000 and 186,000 also were largely removed from the membrane pattern by trypsin digestion of synaptosomes even though they are not labeled by lactoperoxidase. They may be exposed at the external surface of the membrane and are digested by trypsin but do not have groups exposed for lactoperoxidase-catalyzed iodination. It is also possible that these polypeptides either cross the membrane but are exposed primarily at the interior surface or that they are loosely bound to the interior surface and are released from the membrane through changes induced by trypsin. We favor the latter hypothesis for several reasons. First, we have found that these are extrinsic polypeptides which can be removed by extraction of the membranes with 0.6 M KC1 or KI at 0° for 2 h. Second,
plasmasome, soluble polypeptides were not digested by trypsin during incubation of iodinated synaptosomes. Second, it must be demonstrated that only the external membrane surface is labeled. This was accomplished by showing that iodination was entirely enzymatic and did not proceed in the absence of enzyme, and that relatively few major membrane polypeptides were labeled by lactoperoxidase-catalyzed iodination. For this reason, we have not observed such a major polypeptide in our chick membrane preparations. The other surface-exposed polypeptides of rat membranes appear to have counterparts in the chick preparation. This is not surprising since the Coomassie blue-staining patterns of polyacrylamide gels of chick and rat synaptic plasma membranes appear quite similar. Our data using lactoperoxidase also agree with that of Mahler and co-workers in that polypeptides in both membranes with molecular weights of approximately 200,000, 185,000, and 39,000 do not have external surface groups capable of being labeled by lactoperoxidase-catalyzed iodination. However, Mahler reported that a major polypeptide of molecular weight 92,000 is localized on the interior surface of the membrane because it was not iodinated by lactoperoxidase. We find a labeled peak at the same molecular weight in our system. Whether the nature and location of these two polypeptides of similar molecular weight are homologous or not will require additional experimentation.

Contamination of the synaptosomes by other subcellular elements might cause problems in correctly interpreting the results. However, the most likely contaminants such as microsomes, fragments of mitochondrial, myelin, or glial membranes or broken synaptosomes themselves would be more impermeable to lactoperoxidase than synaptosomes. Polypeptides of such contaminants should be highly labeled after lactoperoxidase-catalyzed iodination. This is supported by our finding that all the polypeptides of a typical microsomal preparation contain label after lactoperoxidase-catalyzed iodination (not shown). One would expect many peaks of radioactivity in the gel patterns of polypeptides from intact synaptosomes if substantial levels of contamination persisted in the isolated membranes. High levels of contamination of the purified synaptic plasma membranes probably did not occur because relatively few peaks of radioactivity are observed. Still, the peak-to-background ratios of incorporation we observe are somewhat lower than are usually reported in erythrocyte-labeling experiments. Low levels of contamination by labeled broken synaptosomes or other subcellular organelles provides one possible explanation. Another is that this background may be the result of lactoperoxidase-catalyzed iodination of minor proteins of the synaptosomal external surface. Erythrocyte preparations contain only one cell type, but these synaptosomal preparations are heterogeneous with respect to the transmitters they contain. That such a mixture of synaptosomes could yield a relatively simple polypeptide pattern after poly-
acrylamide gel electrophoresis of their membranes has surprised some workers, but such major bands must represent polypeptides serving a common role in the membranes of all synapses. Structural polypeptides and polypeptides involved in the mechanism of neural excitation-secretion coupling are two possible examples. Polypeptides unique to the external surfaces of synaptosomes utilizing particular transmitters such as presynaptic receptor proteins or transport systems might occur in much smaller amounts. After surface labeling of a synaptosomal mixture the large number of such labeled polypeptides will be observed as a high background on a one-dimensional polyacrylamide gel.

Another problem is the ability of synaptosomes to endocytose portions of their plasma membrane for the purposes of synaptic vesicle re-formation and recycling subsequent to synaptic stimulation and vesicle fusion with the membrane. As described by Ceccarelli et al. (22) and by Heuser and Reese (23) this process requires calcium ions and does not occur when calcium is replaced by magnesium. To avoid the internal labeling which such exocytosis and endocytosis might engender we performed the labeling incubations in calcium-free media and kept the incubation time short (4 to 5 min). Also, Heuser and Reese never observed diffusion of material from vesicles or cisternae into the synaptoplasm during vesicle recycling. For these reasons we feel confident that our results need not be questioned because of synaptic vesicle exocytosis or endocytosis.

It is unfortunate that not every polypeptide which was labeled by lactoperoxidase-catalyzed iodination of intact chick brain synaptosomes was also digested by trypsin. Trypsin rarely removes most of the iodide attached to membranes by lactoperoxidase (6,24). Our purpose in digesting synaptosomes was to demonstrate intactness of the synapto- lactic stimulation and vesicle fusion with the membrane. As mentioned these peaks with molecular weights of 92,000 and 52,000 were simply shoulders of a larger peak. This experience suggests that maximizing the number of surface-exposed polypeptides which are identified requires that experiments be performed at several different levels of labeling intensity.

After submission of this manuscript Wang and Mahler gave a full report of their studies on the topography of the rat synaptosomal membrane (25). Their data are similar to ours and they have now reassigned the polypeptide at 92,000 daltons to the external surface of rat synaptosomal plasma membrane.

REFERENCES

Protein asymmetry in chick synaptosomal plasma membrane.

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