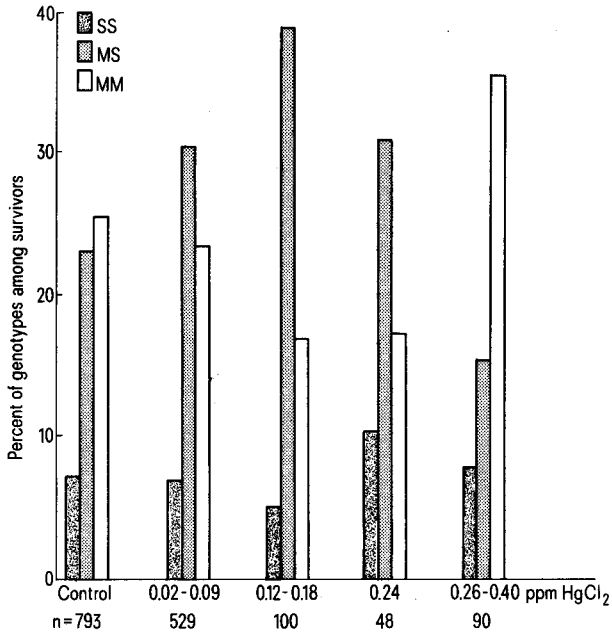

Figure 2. Differential survivorship of three PGM genotypes of *Palaemon elegans* as a function of increasing concentration of $HgCl_2$.

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11 It is likely that differential inhibition of PGM allozymes by mercury is the mechanism involved; see inhibition of PGM by mercury in Milstein, C. Biochem. J. *79*, 591 (1961).

Rhodamine isothiocyanate coupled peanut lectin for quantitative studies of D-galactosyl receptors of neuroblastoma cells[1]

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Summary. Rhodamine-peanut agglutinin conjugate has been obtained without alteration of the lectin activity. This conjugate interacts specifically with terminal D-galactosyl glycoconjugates. Such receptors are found on neuroblastoma differentiated or undifferentiated cell membranes. The density and distribution of these galactosyl sites are different after neuraminidase treatment.

The study of specific interactions involving glycoconjugates is of practical and theoretical importance. The acquisition of glycosylated structures appears to be linked to biological evolution, during ontogenesis and differentiation[2,3]. Their variation may cause the disappearance or the acquisition of certain biological properties. A quantitative study of these variations can be performed using fluorescent labelled lectins. We report the results of the conjugation of peanut agglutinin (PNA), a lectin specific for terminal D-galactosyl residues[4,5], with tetramethylrhodamine isothiocyanate (TRITC), as well as the characterization of the conjugate. Furthermore, a new approach was used to quantify the fluorescence of neuroblastoma cells. These cells provide a well-studied model of in vitro morphological and biochemical differentiation[6,7].

Materials and methods. The seeds of *Arachis hypogaea* were obtained from Lesieur Cotelle S.A. The lectin, specific for D-galactose, was purified by affinity chromatography on desialylated polymerized human red cell ghosts[8]. Hemagglutination assays were performed in microtiter plates with human red blood cells treated with 60 µl/ml of neuraminidase (*Vibrio cholerae* Behring) for 90 min at 37 °C. Polyacrylamide gel electrophoresis was performed on 7.25% slab gels in Tris-glycine buffer, pH 8.9 or in acetate buffer, pH 4.3[9] and on gradient gels, 4-30% in Tris-buffer, pH 4.8 and Tris SDS, pH 7.4[10]. The gels were stained for protein detection with Coomassie brilliant blue. Ouchterlony double diffusion[11] and immunoelectrophoresis[12] were performed in 1.5% agarose in Na-barbiturate buffer, pH 8.6, against anti-peanut lectin obtained against our preparation. To conjugate the lectin with TRITC (isomer R-BBL) we used a method derived from that reported for immunoglobulin[13]. 15 µl of 1 M $NaHCO_3$ was added to 10 ml of the lectin solution in phosphate buffered saline before addition of TRITC solution in dimethylsulfoxide (1 mg/ml). Conjugation was attempted with different quantities of TRITC. The pH was adjusted to 9 with Na_2CO_3M. The mixture was incubated for 3 h, in the dark and at room temperature.

The crude conjugate was chromatographed on Biogel P 10. The first elution peak was either dialyzed against PBS or chromatographed under the same conditions as for lectin purification.

The conjugate was used for specific labelling of neuroblastoma cells (NS20). Cells were grown on glass coverslips in Eagle's minimal essential medium containing 10% foetal calf serum (Eurobio), glutamine (2 mM, Eurobio) and penicillinstreptomycin (100 IU/ml, 100 mg/ml).

Dishes were incubated in an atmosphere of 5% CO_2 - 95% air at 37 °C. Differentiation was induced after 2 days of culture in normal medium either by absence of foetal calf serum[6] or by addition of 2% (vol/vol) dimethylsulfoxide in normal medium[7]. For the quantitative measurement of fluorescence, living cells were labelled with 100 µg of PNA-TRITC in 1 ml of phosphate buffer solution for 30 min at 20 °C and then rinsed 3 times with Hank's balanced salt solution (HBSS). For the enzymatic treatment, cells were preincubated before fluorescent labelling with 0.15 units of

Table 1. Properties and specificity of rhodamine isothiocyanate-peanut lectin

Test	Native PNA	PNA-TRITC
Agglutination of neuraminidase-treated erythrocytes[a]	1000	500
Immunodiffusion and immunoelectrophoresis against anti-PNA	Single precipitate, cross reactivity	
Electrophoresis in polyacrylamide gradient gels	Single band corresponding to a mol.wt of 125,000 and 29,000 in 0.2% SDS	
Gel filtration (P 10)	Elution volumes strictly reproducible	
Absorption spectra	Superposition of the peaks at A_{280} after gel filtration	
Fluorescence labelling of neuroblastoma cells (NS 20)		- PNA-TRITC: + - PNA-TRITC + Gal[b]: − - PNA-TRITC on neuraminidase-treated cells: + +

[a] The titer represents the reciprocal of the greatest dilution which caused full aggregation. Maximum error is one doubling dilution in either direction. [b] [Gal] = 0.05 M.

Table 2. Initial intensity of fluorescence on PNA-TRITC labelled neuroblastoma cells under different culture conditions (all measurements were performed with the same controlled exposure time and excitation intensity)

Neuroblastoma NS 20	PNA-TRITC labelling		
	Labelling	Fluorescence intensity (arbitrary units ± SD)	No. of cells
Undifferentiated cells			
5 h	Diffuse	96 ± 19	13
2 days	Diffuse	32 ± 6	9
2 days, after neuraminidase treatment	Patches 69 ± 16 in diffuse areas	159 ± 57 in patches	12
Differentiated cells			
2 days without serum	Diffuse	54 ± 9	10
2 days with DMSO*	Diffuse	62 ± 16	13
5 days with DMSO*	Diffuse	60 ± 13	12

* DMSO, dimethylsulfoxide.

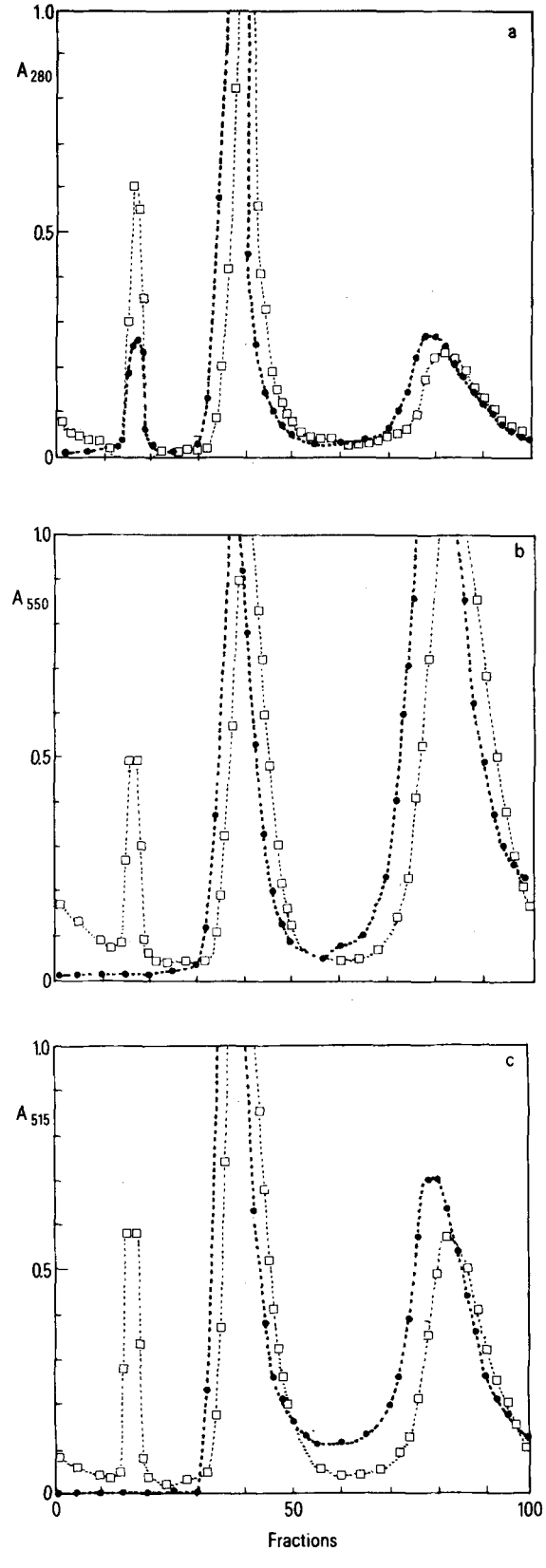

Figure 1. Purification by gel filtration on Biogel P-10 of PNA-TRITC (□ · · · · □) and control studies with free PNA (* —— *) and free TRITC (●·····●). The column (2.6 × 30 cm) was pre-equilibrated and eluted with phosphate buffer saline. Fractions (3 ml) were collected and absorbance at *a* 280 nm, *b* 550 nm and *c* 515 nm were determined.

neuraminidase (*Vibrio cholerae* Behring) in 2 ml of Hank's balanced salt solution for 2 h at 37 °C and washed with HBSS 3 times.

Quantitation of fluorescence was performed on a special apparatus which also allows the measurement of lateral diffusion of membrane components by the 'fluorescence recovery after photobleaching' method[14]. Briefly an argon ion laser beam (Spectraphysics - λ emission = 514.5 nm) was focused through the microscope objective (Leitz orthoplan, × 125) to a small spot with a 1.75 μm e^{-2} radius. Fluorescence was detected with an Emi 9558 photomultiplier tube (Leitz MPV_2). The interfacing to a computer (PDP 11/34) allowed illumination and detection controls and data collection. Each fluorescence measurement lasted 200 msec. The laser beam intensity was recorded with a photometer (UDT). The collected parameters were fluorescence intensity (in arbitrary units) versus time. Fluorescence intensity is proportional to the number of fluorescent sites/illuminated cell membrane location.

Results. The crude protein extract was chromatographed on a column (10 × 1.6 cm) of polymerized desialylated stromas previously equilibrated with 0.2 M NaCl. The lectin was eluted with 0.05 M galactose in 0.2 M NaCl. Fractions containing hemagglutination activity were pooled and sterilized by filtration. The isolated lectin was homogeneous on polyacrylamide gel electrophoresis at pH 8.9 or 4.3. For the conjugation, 2 ratios of TRITC/PNA were tested, 10 and 20 μg per mg of protein. On chromatography on Biogel P 10 the 1st coloured peak contains the conjugate. The other 2 following peaks contained only free fluochrome. The elution volumes of these 3 peaks were strictly reproducible. These results are superimposable with those obtained with free PNA on the one hand and, on the other hand, with those obtained with free TRITC (fig. 1). An initial coupling ratio of 10 μg of TRITC per 1 mg PNA gave a conjugate with an OD_{550}/OD_{280} ratio of 0.25 whereas 20 μg of TRITC per 1 mg PNA gave a conjugate with an OD_{550}/OD_{280} ratio of 0.70.

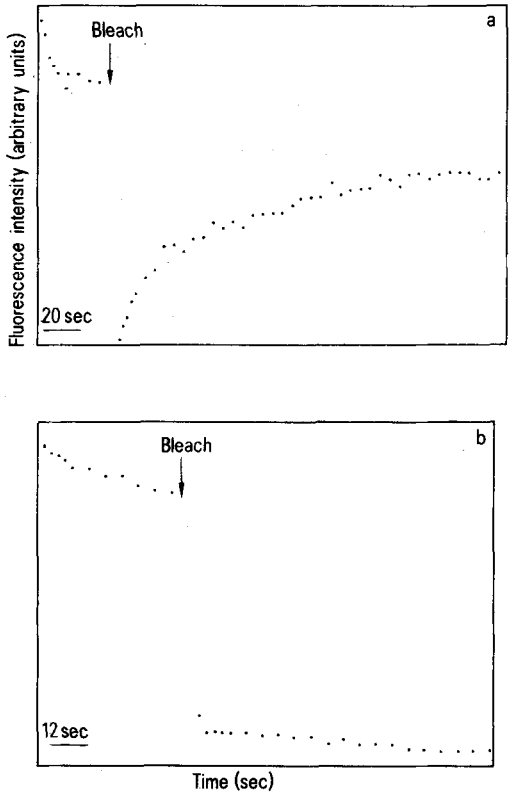

Figure 2. Photobleaching recovery traces of PNA-TRITC bound to undifferentiated neuroblastoma cells. *a* untreated cell: fractional recovery, defined as the total fluorescence recovered by the fluorescence change due to bleaching, is 62%. The initial fluorescence intensity is 85 arbitrary units; *b* neuraminidase-treated cell: the initial fluorescence intensity is 158 arbitrary units. The recorder traces were obtained under same experimental conditions: laser intensity: 120 μW for measurement, 2 mW for bleaching. Each point represents the average value of fluorescence during 200 msec.

When native and conjugate PNA were compared, it appeared that their respective hemagglutination activities, reactivities against anti-native PNA antibodies, elution volumes after gel filtration and electrophoretic mobilities in a polyacrylamide gradient were not significantly modified (table 1). No noticeable difference of activity was found when either PNA-TRITC which had only been dialyzed or the rechromatographed conjugate were used.

A quantitative evaluation of the fluorescence signal was performed on neuroblastoma cells under different culture conditions (table 2). All the untreated cells, differentiated or not, present a diffuse labelling without significant changes of the local fluorescent emission. Pretreatment with neuraminidase leads to a patched pattern of labelling with an enhanced fluorescence intensity. No capping process was seen. Using the 'fluorescence recovery after photobleaching' method, the lateral diffusion of PNA receptors was measured on living neuroblastoma cells. It appears that 55 ± 15% of the receptors are mobile on the experimental time scale, whatever the culture conditions. On neuraminidase-treated cells the receptors were found immobilized in patches (fig. 2). A very low fluorescent signal was detected when the galactose (0.05 M) saturated PNA-TRITC was used either on untreated or on neuraminidase-treated cells. It was nearly at the level of the photomultiplier noise.

Discussion. These results demonstrate that TRITC can successfully be coupled to peanut lectin without much alteration in the lectin activity. The conjugate, if frozen immediately after preparation, is quite stable and need not be rechromatographed before use. Like other authors[15], we have demonstrated 2 peaks of free rhodamine eluted after the conjugate. These peaks were not identified but it seems that the spectral characteristics (absorption at 280, 515 and 550 nm) are different for the 2 components.

Rhodamine derivatives including TRITC show a good light stability, as is shown by the photobleaching recovery traces (fig. 2). Autofluorescence of tissues and cells are considerably reduced by using a green excitation wavelength[13], especially with a laser beam ($\Delta\lambda = 0.5$ nm).

Neuroblastoma cell surfaces, even if not strongly labelled by PNA-TRITC, possess terminal D-galactosyl residues. Using local fluorescent measurements we did not demonstrate drastic quantitative modifications of these residues after differentiation such as had been found for teratocarcinoma cells[3]. However, neuraminidase treatment increases the density of galactosyl sites and modifies their distribution in the presence of PNA. These results suggest that, after neuraminidase treatment, the cross-linking of PNA binding sites is enhanced by their proximity. The differential lateral diffusion properties of PNA receptors may reflect such differences in the topological arrangement of receptors. This rearrangement does not lead to the capping that has been observed for human lymphocytes[16]. Our analysis does not tell us, however, what initial phenomena, such as reorientation of glycosidic chains or increase of lateral mobility of the glycoconjugates, are involved in the modification of the distribution of the galactosyl sites.

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Homologies of *Neurospora* homothallic species using repeated and nonrepeated DNA sequences[1]

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Summary. DNA:DNA hybridization studies of the homothallic species of *Neurospora* showed that the repeated DNA sequences provided no means of distinction among them. Hybridization with nonrepeated DNA sequences, however, showed that the *N. terricola* species was quite unlike the others. These studies suggest that heterothallism might have evolved from homothallism in *Neurospora*.

Morphologically and physiologically, the homothallic species of *Neurospora: N. dodgei* Nelson and Novak, *N. africana* Huang and Backus, *N. lineolata* Frederick, Uecker and Benjamin and *N. galapagosensis* Mahoney and Backus present little or no distinguishing characteristics. *N. terricola* Gochenaur and Backus, another homothallic species with one germ pore instead of two, however, is somewhat different. Unlike the heterothallic and pseudohomothallic species of *Neurospora*, they do not produce conidia but produce perithecia from a single strain. DNA characterizations of these *Neurospora* species were conducted by Williams et al.[2] and Dutta et al.[3] at the molecular level. No distinguishing DNA characteristics between or within the 3 broad *Neurospora* groups were detectable although DNA:DNA homology studies were useful in identifying DNA sequence differences within heterothallic and pseudohomothallic species[3,4]. These latter hybridization studies involved the use of nonrepeated (unique) DNA sequences, but not repeated DNA sequences.

It has been proposed that repeated DNA sequences play a vital role in evolution[5,6]. Very little is known regarding DNA:DNA hybridizations of repeated DNA sequences of *Neurospora* species[7].

In this report we have tried to distinguish between the homothallic species of *Neurospora* by measuring DNA homologies of repeated and unique sequences. The genomes of 2 homothallic species, *N. lineolata* and *N. galapagosensis* were labeled with ^{32}P-isotope and repeated and nonrepeated sequences separated. DNA:DNA hybridization studies were then performed, independently, on an excess of unfractionated unlabeled DNAs of the other 3 homothallic species, *N. dodgei, N. africana, N. terricola* and on themselves, to form hetero- and homo-duplexes, respectively.

Materials and methods. The homothallic strains: *N. africana* (FGSC 1740) *N. lineolata* (FGSC 1910) and *N. dodgei* (FGSC 1692) and the heterothallic species *N. crassa* (FGSC 987) used were obtained from the Fungal Genetics Stock Center (FGSC), Humboldt, California, USA. Cultures of *N. terricola* and *N. galapagosensis* were obtained through the courtesy of Dr Lafayette Frederick of this Department. All homothallic strains of *Neurospora* were cultured 6–7 days under aeration in minimal medium containing 2% sucrose, using small amounts of mycelial fragments as inocula as described by Dutta et al.[3]. Growth of cultures were terminated when heavy deposits of melanin were visible near the mouth of the 4-l flasks in which they were grown.

The modified urea-phosphate method[8] for *Neurospora* DNA isolation was adopted. Cultures meant for ^{32}P-labeled DNA source were grown in phosphate-free medium[9], containing 2 μCi ^{32}P-isotope (New England Nuclear Laboratories) put into 100 ml of growth medium, cultured for 7 days

Compilation of normalized DNA:DNA hybridizations among DNA sequences of *Neurospora* homothallic species

^{32}p-labeled DNA fragments	Unlabeled DNA fragments	Normalized hybridization (%) Nonrepeated	Repeated
N. lineolata	*N. lineolata*	100	100
	N. galapagosensis	97 ± 0.7	96 ± 0.8
	N. africana	91 ± 1.9	97 ± 0.6
	N. dodgei	91 ± 1.4	93 ± 1.1
	N. terricola	83 ± 1.2	87 ± 0.7
	N. crassa	78 ± 1.3	80 ± 0.6
	E. coli	< 0.07	– –
N. galapagosensis	*N. galapagosensis*	100	100
	N. africana	100	97 ± 0.5
	N. dodgei	98 ± 0.7	95 ± 1.6
	N. lineolata	94 ± 1.4	96 ± 1.2
	N. terricola	81 ± 1.8	67 ± 2.1
	N. crassa	53 ± 0.6	60 ± 1.5
	E. coli	< 0.03	< 0.05

All experiments were repeated at least 3 times. Average values ± SD are shown. The basis for the estimation of normalized data is explained in the 'results' section. Average specific activity of repeated ^{32}P-DNA sequences at the time of reactions was 15,000 cpm/μg DNA and that for non-repeated ^{32}P-DNA sequences at the time of reactions was 2,000 cpm/μg DNA.