Peanut agglutinin (PNA) has been shown to be insoluble at low temperatures. This cryoinsolubility has been studied by means of absorption spectroscopy, fluorescence, circular dichroism, and analytical ultracentrifugation. It was found to be dependent on pH, temperature, and protein concentration. No effects on dimer-tetramer equilibrium could be determined nor any conformational changes provoked by exposure of the PNA preparation to low temperatures. The dimer half-molecule apparently does not precipitate. The cryoinsolubility was partially reversible and totally inhibited in the presence of galactosides, the specific ligands of PNA. Their efficacy as inhibitors of cryoinsolubility was related to their affinity for the lectin. The effects of neutral salts and particularly inhibition of the insolubility by strongly chaotropic salts indicate that charge-charge interactions are of little importance and that hydrogen bonds and/or van der Waals interactions are most probably responsible for the formation of the cryoprecipitate.

Groundnut (Arachis hypogea) seeds contain a lectin, peanut agglutinin (PNA), which is able to agglutinate neuraminidase-treated human erythrocytes (1). Of the monosaccharides tested, only D-galactose and α and β-D-galactosides were inhibitors of agglutination. A more effective inhibitory action has been observed for the disaccharide D-Galβ(1 → 3)D-GalNac and desialylated glycoproteins (2-4).

Because of this specificity, PNA has been widely used as a cell surface probe and particularly as a marker for T-lymphocyte subpopulations and immature circulating cells (5-7).

PNA, \( M_w = 98,000-110,000 \), was first isolated by Lotan et al. (4) and is composed of four equal sized polypeptide chains linked by noncovalent bonds (4, 8). Concanavalin A, a lectin which has much in common structurally with PNA, reversibly dissociates from the tetramer form into dimers at low temperature (9, 10). The same behavior is observed for PNA with pH (8), but little is known about its stability at different temperatures. In fact, the saccharide-free protein appears to precipitate at low temperatures. In view of the possible functional implications of the cryoinsolubility of free PNA and particularly the effects on the binding of the lectin to cells, cryoinsolubility is often studied at 4 °C, we considered it to be important to investigate the sensitivity of PNA to low temperatures. In this paper, we describe the kinetics of PNA precipitation from turbidity measurements and an assessment of the conformational stability of the protein as a function of temperature.

Conditions are defined (pH, saccharides, and salts) under which PNA remains soluble at low temperatures and their effects are considered in terms of their influence on the conformation of the protein.
photomultiplier tube distortions were made with rhodamine B using the program developed for the HP 9815 A by Jobin et Yvon.

Sedimentation velocity experiments were carried out at 50,780 rpm in a Beckman model E analytical ultracentrifuge equipped with schlieren optics. Temperature was controlled by a RTIC unit. A partial specific volume, \( \delta \), of 0.73 (8) was used for correction relative to density of solvent. Some experiments at 4 °C in the absence of lactose (PNA 0.25 and 0.45 mg/ml) were done at 50,000 rpm in an MSE Centriscan 75 ultracentrifuge equipped with absorption optics.

pH was measured with a Radiometer model PHM 62 pH meter which was calibrated with Radiumeters buffers. All experiments were performed with solutions passed through Millipore filters (0.45 pm). Guar gum and carbohydrates were from Sigma and neutral salts from Merck of the best grade available. The disaccharide \( \beta\text{-Gal(1 \rightarrow 3)\beta-GalNac} \) was a generous gift of Dr. G. Strecker (Centre National de la Recherche Scientifique, L. A. 217, Villeneuve d'Ascq).

### RESULTS

When the temperature of a PNA solution is decreased from 20 to 4 °C, a progressive development of turbidity can be observed, followed by precipitation after a certain lag time.

The effects of time and concentration on the solubility of PNA at 4 °C and neutral pH are shown in Fig. 1 where the time course of cryosensitivity was monitored by measuring the turbidity of the solution. At and below a concentration of 0.8 mg/ml, the protein was completely soluble throughout the period tested. The time lag before development of turbidity was strongly dependent on protein concentration, as has been found for an IgG-cryoimmunoglobulin (12). The decrease in turbidity after longer periods (vertical arrows on Fig. 1) was due to protein precipitation. The increase in turbidity observed with time as shown in Fig. 1 was reversible if the PNA solutions were rewarmed, provided that precipitation had not yet occurred. In subsequent experiments, the increase in turbidity was taken as the index of cryoinsolubility of PNA.

**Effects of Temperature on the Quaternary Structure of PNA**—In order to ascertain whether low temperatures affect the dissociation-association equilibrium, PNA solutions were subjected to analytical ultracentrifugation at 20 (1.09-4.79 mg/ml) and 4 °C (0.25-0.81 mg/ml). Identical values of \( S_{20,w} \) were found in both experiments (Fig. 2, a and d), i.e. \( S^2 = 6.00 \) S at 20 and 4 °C (the latter after correcting to 20 °C for density and viscosity of solvent). Under these experimental conditions, therefore, temperature apparently does not affect the transition from the tetramer to dimer form of the PNA molecule. With this value obtained for \( S_{20,w} \) and the reported molecular weights of PNA, 98,000 (8) and 110,000 (4), frictional ratios, \( f/f_0 \), of 1.28 and 1.33, respectively, can be calculated. This does not coincide with the findings of Fish et al. (8) who concluded from their \( f/f_0 = 1.16-1.18 \) (s = 6.4 S) that PNA is a globular molecule. Our results indicate that PNA is a rather extended molecule.

**Effects of Temperature on the Secondary and Tertiary Structures of PNA**—Exposure to low temperatures could trigger conformational changes in the PNA molecule. CD spectra were, therefore, measured as a function of temperature in the far (200-250 nm) and near (250-300 nm) UV zones.

At low concentrations (0.049 mg/ml), there was no change in the far UV CD spectrum of PNA between 20 and 4 °C and the shape and size of the spectrum were the same as those observed at room temperature (8).

The shape of the spectrum confirms that PNA belongs to the Class I of lectins (13). According to this classification, the presence of a large negative extremum at 225 nm indicates that PNA contains a very high proportion of \( \beta \)-structure, as is the case in other cryoprecipitable proteins (14). The near UV spectra of 20 and 4 °C were superimposable (Fig. 3) whether low (0.48-0.54 mg/ml) or high (1.82-2.04 mg/ml) concentrations of protein were studied. Even light scattering developed with turbidity at high PNA concentrations did not provoke CD changes.

The intrinsic fluorescence of PNA was examined over a...
temperature range 0.8-77 °C (Fig. 4). The wavelength of emission maximum (λ_{max} corr. = 328 nm) was not modified below 52 °C and the monotonic decline in emission intensity observed with increasing temperature confirms that no structural alteration occurred over this temperature range (15). Deviation from linearity was observed only at temperatures higher than 52 °C. At 77 °C, the wavelength of the emission maximum was 343 nm, which is close to the wavelength observed under denaturing conditions (λ_{max} = 345 nm in the presence of 6 M GdmCl). When the same spectra were recorded at concentrations higher than 1.5 mg/ml, the wavelength of the emission maximum was maintained at 328 nm despite the turbidity of the solutions at low temperatures.

The negative results obtained with CD and fluorescence must, however, be interpreted with caution. In turbid solutions, the scattering molecules could not contribute to the spectra observed.

**pH Dependence of PNA Cryoinsolubility**—The increase in turbidity of the PNA solutions at 4 °C was followed at different pH values (Fig. 5). PNA cryoinsolubility was evident only between pH 4.9 and 9.2. The results obtained at low pH could be interpreted as indicating that the dimer form of the lectin (M_r = 50,000-55,000) does not precipitate, the sharp curve in this pH zone in Fig. 5 overlapping the transition from a molecular weight 98,000-110,000 to that of 50,000-55,000 (8). Nevertheless, this interpretation should be considered with caution since ionizations occur in this pH zone and the apparent effects of pH could correspond to those seen in the presence of neutral salts (see below).

There was no change in the wavelength of the fluorescence maximum (λ = 328 nm) between pH 3.1 and 10.75 either at 20 or 4 °C.

**Effects of Saccharides on the Cryoinsolubility of PNA**—Lactose is known to modify the near UV CD spectrum (8) and, to a lesser extent, the absorption spectrum (16) of PNA. During the routine isolation procedure, PNA was seen to remain soluble at low temperatures in the presence of lactose. This specific ligand was used for the elution of the affinity chromatography step (see "Materials and Methods"). The effects of mono-, di-, and trisaccharides on the cryoinsolubility of PNA were, therefore, examined.

As is shown in Fig. 6, at low concentrations, specific saccharides, namely galactosides, inhibited cryoprecipitation. In addition, their effect was related to their ability to inhibit agglutination. The disaccharide D-GalP(1→3)D-GalNac which has been described as the most effective agglutination inhibitor among the tested galactosides (4) was found to be the most powerful inhibitor of cryoinsolubility of the three sugars. In view of these findings, PNA solutions are stored at 4 °C for months in the presence of lactose (the less expensive sugar).

By contrast, "nonspecific" sugars, like glucose, maltose, saccharose, and maltotriose, inhibited cryoprecipitation only when at very high concentrations (Fig. 7). No apparent correlation could be found between their molecular size and their
inhibitory effect on cryoinsolubility. The curve obtained with lactose has been added to Fig. 7 by way of comparison. Analytical ultracentrifugation was carried out in the presence of 0.1 M lactose. Values of 5.96 and 5.89 S were found for s° at 20 and 4 °C, respectively, after correction for density and viscosity of solvent (Fig. 2, b and d). These values are very close to those obtained for the saccharide-free PNA. As is shown in Fig. 3, the presence of lactose brought about changes in the CD spectra of PNA in the near UV zone at both 4 and 20 °C. However, it should be noted that although these changes coincide with the findings of Fish et al. (8), we did not observe the 40% increase in size of ellipticity bands at 275 and 282 nm reported by these authors. Whatever the temperature, our CD bands did not increase by more than 10% on exposure to 0.1 M lactose.

Effect of Neutral Salts on the Cryoinsolubility of PNA—The effects of a variety of salts on the cryoinsolubility of PNA are summarized in Table I.

The effects of anions were tested in association with Na⁺ as cation and those of cations in the presence of Cl⁻. Neutral salts inhibit cryoprecipitation in an order corresponding to the Hofmeister series of ions. While high concentrations of nonchaotropic neutral salts appeared slightly to increase pre precipitation, the latter was almost totally inhibited by 1 M MgCl₂, NaI, or NaSCN (Fig. 8).

In fact, PNA solutions at concentrations exceeding 10 mg/ml were found to be slightly turbid even at 20 °C. However, it should be noted that although these changes coincide with the findings of Fish et al. (8), we did not observe the 40% increase in size of ellipticity bands at 275 and 282 nm reported by these authors. Whatever the temperature, our CD bands did not increase by more than 10% on exposure to 0.1 M lactose.

**TABLE I**

| Salt    | % | Anion    | % |
|---------|---|----------|---|
| K⁺      | 112 | Cl⁻      | 109 |
| Na⁺     | 109 | Br⁻      | 64  |
| NH₄⁺    | 91  | ClO₄⁻    | 25.7|
| Ca⁺     | 64  | SCN⁻     | 17  |
| Li⁺     | 61  | I⁻       | 8.3 |
| Mn⁺²    | 56  |          |     |
| Mn⁺⁴⁺   | 20  |          |     |
| Mn⁺⁴⁺ + Ca⁺⁺ | 14.2 |
| Mg⁺²    | 7.8 |          |     |

* Each salt was 0.5 M.

**Fig. 8. Effect of chaotrophic neutral salts on the cryoinsolubility of PNA.**

In the presence of MgCl₂, PNA exhibited a near UV CD spectrum with the same shape as that recorded in the absence of salt, but there was a slight but net increase in size of ellipticity bands (Fig. 3). This spectrum was not modified when the temperature was decreased from 20 to 4 °C or after 2 h at 4 °C. With the addition of 0.1 M lactose, the CD spectrum underwent the same transition as that observed in the absence of MgCl₂. No significant (or very little) changes of the far UV CD spectrum were observed in the presence of 1 M MgCl₂ and intrinsic fluorescence revealed none. Analytical ultracentrifugation carried out at 20 °C in the presence of 1 M MgCl₂ showed an alteration in sedimentation coefficient. A value of 4.70 S was found for the s° at this temperature (Fig. 2c) after correction for density and viscosity of solvent (v = 0.73).

**DISCUSSION**

The results reported in this paper clearly show that the solubility of PNA (the lectin from the peanut, A. hypogea) is dependent on temperature. The cryoinsolubility of PNA appears to be partially reversible and the reversibility is related to the time at low temperatures and the PNA concentration. In fact, PNA solutions at concentrations exceeding 10 mg/ml were found to be slightly turbid even at 25 °C.

Several proteins have been demonstrated to be cryoinsoluble: cryoinmunoglobulins from pathological human plasma (17) and C³g, cold-insoluble globulin (18). The cryoinsolubility of PNA has not been mentioned in the literature. This lack of observation may well be because of galactosides present in the preparations of PNA used, as these sugars have been shown to inhibit the cryoinsolubility of the lectin (see "Results"). A
good example is provided by commercially available PNA preparations which apparently do not precipitate at low temperatures. A significant amount (1-6%) of galactose or lactose, probably used as eluting agents for the affinity chromatography step, has been found in two commercial preparations which became cryoinsoluble after extensive dialysis. Another preparation precipitated at low temperatures following a single Sephadex G-100 gel filtration (although it did not after dialysis), which would suggest that it contained saccharide breakdown products of the affinity column itself. The presence of these carbohydrates should be considered very carefully when the binding properties of PNA to small ligands or cells are to be determined. In particular, these carbohydrates can lead to nonlinearity in the Scatchard plots and, hence, to misinterpretation of the types of sites involved or of possible cooperativity between sites on the cell surface.

A number of possibilities can be ignored as causes of the cryoinsolubility of PNA. (a) Lipids possibly present in the preparation would be unlikely candidates since the same behavior has been observed in PNA samples prepared after removal of the lipids from ground seeds with organic solvent. (b) Self-aggregation involving sugar moieties of different molecules can be excluded since PNA is not a glycoprotein. (c) PNA being devoid of cysteine (4), interchain disulfide bonds, as occur in cryoimmunoglobulin WEB (12, 20), are out of the question. (d) The insolubility cannot be attributed to precipitation of a few impurities as, with time, the whole preparation precipitates and, in addition, the pH used in the various experiments was significantly higher than the pI of all of the PNA isoelectrons (21). (e) Although atomic absorption spectroscopy has recently shown that Mg$^{2+}$ and Ca$^{2+}$ are present in the PNA molecule in amount approaching 1 mol/mol of subunit (16), it would be difficult to imagine an absence of constitutive metal(s) being the origin of the cryoinsolubility. The purification procedure does not include any step which might remove metal ions from the protein and, in view of the affinity of metals for lectins (22), any addition of MgCl$_2$ would promote the inhibition of insolubility and even more than is reported in Fig. 8. (f) No evidence has been gained by analytical ultracentrifugation of any change in the amount of polymerization in PNA samples cooled to 4 °C, although concanavalin A converts from tetramer to dimer at low temperatures (10, 23) and cold-insoluble globulin, before precipitating, undergoes a dimer to tetramer transition with cooling (24). (g) It has been reported that conformational changes provoked by low temperatures are a necessary step in the insolubility of some cryoimmunoglobulins (25-27). In at least one case, however, these initial observations proved to be artificial (26, 28) and it is now thought that in most cases cryoimmunoglobulins do not undergo any conformational changes at low temperatures (28, 29). With a number of spectrophotometric methods (absorption, CD, fluorescence), we were unable to determine any conformational changes in our PNA samples during exposure to low temperatures.

By a process of elimination, we must conclude that the cryoinsolubility of PNA is probably due to the individual solubility properties of the protein itself. Presumably, owing to an atypical conformation (compared to other lectins), PNA interacts unfavorably with the solvent, thus maximizing intermolecular associations. This notion is now generally accepted for cryoimmunoglobulins (28, 29). The cryoinsolubility of PNA is marked by a highly concentration-dependent lag time and a "critical" concentration below which the protein is completely soluble. Such features are characteristic of protein associations requiring a nucleation event (30).

A final question was that of the types of interactions involved in the mechanism of the cryoinsolubility and the effects of sugars and salts on these interactions. It is well known that sugars and polyols increase the thermostability of proteins (31). This stabilization is explained in terms of their effects on the structure of water which in turn determines the strength of hydrophobic interactions (32). A similar phenomenon may be responsible for the effects of "nonspecific" sugars on PNA (Fig. 7).

The effects of the "specific" saccharides on the solubility of PNA (Fig. 6) require another explanation. The increased stability of proteins in the presence of ligands has been known for a long time (33). In addition, studies using differential scanning calorimetry to investigate the interactions between concanavalin A and carbohydrates have shown that binding stabilizes the structure of the protein (34), the degree of stabilization being a function of the association constant for each sugar moiety. This could be compared with the effects of specific galactosides on the cryoinsolubility of PNA. These sugars inhibited the development of turbidity in the same order as their affinity as ligands for the lectin (as determined by their inhibition of erythrocyte agglutination). Our results indicate that there is an obvious functional relationship between the two phenomena. The effects of galactosides, as shown by the changes in CD spectra induced by lactose in the near UV zone, even in the presence of MgCl$_2$ can be explained in terms of their action on residues responsible for aggregation. These residues are either localized in the sugar binding site and then masked by galactosides, or in the vicinity of the sugar binding sites and locally reorganized with the binding of the saccharide.

Particularly interesting papers have been published on the solubility of proteins and the lyotropic series of ions (35-37). The latter have been used to analyze the mechanism of cryoprecipitation of IgG McE (38). The lack of inhibition of PNA cryoinsolubility by high concentrations of neutral salts at the salting out end of the Hofmeister series (e.g. KCl, NaCl, and NH$_4$Cl) suggests that charge-charge interactions are not directly responsible for the formation of the cryoprecipitate (35, 38). It should be noted that the increase in turbidity in concanavalin A solutions at neutral pH is counteracted by the use of high NaCl concentrations (9).

The inhibition of cryoprecipitation by strongly chaotropic neutral salts (NaSCN, NaI, and MgCl$_2$) can be due to a direct action on the lectin molecule or to changes in the solvent structure in the presence of these salts which would indirectly affect the protein structure. The sedimentation coefficient, $s = 4.70$ S, found in 1 M MgCl$_2$ could partly be assigned to an effect on the dimer-tetramer equilibrium, assuming no preferential hydration, i.e. no change of $v$. This value is higher than the sedimentation coefficient of the dimer, 3.8 S (8), and does not correspond to an unfolded form of the PNA tetramer. The absence of any meaningful changes in terms of structural modification of the protein, observed by circular dichroism in the near UV zone, is confirmed by the far UV CD spectrum and by intrinsic fluorescence.

The strength of hydrophobic interactions is decreased at low temperatures. The formation of the cryoprecipitate can then be explained by two types of noncovalent interactions: van der Waals interactions and hydrogen bonds. Referring to the study of Klots and Franzen (39) on model peptides in solution, Middaugh and co-workers (28, 29, 38) suggested that

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3 M. Decastel and J.-P. Frénoy, unpublished results.
van der Waals interactions are involved in the formation of the cryoprecipitate of the two cryoimmunoglobulins McE and Gre. It should be pointed out that PNA has the same behavior toward neutral salts as these two proteins (29, 38).

Interpeptide hydrogen bonds are known to be stabilized when the temperature is lowered. The fixation of chaotropic neutral salts to peptide amide dipoles (40) would disrupt these hydrogen bonds and inhibit the cryoprecipitation of PNA. From our data, and although their relative effects cannot be precise, both van der Waals interactions and hydrogen bonds may be involved in the formation of the cryoprecipitate.

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