16 Trakulrungsi, C., Reiter, R. J., Trakulrungsi, W. K., Vaughan, M. K., and Johonson, L. Y., Ann. Biol. anim. Biochem. Biophys. *19* (1979) 1647.
17 Axelrod, J., and Wurtman, R. J., in: Endocrines and the Central Nervous System, p. 220. Ed. R. Levine. Williams and Wilkins, Baltimore 1966.
18 Acuña, D., Rosenstein, R. E., Romeo, H. E., and Cardinali, D. P., Neuroendocrinology *43* (1986) 24.
19 Casanueva, F., Apud, J., Locatelli, V., Martinez-Campos, A., Civati, C., Racagni, R., Cocchi, D., and Müller, E., Endocrinology *109* (1981) 567.
20 Cardinali, D. P., Vacas, M. I., Keller Sarmiento, M. I., Etchegoyen, G. S., Pereyra, E. N., and Chuluyan, H. E., J. steroid Biochem. *27* (1987) 565.
21 Vodicnik, M. J., and Vlaming, V. L., Endocr. Res. Comm. *5* (1978) 199.

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Gradient distribution pattern of muscarinic receptors in N1E 115 mouse neuroblastoma cells

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Summary. The distribution pattern of muscarinic receptors in N1E 115 mouse neuroblastoma cells after linear and non-linear gradient centrifugation was investigated. In untreated cells, at least two forms of the receptors, with different densities, were found.
Key words. Muscarinic receptors; N1E 115 neuroblastoma cells; gradient distribution; concanavalin A.

It has been suggested that receptors for catecholamines are localized in the plasma membrane, and that treatment with agonists causes the internalization of these receptors. This has been demonstrated for β-receptors in frog erythrocytes[1,2], human astrocytoma cells[3,4], mouse lymphoma cells[5] and C6 rat glioma cells[6]. These results were in most cases obtained using either differential[1,2] or gradient centrifugation techniques, combined with incubation in a medium containing concanavalin A[3-6].

As far as the muscarinic receptor is concerned, few data are available. Harden et al.[7] reported the presence of muscarinic receptors in the plasma membrane of human 1321N astrocytoma cells, and the formation of a 'light vesicle' fraction containing muscarinic receptors after carbachol stimulation. To our knowledge, no such data are available for neuroblastoma cell lines. Since these cells are frequently used for the investigation of the interaction of agonists with muscarinic receptors in neuronal cells, we studied the cellular localization of the muscarinic receptors in the N1E 115 mouse neuroblastoma cells. These cells contain a well-defined muscarinic receptor[8] predominantly of the M1 subtype[9]. Using centrifugation techniques comparable to those used for β-receptors, it was found that in non-stimulatory conditions, two forms of the muscarinic receptor, characterized by a difference in density, are present in N1E 115 mouse neuroblastoma cells.

Materials and methods

Reagents. Tritiated quinuclidinylbenzylate (QNB, 36 Ci/mmole) and N-methylscopolamine (NMS, 72 Ci/mmole) were obtained from Amersham. All other reagents were from commercial sources and of the highest purity available.

Cell culture conditions. Mouse neuroblastoma cells, clone N1E 115, passage numbers 45 to 52, were cultured as described before[10]. Briefly, the cells were grown as monolayer in DMEM medium, containing 10% fetal calf serum, 100 µg/ml streptomycin and 100 U/ml penicillin. Cells were kept in 100% relative humidity at 37 °C in 5% CO_2. The cells were subcultured when they had grown to confluency (between days 6 and 10), at a ratio of 1 to 5 or 1 to 10 in disposable plastic culture vessels of 175 cm^2 surface, using 0.025% trypsin in 0.9% NaCl solution. The cells were fed every other day.

The glioma cell line C6, passage numbers 82 to 85, was cultured in DMEM with the same additives as for the neuroblastoma cells. Subculturing at a ratio 1 to 10 was done by transferring the loosely attached cells to new culture vessels.

Pre-incubations and tissue preparations. Cells were harvested when confluent, which took 6 to 10 days. In all cases, they were rinsed twice for 5 min in 0.9% NaCl at room temperature before further manipulations.

Three different tissue preparations were used.

The *lysate* was prepared by rinsing the cells in 1 mM Tris buffer pH 7.4[6]; the cells were subsequently lysed in the same buffer during 20 min at 4 °C. The lysate was centrifuged at 300 g for 5 min and the supernatant was used for gradient centrifugation (see further).

The *microsomal pellet* was prepared by scraping the cells in 0.9% NaCl from the culture surface with a policeman.

The suspension was subsequently homogenized at 4 °C using a Virtis homogenizer at 70% of maximum power (twice 15 s with a 1-min interval). Unbroken cells and the nuclei were removed by centrifuging the homogenate at 300 g for 10 min; the supernatant was further centrifuged at 90,000 g for 60 min to give a microsomal pellet. The pellet was resuspended in 10 mM Tris buffer pH 7.4 containing 8% sucrose.

The *concanavalin A-lysate* (ConA-lysate) was obtained after incubation of the cells with ConA (0.25 or 2.5 mg/ml) for 30 min at 4 °C. The incubation was conducted either in DMEM medium without serum or in 50 mM acetate buffer (pH 6.0) containing 0.9% NaCl and 1 mM of $CaCl_2$, $MgCl_2$ and $MnCl_2$. The cells were subsequently rinsed with 0.9% NaCl (2 times 5 min) and used as described for the lysate. The effect of ConA incubation (0.25 mg/ml) was also evaluated in C6 glioma cells for comparative purposes.

Gradient centrifugation. All solutions for gradient centrifugation were buffered with 10 mM Tris buffer pH 7.4. Two types of sucrose gradients were used: linear gradients in which the sucrose concentration ranged from 15 to 55%, and non-linear gradients, in which the sucrose concentration again ranged from 15 to 55%, but with an intermediate plateau (3 ml) at the concentration of 32% [6]. In each case, 2 ml of either lysate or ConA-lysate (adjusted to 8% sucrose) or resuspended microsomal pellet was layered on the gradients of 10 ml. The gradients were spun in a Beckman centrifuge for 70 min at 90,000 g and collected in 24 fractions of 0.5 ml.

In some experiments with lysate, fractions from the gradient were recentrifuged as follows. Non-linear gradients were spun and collected as described above. Portions of the gradient fractions were assayed for ligand binding, the remainder were combined to form two pools, corresponding to the regions of high (fractions 3 to 8) and low (fractions 10 to 17) sucrose concentrations. These two pools, which contained the two forms of the muscarinic binding sites (see result section), were diluted at least 10-fold with 0.9% NaCl and centrifuged at 90,000 g for 60 min. The pellets were resuspended in the buffer and the suspensions were layered on two separate non-linear gradients identical to the first one. These gradients were handled as before.

Ligand binding. Gradient fractions were incubated for 60 min with, for the N1E 115 neuroblastoma cells, tritiated QNB or NMS (both at 0.08 nM, which represents approximately 80% of the maximal binding, see Buyse et al. [10]) and, for the C6 glioma cells, with [^{125}Iodo] cyanopindolol (^{125}I CYP) (0.1 nM) as radioactive ligand. The fractions were filtered over Whatman GF/c glass fiber filters followed by two rinses with cold 0.9% NaCl. The wet filters were counted by liquid scintillation counting and the results were expressed in counts per minute. Two series of control experiments were performed. First, it was checked whether the sucrose concentrations influenced the binding. A constant number of QNB binding sites (as cell homogenate, prepared according to Buyse et al. [10]) was added to the fractions of an unloaded linear gradient, and the fractions were assayed for ligand binding as before. In a second series of experiments, the influence of ConA on the ligand binding, and/or the binding of ConA to membranous glycoproteins, was examined using α-methylmannoside, a carbohydrate known to dissociate the ConA-glycoprotein complex. After gradient centrifugation of ConA-lysate, α-methylmannoside was added to the fractions (50 mM final concentration) and the ligand binding was carried out as before.

In a separate series of experiments, the intact cell monolayer was incubated with NMS for 30 min at 37 °C in serum-free DMEM. After incubation, the cells were thoroughly rinsed with 0.9% NaCl (3 times 5 min) and used for the preparation of the lysate (see above). After centrifugation on a non-linear gradient and collection, 100 µl of each fraction was counted for radioactivity (referred to as pre-incubation samples) and 400 µl was used for ligand binding with NMS as described above (referred to as post-incubation samples).

The recovery for the gradient centrifugation method was calculated as follows. The sum of the tissue-bound radioactivity of the different fractions was expressed as a percentage of the tissue bound radioactivity determined in the non-fractionated sample. The recovery was 80.4 ± 10.6% for 6 samples without pre-incubation with ConA and 77.8 ± 5.6% for 6 samples which had been pre-incubated with ConA ($p > 0.05$ Mann-Whitney U-test). Gradient distributions are presented in counts per minute in representative examples. Mean values ± SEM for the distribution are given in the text.

Determinations. Alkaline phosphatase was determined according to Kind and King [11]. Samples from the gradient fractions were diluted 10- to 20-fold in 10% diethanolamine buffer pH 9.8 and incubated with paranitrophenol (1 mg/ml final concentration) for 30 min. The enzyme activity is expressed in optical density units. The influence of the sucrose concentration of the gradient on the enzyme activity was investigated in an experiment similar to the control experiment described above for the binding. Statistical differences were calculated with the Mann-Whitney U-test; $p < 0.05$ was considered as significant.

Proteins were measured with the dye binding method of Bradford [12]. The sucrose concentrations were measured with the aid of an Abbé refractometer, and were expressed as optical rotatory dispersion units.

Results

Figure 1 shows representative examples of the gradient distribution pattern of the QNB binding sites, the alkaline phosphatase enzyme activity and the density in the fractions of the gradient after centrifugation of lysates

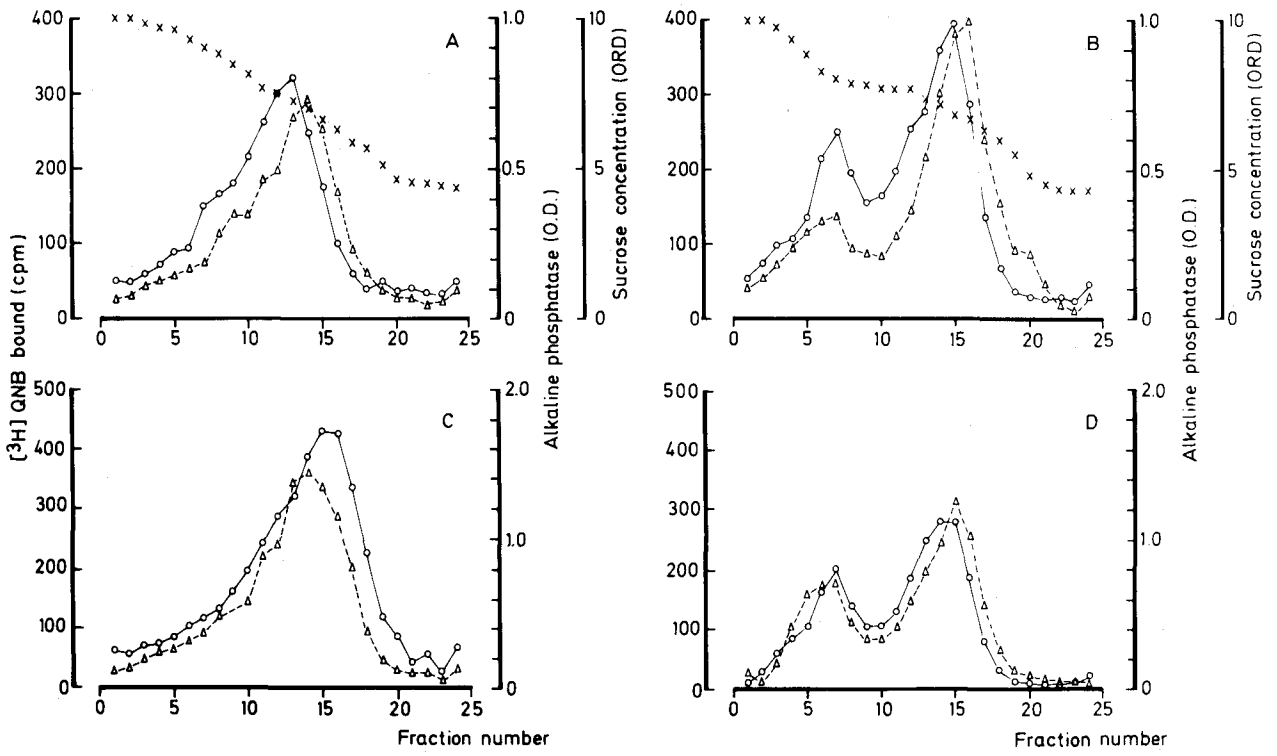

Figure 1. Gradient distribution pattern of a lysate (*A, B*) and a microsomal pellet (*C, D*) in linear (*A, C*) or non-linear (*B, D*) gradients. The data shown (mean of duplicate determinations) are representative of those obtained in 3 similar experiments. △, QNB binding; ○, Alkaline phosphatase enzyme activity; x, Sucrose concentration.

(upper panel), and of microsomal pellets (lower panel), of N1E 115 neuroblastoma cells.

The distribution pattern is essentially identical for lysates and microsomal pellets. For the linear gradient (left side of fig. 1.), a broad peak of QNB binding was obtained with both tissue preparations. When non-linear gradients were used (right side of fig. 1), a clear-cut separation of the QNB binding sites into two fractions was observed. In this first series of experiments (n = 4) 36.2 ± 1.3% of the QNB binding sites were present in the region of high sucrose concentration of the gradient, the remaining of the binding sites (62.8 ± 1.2%) was situated at a lower sucrose concentration. Alkaline phosphatase enzyme activity coincided closely with the distribution of the QNB binding in both types of gradients and with both tissue preparations. Identical results were obtained when NMS was used as ligand instead of QNB (n = 3); similarly, using 0.04 (n = 2) or 0.16 (n = 2) nM of QNB (instead of 0.08 nM) did not affect the distribution pattern in non-linear gradients of the lysate.

Addition of a constant number of binding sites (taken as 100%) to the fractions of an unloaded gradient, followed by ligand binding and determination of the alkaline phosphatase enzyme activity in the fractions, showed that on average, 99.5 ± 2.4% (range 89 to 112%) of the binding sites and 100.1 ± 1.6% (range 93 to 105%) of the enzyme activity (n = 2 gradients of 24 fractions) were found.

Recentrifugation of both QNB binding sites separately showed that for the QNB binding isolated in the region of high sucrose concentration, a redistribution was obtained (fig. 2). 60% of the binding sites were found in the same fractions as in the original gradient; 20% of the binding sites were found in the low density region of the gradient, and 20% of the binding sites were recovered at the bottom of the tube, i.e. at the highest sucrose concentration. Recentrifugation of the low density fraction yielded a unimodal distribution with the QNB binding in the low sucrose region.

Incubation of C6 glioma cells with ConA in DMEM medium or in acetate buffer caused a shift in the distribution pattern of the ^{125}I CYP binding towards the region of high sucrose concentration (fig. 3). Since the C6 cell line was only used for comparative purposes, a limited number of experiments were performed, using linear gradients only. Figures 4 and 5 summarize the results obtained when incubating the N1E 115 cells with ConA. When the pre-incubation was done in DMEM medium (fig. 4), no effect on the distribution pattern of QNB binding was seen, either with a linear or a non-linear gradient. Increasing the concentration of ConA to 2.5 mg/ml did not change the results (not shown, n = 4 for linear gradients only). When acetate buffer was used (fig. 5), a significant shift in distribution pattern towards the high sucrose region of the gradient was observed in both types of gradients. In the non-linear gradients

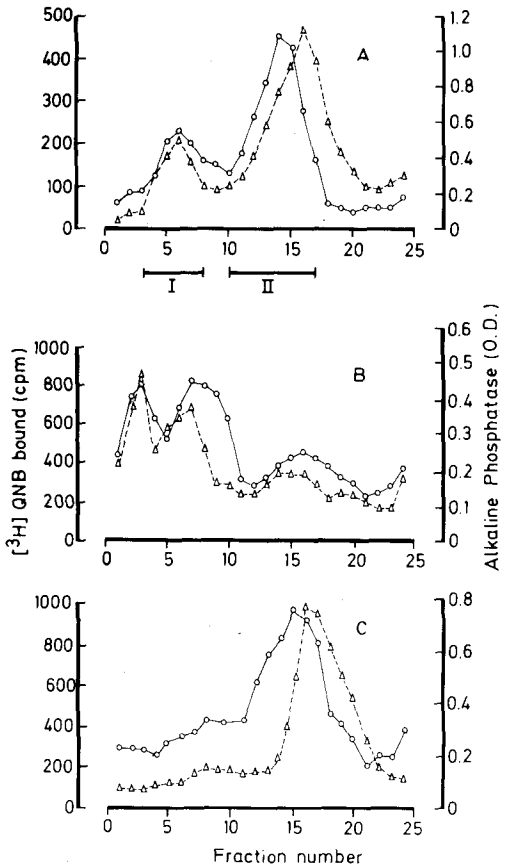

Figure 2. Gradient distribution pattern of a lysate (*A*) and of the corresponding recentrifuged fractions I (region of high sucrose concentration, *B*) and II (region of low sucrose concentration, *C*). (The data shown are representative of those obtained in 3 similar experiments). △, QNB, binding; ○, Alkaline phosphatase enzyme activity.

(n = 7), the percentage of QNB binding sites increased from 38.4 ± 1.3 to 51.5 ± 1.1% in the region of high sucrose concentration ($p < 0.05$), while in the region of low sucrose concentration, a decrease from 61.6 ± 1.4% to 48.5 ± 1.1% ($p < 0.05$) was observed upon ConA incubation. Adding α-methylmannoside to the fractions of the gradient during the incubation with QNB did not influence the distribution pattern (result not shown, n = 2 for non-linear gradients).

The results of the experiments in which the cells were pre-incubated with NMS before gradient centrifugation are shown in figure 6. The distribution pattern was identical to what is shown in previous results (fig. 1 B). Furthermore, it was found that the distribution pattern was similar whether the fractions from the gradient were counted directly or were further post-incubated with the same ligand for another 30 min, filtered and counted.

Discussion

To our knowledge, no data on the subcellular localization of muscarinic receptors in N1E 115 neuroblastoma cells in vitro are available. Some studies, however, have been done with β-receptors demonstrating the occurrence of two β-adrenergic binding sites with different densities. The site with the highest density is tentatively identified as being part of the plasma membrane and the binding site with the low density is often referred to as occurring in a 'light vesicle fraction'. The latter fraction is generated upon agonist stimulation and it is generally accepted that this site represents internalized binding sites[1–6]. Specific experimental conditions, including

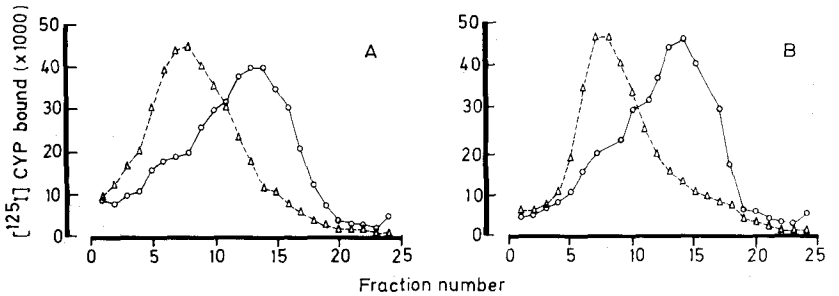

Figure 3. Effect of pre-incubation of C6 glioma cells with ConA on the distribution pattern of β-receptors in either DMEM (*A*) or in acetate buffer (*B*). (The data shown are representative of those obtained with 2 gradients with and 2 gradients without ConA for each type of gradient). ○, ^{125}I CYP binding without ConA pre-incubation; △, ^{125}I CYP binding after ConA pre-incubation.

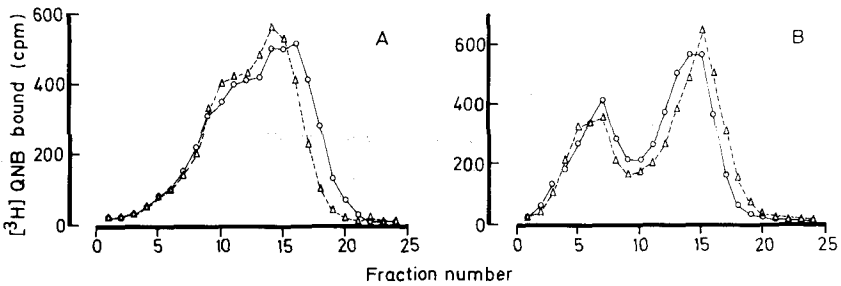

Figure 4. Effect of pre-incubation of N1E 115 neuroblastoma cells with ConA in DMEM using either linear (*A*) or non-linear (*B*) gradients (The data shown are representative of those obtained in 3 gradients with and 3 without ConA incubation for each type of gradient). ○, QNB binding without ConA pre-incubation; △, QNB binding with ConA pre-incubation.

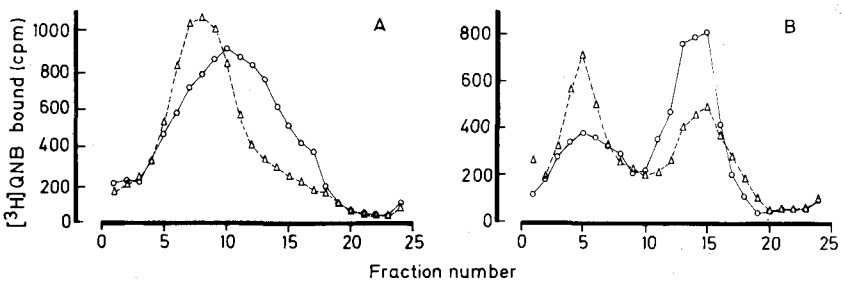

Figure 5. Effect of pre-incubation of N1E 115 neuroblastoma cells with ConA in acetate buffer using either linear (*A*) or non-linear (*B*) gradients. (The data shown are representative of those obtained in 4 gradients with and 4 gradients without ConA for *A* and 7 gradients with and 7 without ConA for *B*). ○, QNB binding without pre-incubation with ConA; △, QNB binding with pre-incubation with ConA.

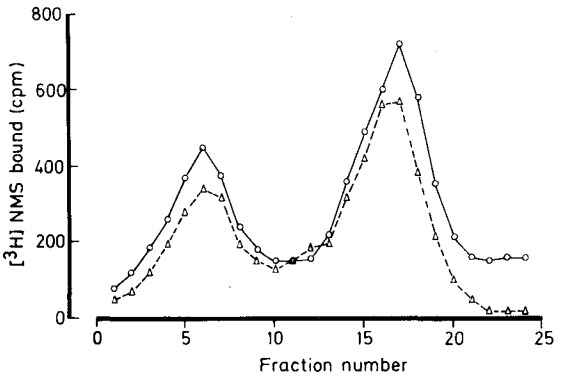

Figure 6. Gradient distribution pattern of a lysate prepared from cells, pre-incubated with NMS. (The data shown are representative of those obtained in 3 similar experiments). ○, without post-incubation; △, corresponding fractions with post-incubation with NMS.

non-linear gradient centrifugations[6], and incubations with ConA[3-6] in order to increase the density of vesicles derived from the plasma membrane[13,14], are required for the detection of the two sites. It has been reported[5] that two forms of β-receptors can be separated without ConA but no further details are given. When linear gradients are used, only one single binding site for β-receptors are found[3-5].

Our results demonstrate that in a linear gradient a broad, ill-defined peak of QNB binding is present; this is comparable to the data in the literature for β-receptors[3-5], and for muscarinic receptors[7]. However, in non-linear gradients without ConA incubation, at least two types of QNB binding sites are present in untreated cells. Approximately 40% of the QNB binding is located in the region of high sucrose concentration of the gradient and about 60% is found in the region of low sucrose concentration. Alkaline phosphatase activity coincided closely with both peaks of QNB binding activity. Since this enzyme is considered to be mainly located in the plasma membrane, we suggest that both peaks with QNB binding activity are derived from the plasma membrane. It was found that the distribution pattern was identical in gradients from lysate or from microsomal pellet. This suggests that, although only part of the total receptor population is studied when using the lysate, it is representative for the total population. In all further experiments, the lysate was used, since the protein concentration was lower, allowing optimal separation, and the number of manipulations is minimal. For similar studies with β-receptors, a cell lysate has also frequently been used[1-6].

The results of the recentrifugation experiment suggest that the peak of QNB binding at the low sucrose region of the gradient is possibly contaminated with QNB binding from the high sucrose region. Indeed, some of the recentrifuged QNB binding sites isolated from the region of high sucrose concentration are found in the low sucrose region of the gradient. This cannot, however, explain the ratio between the two forms of QNB binding, since only one fifth of the recentrifuged peak was redistributed in the low sucrose region, which represents roughly 8% of the total QNB binding sites. Concerning the QNB binding sites redistributed at the bottom of the tube, we believe that this is due to formation of large aggregates during the manipulations, not representing a vesicular structure like those observed before.

The results with ConA require further comment. When using DMEM as incubation medium for ConA, no effect on the distribution pattern of QNB binding in N1E 115 cells was seen, whereas in acetate buffer, a moderate but significant shift in QNB binding in favor of the binding sites in the high sucrose region of the gradient is found, without a change of the positions of the ligand binding sites. Since pre-incubation of C6 glioma cells with ConA in DMEM led to the expected shift of β-receptor binding sites[6], the absence of effect in the N1E 115 cells when DMEM is used is considered to be cell-specific, and due to poor reactivity of the membranous glycoproteins in the N1E 115 cells in our culture conditions. Wide variability between different cell lines, as far as the reaction with ConA is concerned, has been reported before[15].

Post-incubation of the gradient fractions with α-methylmannoside, a molecule which breaks the bond between ConA and glycoproteins, does not modify the results, suggesting that phenomena such as steric hindrance do not influence the results.

Our experiments demonstrated that in unstimulated cells, binding sites are present in the sucrose region where the light, intracellular, membranous vesicles containing

β-receptors are found. The next question is whether the vesicles observed in the low sucrose region of the gradient are similar to the light density vesicles described by others for β-receptors. After pre-incubation of intact cells with tritiated NMS, which does not cross the plasma membrane[16], followed by gradient centrifugation, a distribution pattern is found which is identical to that found when the gradient fractions are incubated with either QNB or NMS. Furthermore, upon post-incubating the fractions of the gradient obtained with pre-incubated cells in monolayers, no further increase of bound radioactivity was found. This means that all binding sites are already occupied during the first incubation. If the low-density fractions in the unstimulated cells were to consist of intracellular vesicular binding sites, NMS would not have labeled these binding sites and, consequently, post-incubation would result in an increase of the bound radioactivity in this region of the gradient. That this is not the case suggests that the QNB binding site in the low sucrose region of the gradient is not associated with an intracellular particle, but present at the outer surface of the plasma membrane.

We conclude that at least two different QNB binding sites are present in N1E 115 mouse neuroblastoma cells. These forms are separated by non-linear gradient centrifugation methods. We suggest that the binding site in the low sucrose region of the gradient is localized in a vesicle of membranous origin but not by a process of sequestration. These vesicles could originate by a process called 'vesiculization' or 'membrane shedding'. This process, described by Scott[17,18], results in extracellular particles. They contain part of the membrane and membranous components and are shed by the cells through an energy-dependent mechanism. Transformed cells are extremely active in this respect, non-transformed cells much less so. Further experimentation will be needed to establish the exact nature of the low-density ligand binding.

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** Bursary of the IWONL.

1 Stadel, M. M., Strulovici, B., Nambi, P., Lavin, T. N., Briggs, M. M., Caron M. G., and Lefkowitz, R. J., J. biol. Chem. *258* (1983) 3032.
2 Strader, C. D., Sibley, D. R., and Lefkowitz, R. J., Life Sci. *35* (1984) 1601.
3 Waldo, G. L., Northup, J. K., Perkins, J. P., and Harden, T. K., J. biol. Chem. *258* (1983) 13900.
4 Toews, M. L., Waldo, G. L., Harden, T. K., and Perkins, J. P., J. biol. Chem. *259* (1984) 11844.
5 Clark, R. B., Friedman, J., Prashad, N., and Ruoho, A. E., J. cycl. Nucl. Res. *10* (1985) 97.
6 Kassis, S., and Sullivan, M., J. cycl. Nucl. Res. *11* (1986) 35.
7 Harden, T. K., Petch, L. A., Traynelis, S. F., and Waldo, G. L., J. biol. Chem. *260* (1985) 13060.
8 McKinney, M., and Richelson, E., A. Rev. Pharmac. Toxic. *24* (1984) 121.
9 Fraeyman, N., and Buyse, M.-A., Fund. clin. Pharmac. (1989) submitted.
10 Buyse, M.-A., Lefebvre, R., and Fraeyman, N., Tissue Cell (1989) submitted.
11 Kind, P. R., and King, E. J., J. clin. Path. *7* (1954) 322.
12 Bradford, M. M., Analyt. Biochem. *72* (1976) 248.
13 Scarborough, G. A., J. biol. Chem. *250* (1975) 1106.
14 Lutton, J. K., Frederich, R. C., and Perkins, J. P., J. biol. Chem.
15 Wallach, D. F. H., in: Membrane Molecular Biology of Neoplastic cells, p. 435. Ed. D. F. H. Wallach. Elsevier Scientific Publ., Amsterdam 1975.
16 Lee, J.-H., and El-Fakahany, E. E., Eur. J. Pharmac. *110* (1985) 263.
17 Scott, R. E., Science *194* (1976) 743.
18 Rademaker, B., Kramer, K., Stroes, J. W., Vlug, J., Krielaart, M., and Zaagsma, J., Eur. J. Pharmac. *111* (1985) 31.

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Increased susceptibility to lipid peroxidation in skeletal muscles of dystrophic hamsters

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Summary. The results showed that the total content of lipids, which could be peroxidized with Fe(2 +)/ascorbate stimulation in vitro, was 45.4% and 53.7% higher than normal in the dystrophic hamster muscle at the age of 1 and 3 months, respectively. Correspondingly, the susceptibility to lipid peroxidation (stimulated by ADP-chelated iron at 37 °C) was 38.6–74.3% higher in dystrophic muscles. The increases were not related to necrotic lesions and inflammation observed. The activities of glucose-6-phosphate dehydrogenase, glutathione reductase, thioredoxin reductase and catalase were increased in dystrophic muscles but those of superoxide dismutases and glutathione peroxidase were unaffected.
Key words. Lipid peroxides; antioxidants; muscular dystrophies; skeletal muscles; hamster.

The mechanism of cellular damage in genetic muscular dystrophies is unknown, although human cDNA and the protein product, dystrophin, of the Duchenne muscular X-linked locus have recently been identified[1]. Dystrophin is localized in the sarcolemma[2–4] and t-tubular membranes[3] of muscle fibers. One possible cause for