Proc. Nat. Acad. Sci. USA
Vol. 70, No. 6, pp. 1682–1687, June 1973

Glycopeptides from Surface Membranes of Neuroblastoma Cells

(trypsinization/axon-forming cells)

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Communicated by Marshall Nirenberg, March 26, 1973

ABSTRACT **Sequential removal of surface glycopeptides was achieved by subjection of mouse neuroblastoma cells to a two-step trypsin treatment under different conditions. The glycopetides released by each trypsinization step were digested by Pronase and examined on columns of Sephadex G-50. Different chromatographic patterns were found for the two digests. Thus, several groups of glycopeptides can be distinguished by the trypsinization procedure. One group is readily removed and appears to be at a more accessible location on the cell surface. Among the four neuroblastoma clones examined, the glycopeptide patterns from axon-forming cells differed from those of axon-minus cells.**

It is generally accepted that the conductance of impulse in nerve cells depends on specific changes in the ionic permeability of the surface membrane. These membranes are involved in the formation of axons and dendrites, the specificity of neuron junctions, and the formation of the synaptic structure.

Glycoproteins appear to be on the outermost surface of all cells examined (1–3) and have been implicated in cell recognition (4, 5) and contact (6, 7). It has been suggested that sialic acid, a terminal component of glycoproteins and glycolipids may be fundamental in processes occurring during excitation of nerve cells (8).

As part of an approach to these problems, we have examined the glycoproteins from the cell surface of clones isolated from a mouse neuroblastoma, C-1300. These clones differ in their capacity to extend neurites, to synthesize various neurotransmitters, and to respond to electrical stimuli (9). In this study a trypsinization procedure has been developed by which several groups of glycopeptides are distinguished. One group is readily removed and appears to be at a more accessible location on the cell surface. A preliminary report has been published (10).

MATERIALS AND METHODS

Materials. L-[^{14}C]Fucose (50 Ci/mol) and L-[^{3}H]fucose (4.8 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass., and D-[^{14}C]glucosamine (318 Ci/mol) and D-[^{3}H]glucosamine (6.2 Ci/mmol) were obtained from the Radiochemical Center, Amersham, England. Lyophilized, three-times-crystallized trypsin (180 units/mg) and purified soybean trypsin inhibitor were obtained from Worthington Biochemicals, Freehold, N.J. Pronase was obtained from Calbiochem.

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Cell Cultures. Clones N-18, NS-20, N-1, and N1A-103 derived from a mouse neuroblastoma C-1300 (9), were obtained from Dr. M. Nirenberg, NIH, Bethesda, Md. Clone N-18, which is inactive with respect to tyrosine hydroxylase (EC 1.14.3a) and choline acetyltransferase (EC 2.3.1.6), contains acetylcholinesterase (EC 3.1.1.7) and is able to extend neurites in the absence of serum. Clone NS-20, a cholinergenic clone, contains choline acetyltransferase and acetylcholinesterase and is able to form neurites but does not contain detectable activity of tyrosine hydroxylase. Clone N-1 contains low activity of tyrosine hydroxylase, choline acetyltransferase, and acetylcholinesterase and does not form neurites. Clone N1A-103, a subclone of N-1, is almost devo'd of tyrosine hydroxylase and choline acetyltransferase activities, has a low acetylcholinesterase activity, and is an axon-minus line (9).

The cells were grown in Dulbecco modified Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.) containing 1.2 g of $NaHCO_3$ per liter and supplemented with 10% fetal-calf serum (Colorado Serum Co.), penicillin (100 U/ml), and streptomycin (100 μg/ml), in an atmosphere of 5% CO_2–95% air with 100% humidity at 37°. 5×10^5 cells in the exponential phase of growth were seeded on 90-mm petri dishes (NUNC, Denmark) and grown in 10 ml of medium. The medium was replaced every second day until the cells reached confluency. At this time the medium was replaced with 7 ml of fresh medium containing 3 μCi of radioactive L-fucose or D-glucosamine. After 48 hr the labeled medium was removed from each culture dish and the adhering cells were washed 4–5 times with the specified solution.

Differential Removal of Surface Glycopeptides. The radioactive cells were subjected to a two-step trypsin treatment under different conditions. The material obtained from each of the steps was designated "trypsinate."

Trypsinate A—Cells were removed from the monolayers by a modification of a previous trypsinization method (11). After the cells were washed with 5 ml of 0.16 M NaCl, 2 ml of unbuffered (about pH 6.5) trypsin solution (25 μg/ml in 0.16 M NaCl) was added to each culture dish and incubated for 10 min at room temperature (25°). At the end of this period, 1 ml of soybean trypsin-inhibitor solution (50 μg/ml in 0.16 M NaCl) was added; the cells were cooled and centrifuged at $65 \times g$ for 5 min. The radioactive supernatant solution was collected and designated "trypsinate A."

Trypsinate B—The pellet of cells (about 5×10^7) from trypsinate A was resuspended in 1 ml of cold 0.15 M NaCl–0.02 M Tris·HCl (pH 7.5) (TBS) containing 0.5 mg of trypsin. After 2 min at room temperature, 0.5 ml of soybean trypsin

inhibitor (1 mg/ml of TBS) was added and the cells were centrifuged for 5 min at 65 × *g*. The radioactive supernatant solution was designated "trypsinate B."

Standard Procedure for Removal of Surface Glycopeptides. This trypsinization procedure has been used previously to remove glycopeptides from the surfaces of various cells (12, 13).

Trypsinate S—The cells were washed on the monolayer with 5 ml of TBS and treated with 1 ml of trypsin (3 mg/10 ml of TBS) for 4 min at room temperature according to the procedure of Buck *et al.* (12). The radioactive supernatant solution was designated "trypsinate S."

Treatment of Trypsinates and Cells. The various trypsinates were centrifuged at 49,000 × *g* for 20 min, and the supernatant solution was lyophilized and dissolved in water before use.

Cells remaining after the removal of trypsinates A and B and the cells from trypsinate S were washed 3 times with 0.16 M NaCl and aliquots were removed for scintillation counting.

Surface Membranes. Surface membranes were prepared by the Zn-ion procedure (14) from the saline-washed pellet of cells after the removal of trypsinate S.

Gel Filtration of Surface Glycopeptides. Whenever two trypsinates were compared they were combined and digested with Pronase before chromatography on a Sephadex G-50 column (12). The column fractions (0.65 ml) were mixed with 0.5 ml of H_2O and 9 ml of toluene–Triton X-100 scintillation mixture and counted.

RESULTS

Surface-membrane glycopeptides

The radioactive glycopeptides were removed from the surface of clone N-1 cells by trypsinization (trypsinate S), and surface membranes were isolated from the cells of the same experiment from which the trypsinate was obtained. The trypsinate and surface-membrane fractions were treated with Pronase and then examined by chromatography on Sephadex G-50 columns. Fig. 1*a* demonstrates that the glucosamine-containing compounds from trypsinate S were eluted in three major regions. The first region consists of material that was eluted with the blue dextran marker and may contain some mucopolysaccharides. The material eluted in the middle region of the profile (fractions 20–40) showed a major component followed by a shoulder. The enrichment with more rapidly-migrating material (fractions 20–33) in this region is typical for the pattern obtained from virus-transformed cells (12). The third region consists of material migrating ahead of the phenol red marker.

The fact that the glycopeptides that were eluted in the second region are found at the cell surface was shown by examining the glycopeptides of isolated surface membranes. Fig. 1*b* shows that the pattern from the isolated surface membranes is similar to that obtained from the trypsinate. It further shows that the third region (fractions 50–65), which may contain free glucosamine, is not found in the surface membranes.

Differential removal of glycopeptides

The possibility of differentiating between various surface glycopeptides was investigated. It was found that by varying the standard trypsinization conditions (trypsinate S), two

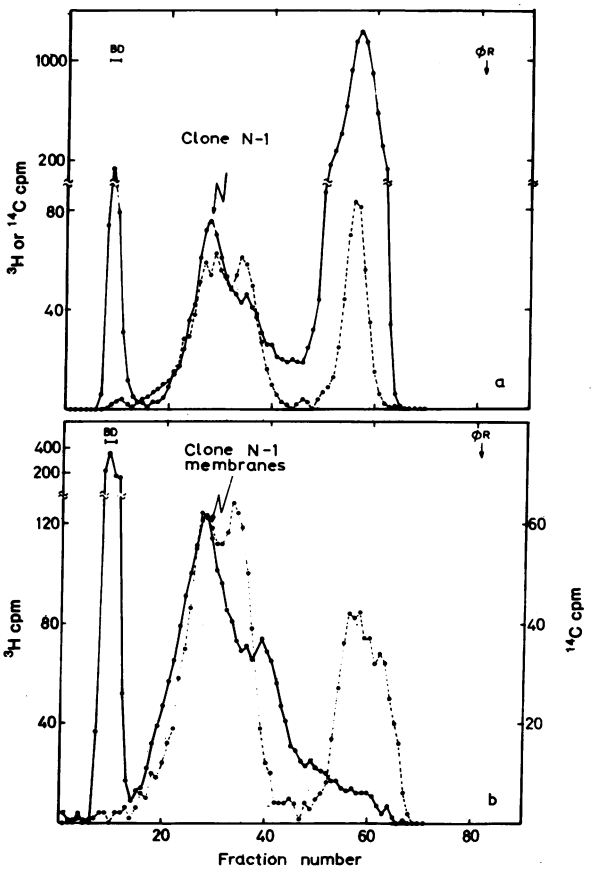

FIG. 1. Chromatography on Sephadex G-50 of Pronase-digested trypsinate S or surface membranes from clone N-1 cells. Cells were grown, labeled, harvested, trypsinized, and digested by Pronase as described in *Methods*. A Sephadex G-50 fine column (0.8 × 100 cm) was developed with 0.1 M Tris-acetate (pH 9.0)–0.1% Na dodecyl SO_4–0.1% 2-mercaptoethanol–0.01% EDTA. *BD* represents fractions in which the blue dextran 2000 was eluted. *φR* represents the first tube in which the phenol-red marker appeared. (*a*) Profile obtained by cochromatography of Pronase digests of pooled trypsinate S from clone N-1 cells grown in presence of D-[^{3}H]glucosamine and hamster-embryo cells transformed by polyoma virus grown in presence of L-[^{14}C]fucose. The latter served as reference marker. (*b*) Profile obtained by cochromatography of Pronase-digested surface membranes from clone N-1 cells grown in presence of D-[^{3}H]-glucosamine and the reference marker as used in (*a*). ^{3}H, ●——●; ^{14}C, O- - -O.

groups of glycopeptides can be sequentially removed. The first group is removed by treating the cells with very low concentrations of trypsin in unbuffered saline (trypsinate A), whereas, the second group is removed in the subsequent step that uses higher concentrations of trypsin in TBS (trypsinate B). It should be noted that conditions for trypsinization used in the second step are essentially the same as those used with trypsinate S.

Glucosamine-Containing Glycopeptides. The chromatographic patterns of the Pronase-digested trypsinates A and B of N-1 cells are shown in Fig. 2*c* and *d*. Comparison between the two patterns reveals that the most striking difference is found in the middle region of the profile (fractions 20–40). Fig. 2*c* shows that the first trypsinization step (trypsinate A) removes two groups of glycopeptides from the cell surface

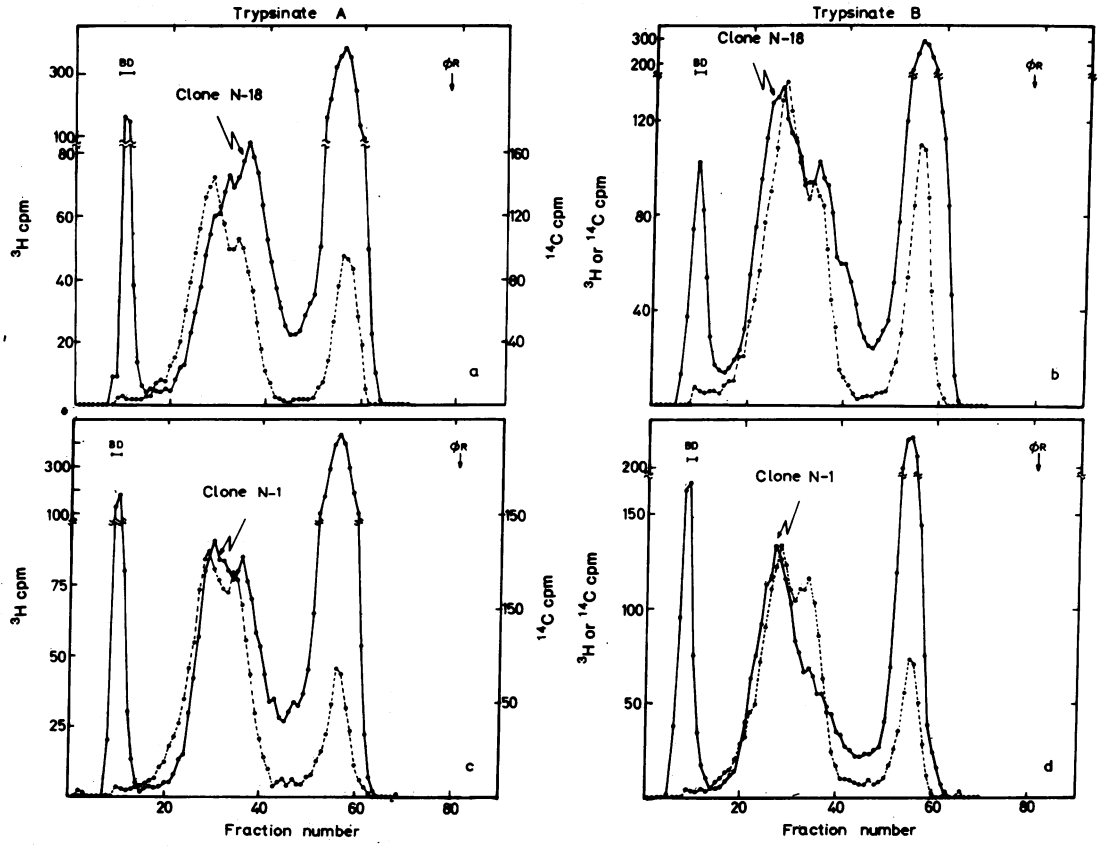

FIG. 2. Chromatography on Sephadex G-50 of Pronase-digested trypsinates A or B from cells of clone N-1 or N-18 grown in the presence of D-[^{3}H]glucosamine. The trypsinates and Pronase digests were prepared as described in *Methods*. Cochromatography of Pronase digests of (*a*) trypsinate A or (*b*) trypsinate B of cells of clone N-18 (●——●); or (*c*) trypsinate A or (*d*) trypsinate B of cells of clone N-1 (●——●). Before Pronase digestion each of these trypsinates was combined with the reference marker (○- - -○) as described in Fig. 1*a*.

that were eluted in fractions 20–32 and 33–40. In contrast, trypsinate B (Fig. 2*d*) contains very little of the slower moving components (fractions 33–40).

Of the total radioactive glucosamine incorporated into the cells of clone N-1, 37% was found on the cell surface (Table 1). The first trypsinization (trypsinate A) removed 7% of the total radioactivity while the subsequent trypsinization removed an additional 3%. Similar results were obtained for clone N-18 cells. Trypsinate S removed 16% of the incorporated radioactivity, which is somewhat more than the total amount of trypsinates A and B.

Fucose-Containing Glycopeptides. The chromatographic patterns of the fucose-containing glycopeptides from N-1 cells are shown in Fig. 3*c* and *d*. The radioactivity is distributed mainly in two regions. The distribution of radioactivity in the middle region (fractions 20–40) is similar but not identical to that obtained when labeled glucosamine was used as a precursor (Fig. 2*c* and *d*). As in the latter case, the slower-migrating components (fractions 33–40, Fig. 3*c*) are more pronounced in the patterns of trypsinate A than those of trypsinate B. Thus, varying the conditions of trypsinization removes different groups of glycopeptides from the cell surface.

Comparison of the glycopeptides from the surface of various neuroblastoma clones

The glycopeptides of some neuroblastoma clones were examined by growing the cells in the presence of radioactive D-glucosamine or L-fucose and subjecting them to the two-step trypsin treatment. Figs. 2 and 3 compare the chromatographic patterns of the Pronase digests of trypsinates from the surface of clone N-18 to those obtained from clone N-1 cells. Fig. 4 compares the patterns of clones N1A-103 and NS-20 cells to those of N-18 cells.

Different chromatographic patterns were found for trypsinates A and B regardless of the radioactive precursor or the clone used. It is also clear that the slower-migrating components in the middle region (fractions 33–40) are removed more readily and appear mainly in the trypsinate A digest. In clones N-1 and N1A-103, substantial amounts of the faster-migrating components (fractions 23–32) are also removed in the first trypsinization.

Fig. 2 shows that when glucosamine was used to label the glycoproteins, different patterns were obtained for axon-minus N-1 and inactive N-18 cells. More of the faster-moving glycopeptides (fractions 23–32) were found in trypsinate A of N-1 than in N-18 cells. A similar difference was observed in trypsinate A of L-fucose-labeled cells (Fig. 3*a* and *c*). In addition, trypsinate B of N-18 cells had more of the slower-migrating component (tubes 33–40) when compared to N-1 cells.

The patterns for the fucose-containing glycopeptides of the cholinergic NS-20 cells are rather similar to that found for the inactive N-18 cells. On the other hand, the profiles from clone N-1 (Fig. 3) and its subclone N1A-103, both axon-minus cells, resemble each other and differ from those of N-18 and NS-20

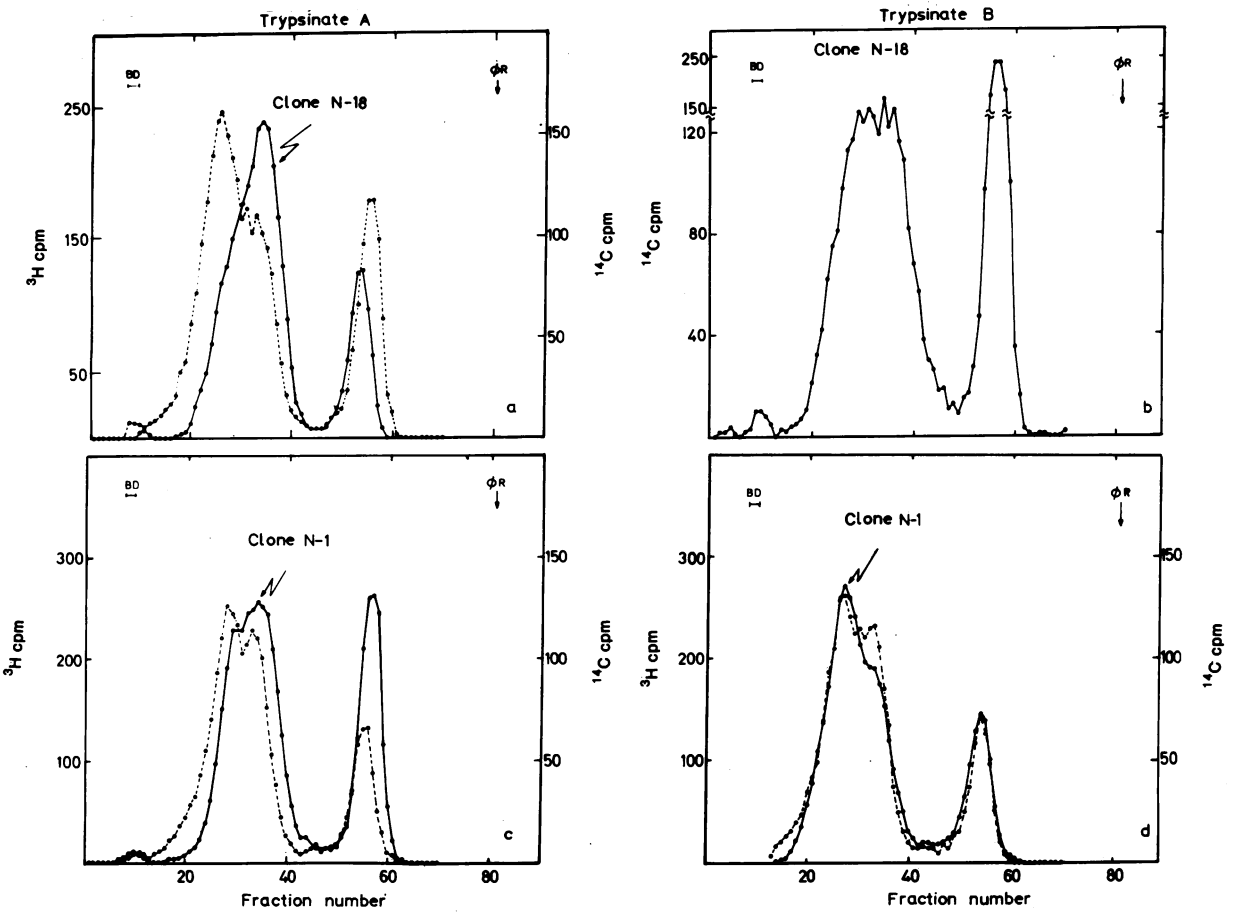

FIG. 3. Chromatography on Sephadex G-50 of Pronase-digested trypsinates A or B from cells of clones N-1 or N-18 grown in the presence of L-[^{3}H]- or [^{14}C]fucose. Cochromatography of Pronase digests of (*a*) trypsinate A or (*b*) trypsinate B of cells of clone N-18 (●——●), or (*c*) trypsinate A or (*d*) trypsinate B of cells of Clone N-1 (●——●). Before Pronase digestion, each of these trypsinates with the exception of (*b*) was combined with the reference marker (○- - -○) described in Fig. 1*a*.

cells (Fig. 4). Similar results were obtained with the glucosamine-containing glycopeptides.

DISCUSSION

We have devised a two-step trypsinization method, the purpose of which is to remove sequentially different groups of glycopeptides from the cell surface. This method appears to be a general one and has been used successfully to remove specific glycopeptides not only from the surface of neuroblastoma cells but also from hamster embryo cells transformed by polyoma virus (10).

To examine more closely the carbohydrate moieties in the material removed by trypsin (trypsinates), the peptide portions of the glycopeptides were subjected to extensive digestion with Pronase, making it possible to obtain the carbohydrate units of these molecules with only a few amino acids attached. These glycopeptides can be divided into several groups by chromatography on Sephadex G-50 columns. They may arise, for example, by cleavage of one glycoprotein molecule carrying several different branched groups or by proteolysis of different glycoprotein molecules residing on the cell surface.

By these procedures our results indicate that, indeed, the surface-membrane glycopeptides of all the neuroblastoma clones tested can be subdivided into several groups, one of which (trypsinate A) is more readily removed from the cell surface. It is reasonable to assume that this group is at a more accessible location on the cell membrane. The second group that is removed after a subsequent trypsinization (trypsinate B) is probably buried more deeply in the surface membrane or, perhaps, is folded in a configuration not available for trypsin action under the particular condition used. These differences can undoubtedly be used to give further insight into membrane structure.

The surface-membrane glycopeptides from virus-transformed fibroblasts have been found to differ from those of their normal counterparts (12, 13). As shown in the present work, the patterns of the glycopeptides obtained from the

TABLE 1. *Percentage of radioactive glucosamine found in cell fractions after trypsin digestion*

Clone	Trypsinate A	Trypsinate B	Trypsinate S	Surface membranes	Wash	Remainder
	Percentage of total counts/min					
N-1	—	—	16	21	ND*	63
N-1	7	3	—	—	4	86
N-18	6	4	—	—	4	88

* ND denotes not done

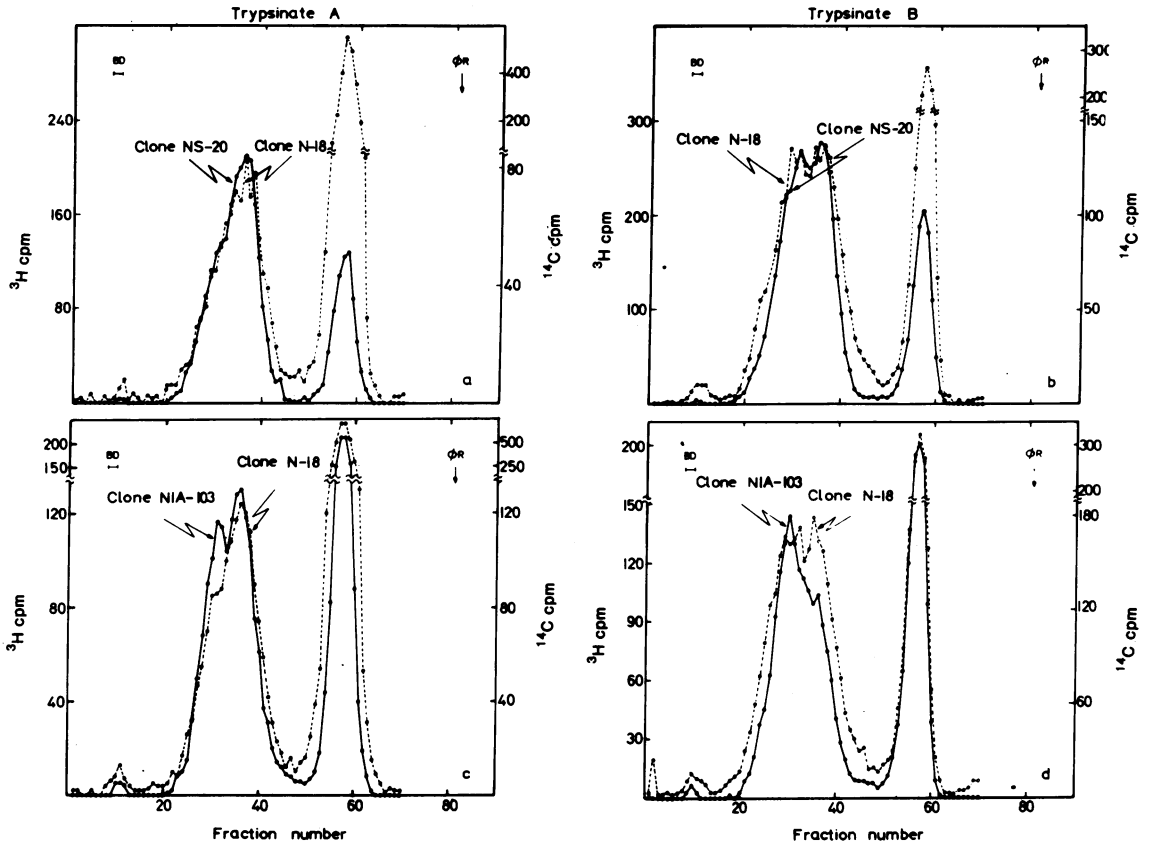

FIG. 4. Cochromatography on Sephadex G-50 of Pronase-digested trypsinates from clones N-18, NS-20, and N1A-103 cells grown in presence of L-[^{14}C]- or [^{3}H]fucose. Cochromatography of Pronase digests of pooled (*a*) trypsinates A or (*b*) trypsinates B from cells of clone N-18 (O- - -O) and NS-20 (●——●); or (*c*) trypsinates A or (*d*) trypsinates B from cells of clone N-18 (O- - -O) and N1A-103 (●——●) grown in the presence of L-[^{14}C]fucose (O- - -O) or L-[^{3}H]fucose (●——●).

surface of neuroblastoma clones resemble those of virus-transformed cells rather than those of nontransformed cells. However, the clones of neuroblastoma cells that form axons show less of the virus-characteristic glycopeptides than observed in the axon-minus clones. Thus, the clones that are capable of differentiating show a more "normal" glycopeptide pattern. It should be noted that by the two-step trypsinization procedure, the first group of glycopeptides removed (trypsinate A) shows a chromatographic pattern similar to that observed for the nontransformed cells. Only the second trypsinization step (trypsinate B) reveals the glycopeptide groups more characteristic of the virus-transformed cells.

Certain clones of neuroblastoma cells can be induced to differentiate. It was shown that the differentiated state of these cells is characterized by high activities of certain enzymes, the extension of axons, and the development of an excitable membrane (9, 15). It was also shown that all of these characteristics are not necessarily genetically linked (15). It is possible that the process of differentiation of neuroblastoma cells may be accompanied by a change in the surface glycopeptides (16). However, it has been observed recently that the patterns of surface glycopeptides vary for baby hamster kidney cells maintained in log phase or at confluency (17). Such differences have also been observed by us for neuroblastoma cells (unpublished results). Since the confluent neuroblastoma cells are closer in some of their properties to the differentiated state (18), we performed our present studies with cells in confluency in an attempt to magnify the differences in the various clones. Indeed, less differences were observed in the surface-glycopeptide patterns of log-phase cells (unpublished results).

Comparison of the surface glycopeptides of the various clones reveals certain trends. The glycopeptide pattern from cells (NS-20) that have a high capacity to synthesize acetylcholine has a great resemblance to that obtained from cells (N-18) that are inactive in their ability to synthesize adrenergic or cholinergic transmitters. Therefore, no conclusion can be made, as yet, about the correlation between enzymic capacities of the cells from different clones and their surface-glycopeptide pattern. However, of more interest is the fact that these two clones, which have a similar glycopeptide pattern, are able also to extend axons and develop an excitable membrane. Moreover, the glycopeptide patterns of the two axon-minus clones (N-1 and N1A-103) resemble each other and are significantly different from those obtained from the axon-forming cells (N-18 and NS-20). Thus, it may be possible that a positive correlation exists between the surface glycopeptides and axon formation.

We thank Dr. M. Nirenberg, NIH, for the clones of neuroblastoma cells. The excellent technical assistance by Mrs. J. Barak and Mr. D. Giveon is acknowledged. This investigation was supported in part by American Cancer Society Grant PRA-68 and by U.S. Public Health Service Agreement no. 455114.

1. Winzler, R. J. (1970) *Int. Rev. Cytol.* **29**, 77–125.

2. Bretscher, M. S. (1971) *Nature New Biol.* **231**, 229–232.
3. Poduslo, J. F., Greenberg, C. S. & Glick, M. C. (1972) *Biochemistry* **11**, 2616–2621.
4. Gesner, B. M. & Ginsberg, V. (1964) *Proc. Nat. Acad. Sci. USA* **52**, 750–755.
5. Morell, A. G., Gregoriadis, G., Scheinberg, I. H., Hickman, J., Jr. & Ashwell, G. (1971) *J. Biol. Chem.* **246**, 1461–1467.
6. Moscona, A. A. (1965) in *Cells and Tissues in Culture*, ed. Wilmer, E. N. (Academic Press, New York), Vol. I, pp. 489–529.
7. Roth, S. & White, D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 485–489.
8. Lehninger, A. (1968) *Proc. Nat. Acad. Sci. USA* **60**, 1069–1080.
9. Amano, T., Richelson, E. & Nirenberg, M. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 258–263.
10. Glick, M. C., Kimhi, Y. & Littauer, U. Z. (1973) *Fed. Proc.* **32**, 1714 abstr.
11. Littauer, U. Z., Minna, J., Thompson, E., Peacock, J. & Nirenberg, M. W. (1973) *Proc. Nat. Acad. Sci. USA* **70**, in press.
12. Buck, C. A., Glick, M. C. & Warren, L. (1970) *Biochemistry* **9**, 4567–4576.
13. Buck, C. A., Glick, M. C. & Warren, L. (1971) *Science* **172**, 169–171.
14. Warren, L. & Glick, M. C. (1969) in *Fundamental Techniques in Virology*, eds. Habel, K. & Salzman, N. P. (Academic Press, New York), pp. 66–71.
15. Minna, J., Glazer, D. & Nirenberg, M. (1972) *Nature New Biol.* **235**, 225–231.
16. Brown, J. C. (1971) *Exp. Cell Res.* **69**, 440–442.
17. Buck, C. A., Glick, M. C. & Warren, L. (1971) *Biochemistry* **10**, 2176–2180.
18. Blume, A., Gilbert, F., Wilson, S., Farber, J., Rosenberg, R., Jr. & Nirenberg, M. (1970) *Proc. Nat. Acad. Sci USA* **67**, 786–792.