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Effect of physical environment on the conformation of ricin

Influence of low pH*

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The molecular properties of ricin (the toxic lectin from *Ricinus communis* seeds, RCA II or RCA 60) were evaluated by analytical ultracentrifugation, viscosimetry, c.d., fluorescence and equilibrium dialysis. Measurements of sedimentation ($s^0_{20,w} = 4.60$ S) and viscosity ($\eta = 2.96 \times 10^{-2}$ dl/g) indicated that, at neutral pH, the ricin molecule is very compact. Various transitions were explored, and a pH-triggered change in the ricin conformation was observed between pH 7 and 4. In this range, the sedimentation coefficient, far-u.v. c.d. and fluorescence altered simultaneously without unfolding. Below pH 7 the change in the ricin conformation was accompanied by a decrease in the affinity of ricin for galactosides, and at pH 4.0 by an alteration in its binding capacity. These effects of low pH are discussed in relation to the physical conditions encountered by ricin molecules during their entry into living cells.

INTRODUCTION

Castor-bean (*Ricinus communis*) seeds contain two closely related lectins: the haemagglutinin (RCA I or RCA 120) and ricin (RCA II or RCA 60), with M_r values of 120000 and 60000 respectively. Both lectins are glycoproteins and have similar specificities for galactose and galactosides (Pardoe *et al.*, 1969; Nicolson & Blaustein, 1972; Olsnes *et al.*, 1974).

Ricin is a cytotoxic lectin (Nicolson & Blaustein, 1972) possessing anti-tumour activity against various sarcomas in rat and mouse (Lin *et al.*, 1970). It is composed of an A and a B polypeptide chain (M_r approx. 30000 each) joined by a disulphide bond. The B-chain has two sites that both bind galactosides and galactosaminides (Zentz *et al.*, 1978; Villafranca & Robertus, 1981), and binds to the membrane of sensitive mammalian cells. Once the ricin has entered the cell, the A-chain specifically inhibits protein synthesis by decreasing the affinity of the 60 S ribosomal subunit for the elongation factors (Olsnes & Sandvig, 1983).

The interest in ricin recently increased still further with the coupling of the A-chain with cell-specific binding proteins for use as a 'cell-killer' in the preparation of selective chimeric toxins known as immunotoxins (Uhr, 1984).

The pH-dependent interaction between 8-anilinonaphthalene-1-sulphonic acid and ricin (Houston, 1980) as well as the existence of different transitions in the effect of temperature on the binding of this toxic lectin to HeLa cells and on its subsequent release (Sandvig & Olsnes, 1979) might indicate that ricin undergoes pH- and temperature-dependent conformational changes. Investigation of these changes would help to explain the mechanism of action of ricin in molecular terms.

In endocytotic vesicles, ligand–receptor complexes are exposed to mildly acidic conditions, and acidification is connected with the mechanism of receptor segregation and recycling during endocytosis (Wileman *et al.*, 1985). pH has also been said to affect the entry of ricin into the cells and the expression of its toxic activity (Sandvig *et al.*, 1976; Sandvig & Olsnes, 1982; Ghosh *et al.*, 1985). In addition, sialic acid residues of cell-surface glycoconjugates determine acidic microenvironments (Bonfils *et al.*, 1981) and could thus modify the affinity for ricin of binding sites containing galactose and the conformation of the toxic molecule itself.

In the present investigation the molecular structure and stability of ricin have been evaluated, particularly in acid media, by analytical ultracentrifugation and several spectroscopic methods.

MATERIALS AND METHODS

A homogeneous preparation of ricin was obtained from *Ricinus communis* (var. *sanguineus*) seeds (Vilmorin-Andrieux, Paris, France) by the procedure of Nicolson & Blaustein (1972). The preparation was homogeneous when tested by polyacrylamide-gel electrophoresis at pH 7.5, ultracentrifugation and immunoelectrophoresis. Its haemagglutinating activity was checked against human O^- erythrocytes and was 17 μg (minimum haemagglutinating dose). The ricin concentration was determined by absorbance measurements at 280 nm, at pH 7, by using $a = 1.4$ litre$\cdot$g$^{-1}\cdot$cm^{-1} (Zentz *et al.*, 1978).

Sedimentation-velocity experiments were carried out at 59780 rev./min in a Beckman model E instrument equipped with schlieren optics. The temperature was controlled by an R.T.I.C. unit. A partial specific volume, $\bar{v}$, of 0.715 was calculated from the amino acid (Nicolson *et al.*, 1974) and carbohydrate (J. Font, unpublished work) compositions of ricin in accordance with Cohn & Edsall (1943) and Gibbons (1971) respectively, and was used for corrections relating to solvent density.

C.d. spectra were measured with a Jobin et Yvon mark V Dichrograph. In the near u.v. cells with pathlengths of 0.2 and 1 cm were used for ricin concentrations of 5.31 and 1.10 mg/ml respectively, and in the far u.v. a cell

* In memory of Harold Edelhoch (National Institutes of Health, Bethesda, MD, U.S.A.), who died on 15 January 1986.

with a pathlength of 0.5 cm was used for a ricin concentration of 0.042 mg/ml. Mean residue ellipticity $[\theta]$ at any wavelength (λ) was calculated by using the equation:

$$[\theta]_\lambda = \theta_\lambda \times 109.6/10 \times l \times c$$

where θ_λ is the observed ellipticity, l the pathlength (in cm), c the protein concentration (in g/ml), and 109.6 the mean residue weight of ricin calculated from its amino acid composition. No correction was made for the refractive index of lactose.

Fluorescence spectra were obtained with a Jobin et Yvon JY3 fluorimeter coupled to a Hewlett–Packard 9815 A calculator and 9862 A plotter, and thermostatically controlled with a Haake F3K cryothermostat. Wavelength-dependent corrections for optical and photomultiplier tube distortions were made with Rhodamine B by using the program developed for the Hewlett–Packard 9815 A calculator by Jobin et Yvon. The excitation wavelength was 280 or 295 nm (2 nm bandwidth), and emission was monitored through 4 nm bandwidths. The samples underwent continuous magnetic stirring and the temperature was directly measured in the sample cuvette (1 cm × 1 cm × 4.5 cm) with a YSI 729 probe (Yellow Springs Instruments) coupled to a Digitec 5810 thermometer.

Viscosity was measured in a Fica Viscomatic M-S apparatus equipped with Ubbelohde semi-microdilution viscometers. Constant temperature was obtained with a Haake FK thermostat, and the temperature was set at 20.07 ± 0.02 °C. Solvent and protein solutions were filtered through a sintered-glass filter (no. 3) just before each experiment. Flow time was 207.8 s for solvent with a viscometer 0.46 mm in diameter. Each flow-time value was an average for at least five measurements with a maximal deviation of less than ±0.3 s.

Equilibrium dialysis was performed as previously described (Zentz *et al.*, 1978), over a period of 48 h, with Dianorm 250 μl cells. Thermostatic control at 4 ± 0.1 °C was obtained with a Huber Variostat cryothermostat. [^{3}H]Galactose was from Amersham International and its specific radioactivity was 385 GBq/mmol. At pH 7.0, 6.5 and 6.1 experiments were performed in 0.025 M-sodium phosphate buffer containing 0.15 M-NaCl, and at pH 5.4, 4.7 and 4.0 in 0.05 M-sodium acetate buffer containing 0.15 M-NaCl. I checked that at pH 6.1 sodium acetate did not modify the affinity of ricin for galactose.

For titration experiments, the pH was adjusted by adding small volumes of either concentrated acid or base through a fine polyethylene tube connected to a micrometer-driven Agla syringe while the solution was stirred with a magnetic stirrer. The pH was measured by a Radiometer model PHM 62 pH-meter, calibrated with Radiometer buffers. All experiments were performed with solutions passed through Millipore filters (0.45 μm pore size). All chemicals used were of reagent grade except guanidinium chloride, which was a Serlabo ultra-pure product.

RESULTS

At pH 6.9 the sedimentation coefficient of the preparation in 0.01 M-sodium phosphate buffer containing 0.15 M-NaCl was concentration-independent for ricin concentrations between 0.5 and 2.4 mg/ml (Fig. 1*a*). An $s^0_{20,w}$ of 4.60 S was determined after correction for density and viscosity of the solvent. When $s^0_{20,w}$ is combined with the M_r value of 60000 reported for ricin (Zentz *et al.*, 1978) a frictional ratio f/f_0 of 1.28 can be calculated, indicating a globular molecule, even though somewhat ellipsoid.

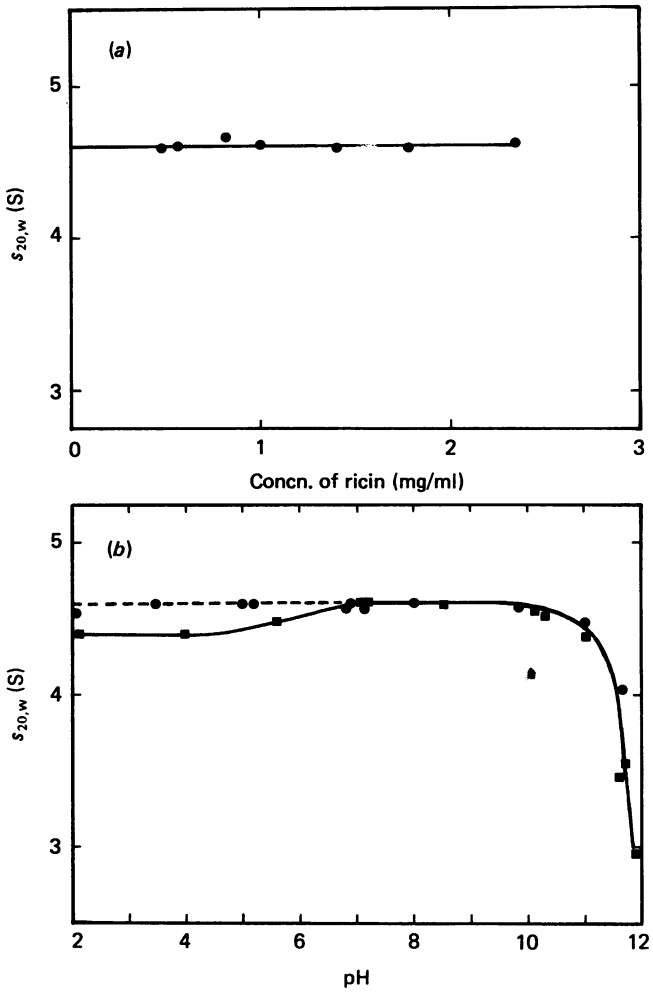

Fig. 1. Sedimentation of ricin at 20 °C

(*a*) Sedimentation coefficient of ricin as a function of initial protein concentration. Experiments were performed in 0.01 M-sodium phosphate buffer, pH 6.9, containing 0.15 M-NaCl. (*b*) Effect of pH on the sedimentation coefficient of ricin. Experiments were performed at ricin concentrations 2.60–2.85 mg/ml in 0.01 M-sodium phosphate buffer in the absence (■) or in the presence (●) of 0.15 M-NaCl. Values are corrected for density and viscosity of the solvent.

At pH 7.1 and 20 °C in 0.01 M-sodium phosphate buffer, the intrinsic viscosity was 2.96×10^{-2} dl/g (experiment not shown), indicating that the ricin molecule is very compact.

The corrected fluorescence-emission spectrum of native ricin was maximal at 334 nm when excited at 280 nm, and the spectrum did not change significantly when the excitation wavelength was 295 nm and only the tryptophan residues were excited (see Fig. 4*b* inset). Tyrosine emission was therefore either completely quenched by non-radiative collision, or transferred to neighbouring emitting tryptophan residue(s) in this native ricin molecule.

In 6 M-guanidinium chloride, where very little or no

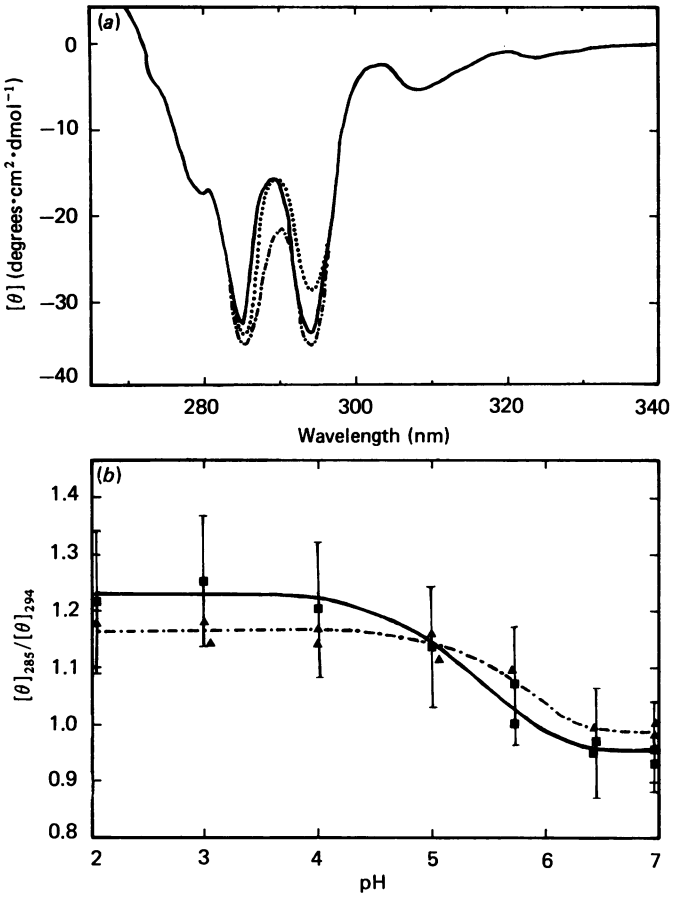

Fig. 2. Near-u.v. c.d. of ricin at 20 °C

(*a*) Near-u.v. c.d. spectra at pH 7.0 (——) and 4.0 (·····) in 0.01 M-sodium phosphate buffer at pH 7.0 in the presence of 0.1 M-lactose (–·–·–); the ricin concentration was 1.10 mg/ml. (*b*) Effect of pH on the ratio of the mean-residue ellipticities at 285 and 294 nm. Experiments were performed in 0.01 M-sodium phosphate buffer in the absence (■) or in the presence (▲) of 0.1 M-lactose.

organized structure is left, a discrete shoulder at 305 nm indicated the tyrosine fluorescence, and the tryptophan peak was at about 350 nm. The lower-wavelength peak of the native protein indicates that some of the tryptophan residues are exposed either incompletely or not at all to solvent molecules and are therefore in a hydrophobic environment.

In the presence of 0.1 M-lactose, very weak enhancement and blue-shift ($\leqslant$ 1 nm) of the ricin fluorescence spectrum were observed. It should also be remembered that an excess of lactose does not affect the M_r of ricin (Zentz *et al.*, 1978).

Thermal stability

Sedimentation coefficients of 2.84 and 2.86 S were measured at 4 °C and pH 6.9 for ricin concentrations of 1.79 and 1.35 mg/ml respectively. These coefficients correspond to 4.58 and 4.62 S after correction for solvent density and viscosity, and were indistinguishable from the $s^0_{20,w}$ obtained at 20 °C.

Between 4 and 65 °C the fluorescence-emission intensity of the ricin diminished regularly, and only a small red-shift in the emission maximum from 334 to 336 nm was observed above 50 °C. Above 65 °C the significant increase in the wavelength of the emission peak indicated increased exposure to the solvent of ricin tryptophan residues, i.e. progressive denaturation.

The effect of temperature enhancement on the near-u.v. c.d. spectrum of ricin (Fig. 2*a*) was characterized by a small gradual widening of the two peaks centred at 285 and 294 nm but no significant changes in their relative intensity. At neutrality, the strongest c.d. peak of ricin was near 294 nm and therefore, according to Strickland (1974), can only belong to a tryptophan residue. The red-shift to 294 nm indicates low environmental polarity for this residue, compared with tryptophan compounds, with a peak centred at 288–292 nm (Edelhoch *et al.*, 1967; Strickland, 1974). The second major peak at 285 nm mainly represents a tyrosine chromophore in a very non-polar environment, as shown by mild acetylation of ricin (Shimazaki *et al.*, 1975; see also below, under 'pH stability').

I observed a c.d. spectrum between 200 and 250 nm similar to that reported by Shimazaki *et al.* (1975). No changes in the shape or size of the far-u.v. c.d. peak occurred between 4 and 20 °C, but at 29 °C an 8% decrease in peak intensity was observed (Fig. 3). At 49 °C the signal intensity was 89% of that measured at 20 °C. This probably reflects a partial loss of the organized secondary structure of ricin when the temperature rises.

pH stability

When the pH of a solution of ricin in 0.15 M-NaCl was lowered from neutrality, the sedimentation coefficient

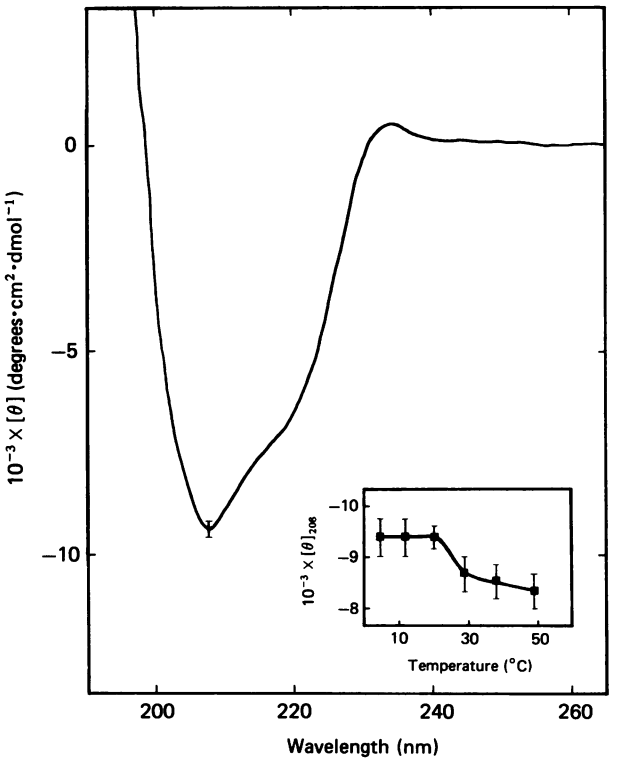

Fig. 3. Far-u.v. c.d. spectrum of ricin (0.042 mg/ml) at 20 °C in 0.01 M-sodium phosphate buffer, pH 7.0

The inset indicates the temperature-dependence of the mean-residue ellipticity at 208 nm.

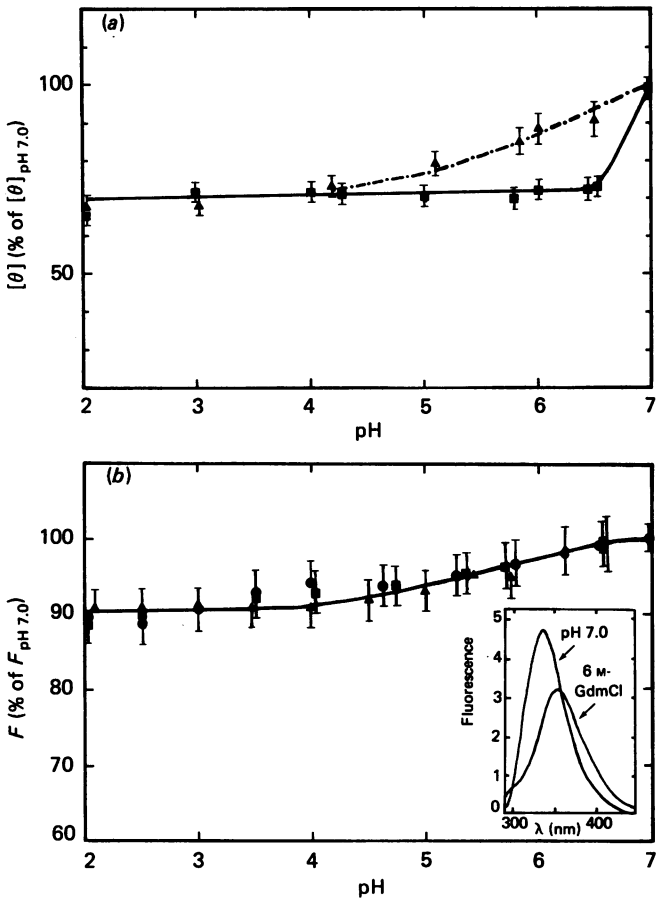

Fig. 4. Effect of pH on the mean-residue ellipticity at 208 nm (*a*) and tryptophan fluorescence (*b*) of ricin

Experiments were performed at 20 °C in 0.01 M-sodium phosphate buffer alone (■), and in the presence of 0.15 M-NaCl (●) or 0.1 M-lactose (▲). Results are expressed as the percentage of ellipticity ($[\theta]_{pH\,7.0}$) or fluorescence intensity ($F_{pH\,7.0}$) measured at pH 7.0. The inset in (*b*) shows the fluorescence-emission spectra in 0.01 M-sodium phosphate buffer containing 0.15 M-NaCl and in 6 M-guanidinium chloride (GdmCl) at pH 6.9. The ricin concentration was 0.057 mg/ml and the temperature 20 °C. Excitation was 280 nm.

was maintained (Fig. 1*b*). A small decrease was observed between pH 7 and 4 in the absence of NaCl, i.e. in 0.01 M-sodium phosphate buffer. The sedimentation coefficient of ricin was constant between pH 7 and 11 but fell reversibly at pH values above 11, in the presence and particularly in the absence of 0.15 M-NaCl (Fig. 1*b*). At pH 11.8 in 0.01 M-sodium phosphate buffer, the reduced viscosity of ricin was 6.82×10^{-2} dl/g.

In the absence and in the presence of NaCl, the fluorescence intensity of ricin fell by 9% between pH 6.5 and 4.5 but the decrease was not accompanied by a shift in the emission peak. The same results was obtained in the presence of 0.1 M-lactose (Fig. 4*b*). Below pH 6.5, the fluorescence-intensity change was concentration-independent at ricin concentrations between 0.004 and 0.09 mg/ml.

Tryptophan emission was continuously quenched by 50% between pH 8 and 11 (results not shown). Since ricin was stable in this pH range, this quenching was probably due to energy transfer from tryptophan to tyrosine residues as the latter ionize.

When the pH of the ricin solution was lowered to below 7.0, the c.d. peak at 285 nm gradually became the largest, as shown in Fig. 2(*b*), where the ellipticity ratios at 285 and 294 nm are plotted versus pH. In the presence of 0.1 M-lactose, when both peaks were enhanced at neutral pH and reached the same intensity (Fig. 2*a*), the peak at 285 nm again became the largest when the pH was lowered.

However, at pH 10 and above, when tyrosine residues ionize, the peak at 285 nm gradually diminished, confirming that it should essentially be assigned to a tyrosine chromophore (see above under 'Thermal stability').

The shape of the far-u.v. c.d. spectrum of ricin was the same between pH 2.0 and 11.0. However, although the intensity of the signal was not modified when the pH was raised above 7, a sharp decrease in the dichroic signal was observed between pH 7.0 and 6.5 (Fig. 4*a*). At pH 6.5, intensity was 72% of that at pH 7.0, and was maintained at this value at pH 6.0 and below. A decrease in the dichroic signal, which was of similar magnitude but was more gradual with the lowering of the pH (Fig. 4*a*), was also observed in the presence of 0.1 M-lactose. This might indicate a change in the secondary structure of ricin to a conformation with a small amount of additional unordered conformation.

Equilibrium dialysis

As the above results suggested the existence of two pH-dependent transitions for ricin molecules in the pH ranges of 7.0–6.5 and 6.5–4.0, I decided to evaluate the effect of pH changes on the carbohydrate-binding properties of ricin. The results of equilibrium dialyses with [^{3}H]galactose at pH 6.5, 6.1, 5.4, 4.7 and 4.0 were therefore compared with those for dialysis at pH 7.0. The Scatchard plots in Fig. 5 definitely show that at pH 6.1 and above, this batch of ricin was able to bind a second galactose molecule, but with a low affinity. [The ricin molecule has been shown by X-ray crystallography to contain two different binding sites for galactosides (Villafranca & Robertus, 1981). By equilibrium dialysis, we previously observed that two molecules of lactose but only one of galactose were bound to each ricin molecule (Zentz *et al.*, 1978), a result again found by Shimoda & Funatsu (1985), despite the fact that Houston & Dooley (1982) reported equivalent binding of two molecules of 4-methylumelliferyl galactosides. In the present experiments, in which I used a batch of ricin that was different from the one used in 1978 but was prepared by the same method from seeds of the same variety and commercial origin, deviation from the linear Scatchard curve at high galactose concentrations showed that a second molecule was bound but with a low affinity. These observations indicate that ricin batches greatly vary as regards their potentiality for galactose binding. Although the binding of this second molecule affects the measurement of the affinity for the first site from the initial slope of the Scatchard plots, the apparent difference between the association constants for these two sites allows a reasonable estimation of $K_{a1} = 1.1 \times 10^4$ M^{-1} at pH 7.0 comparable with the $K_a = 6.9 \times 10^3$ M^{-1} formerly determined at pH 6.9 (Zentz *et al.*, 1978).] When the affinity of galactose for the high-affinity site of ricin was estimated as $K_a = 1.1 \times 10^4$ M^{-1} at pH 7.0, K_a

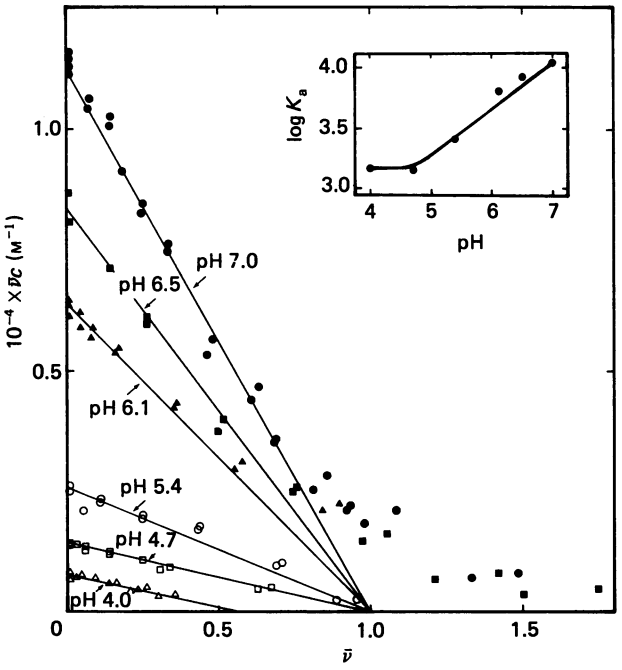

Fig. 5. Equilibrium-dialysis data for the binding of galactose to ricin at 4 °C

Experiments were performed in 0.025 M-sodium phosphate buffer containing 0.15 M-NaCl at pH 7.0 (●), pH 6.5 (■) or pH 6.1 (▲) for ricin concentrations 133–167, 179 and 231 μM respectively. At pH 5.4 (○), pH 4.7 (□) and pH 4.0 (△) ricin concentrations were 248, 295 and 345 μM in 0.05 M-sodium acetate buffer containing 0.15 M-NaCl. The inset shows the pH-dependence of the association constant for the high-affinity site.

values of 8.3×10^3, 6.4×10^3, 2.5×10^3 and 1.4×10^3 M^{-1} were found at pH 6.5, 6.1, 5.4 and 4.7 respectively.

At pH 4.0, where the transition affecting the tertiary structure is complete, ricin still bound galactosides with the same affinity as at pH 4.7 ($K_a = 1.5 \times 10^3$ M^{-1}), but its binding capacity diminished in this acid solution and only 0.55 site per ricin molecule was detected by equilibrium dialysis (Fig. 5)

DISCUSSION

The present results for the toxic lectin ricin, obtained by using sedimentation-velocity, equilibrium-dialysis and several spectrophotometric techniques, indicate that in some respects it is temperature- and more particularly pH-dependent. These characteristics have not so far been reported and might well affect this protein's toxic efficiency.

From X-ray-crystallographic analyses, Villafranca & Robertus (1981) claimed that the B-chains of ricin were composed of two domains, each containing one of the two sugar-binding sites previously demonstrated for lactose by microcalorimetry (Zentz *et al.*, 1977) and equilibrium dialysis (Zentz *et al.*, 1978). In the present study, a frictional ratio of 1.28 and an intrinsic viscosity of 2.96×10^{-2} dl/g were determined for ricin. It is clear from these results that ricin is a globular and compact molecule and consequently that its structural domains are very tight. This conclusion is in accordance with data indicating that ricin is extremely resistant to treatment with proteolytic enzymes (Olsnes *et al.*, 1975) and that the interchain disulphide bond is of difficult access to reducing agents larger than 2-mercaptoethanol (Lappi *et al.*, 1978).

By analogy with the results obtained with *Ricinus communis* haemagglutinin (see the following paper, Frénoy *et al.*, 1986), it is unlikely that the reversible decrease observed in the ricin sedimentation coefficient at pH values above 11 is due to degradation of the protein interchain disulphide bond, which sometimes occurs in alkali (Florence, 1980). What is more, the simultaneous doubling of intrinsic viscosity can probably be related to an increase in the molecular asymmetry of ricin rather to any enhancement of hydration. This does not correspond to denaturation, since at pH 11.8 the maximum ricin fluorescence emission is at 337 nm and is not normalized to an emission corresponding to that of the fully denatured protein in 6 M-guanidinium chloride (i.e. 350 nm).

The most important and interesting effect exerted by pH on the ricin molecule was the one that occurred at pH below 7 on both its conformation and affinity for galactosides.

As the changes in the sedimentation coefficient, far-u.v. c.d. and fluorescence spectra all occurred simultaneously, it is clear that the conformation of ricin alters and that the effect of pH upon fluorescence does not arise from the protonation of the carboxylate groups located near one or more of the tryptophan residues (Steiner & Edelhoch, 1963; Edelhoch *et al.*, 1967). As observed here by far-u.v. c.d., the change in the secondary structure occurred early, when the pH decreased, at least in the absence of lactose.

The apparent enhancement of 8-anilinonaphthalene-1-sulphonic acid binding to ricin at low pH was related to a conformational change in this molecule that revealed hydrophobic zones (Houston, 1980). Although it could be argued that dimerization of 8-anilinonaphthalene-1-sulphonic acid occurred at low pH under experimental conditions that altered the results, the present work undoubtedly shows conformational change(s) in the ricin molecule in the range of pH below neutrality.

When the pH is lowered from neutrality, the affinity of ricin for galactosides decreased concomitantly with the change(s) in molecule conformation; subsequently, at a lower pH, this decrease was accompanied by an alteration in binding capacity. From u.v. difference-spectroscopy measurements Yamasaki *et al.* (1985) reported a decrease with pH of the ricin–galactose association constant that was analogous to the decrease that I found, although it was larger.

In endocytotic vesicles, ligand–receptor complexes are exposed to mildly acidic conditions, i.e. to pH between 5 and 5.5 (Geisow & Evans, 1984). These conditions may produce dissociation of ligands from their receptors and changes in receptor and/or ligand conformation (Di Paola & Maxfield, 1984); these conformational changes are thought to facilitate the fusion of the receptor and/or ligand with the membrane.

It is generally assumed that ricin and other toxins enter mammalian cells by receptor-mediated endocytosis (Olsnes & Sandvig, 1983; van Deurs *et al.*, 1985, 1986), and evidence exists that A- and B-chains penetrate these cells (Ishida *et al.*, 1983). According to Olsnes and co-workers, a pH between 6 and 8 does not affect the

total binding of ricin to the galactose-containing binding sites of the cell surface, or at least not very much (Sandvig *et al.*, 1976; Sandvig & Olsnes, 1982). However, these authors showed that lowering the external pH of cells incubated with ricin decreases the toxicity of this molecule (Sandvig & Olsnes, 1982). They therefore concluded that ricin does not enter the cells via an acidic compartment to exert its activity. Recent and apparently contradictory results of intracellular K^+-depletion experiments were interpreted as proof either that a hitherto unknown pathway for ricin exists inside the cell (Moya *et al.*, 1985) or that a high intracellular pH favours the toxic activity of ricin (Ghosh *et al.*, 1985).

These interpretations are open to question since, from the present work, it is obvious that both the affinity of ricin for galactosides and the number of galactoside-binding sites on the lectin molecule diminish with pH and that concomitantly ricin undergoes a conformational change in the mildly acidic conditions encountered by this molecule in endocytotic vesicles.

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REFERENCES

Bonfils, C., Nato, F., Bourrillon, R. & Balny, C. (1981) FEBS Lett. **123**, 222–224
Cohn, E. F. & Edsall, J. T. (1943) Proteins, Amino Acids and Peptides, pp. 370–381, Reinhold, New York
Di Paola, M. & Maxfield, F. R. (1984) J. Biol. Chem. **259**, 9163–9171
Edelhoch, H., Brand, L. & Wilchek, M. (1967) Biochemistry **6**, 547–549
Florence, T. M. (1980) Biochem. J. **189**, 507–520
Frénoy, J.-P., Tran, A.-T. & Bourrillon, R. (1986) Biochem. J. **240**, 227–231
Geisow, M. J. & Evans, W. H. (1984) Exp. Cell Res. **150**, 36–46
Ghosh, P. C., Wellner, R. B., Cragoe, E. J., Jr. & Wu, H. C. (1985) J. Cell Biol. **101**, 350–357
Gibbons, R. A. (1971) in Glycoproteins (Gottschalk, A., ed.), pp. 31–140, Elsevier, Amsterdam
Houston, L. L. (1980) Biochem. Biophys. Res. Commun. **92**, 319–326
Houston, L. L. & Dooley, T. (1982) J. Biol. Chem. **257**, 4147–4151
Ishida, B., Cawley, D. B., Reue, K. & Wisnieski, B. J. (1983) J. Biol. Chem. **258**, 5933–5937
Lappi, D. A., Kapmeyer, W., Beglau, J. M. & Kaplan, N. O. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 1096–1100
Lin, J. Y., Tserng, K. Y., Chen, C. C., Lin, L. T. & Tung, T. C. (1970) Nature (London) **227**, 292–293
Moya, M., Dautry-Varsat, A., Goud, B., Louvard, D. & Boquet, P. (1985) J. Cell Biol. **101**, 548–559
Nicolson, G. L. & Blaustein, J. (1972) Biochim. Biophys. Acta **266**, 543–557
Nicolson, G. L., Blaustein, J. & Etzler, M. E. (1974) Biochemistry **13**, 196–204
Olsnes, S. & Sandvig, K. (1983) Receptor Mediated Endocytosis: Recept. Recognit. Ser. B. **15**, 188–236
Olsnes, S., Saltvedt, E. & Pihl, A. (1974) J. Biol. Chem. **249**, 803–810
Olsnes, S., Refsnes, K., Christensen, T. B. & Pihl, A. (1975) Biochim. Biophys. Acta **405**, 1–10
Pardoe, G. I., Bird, G. W. & Uhlenbruch, G. (1969) Z. Immunitätforsch. **137**, 442–457
Sandvig, K. & Olsnes, S. (1979) Exp. Cell Res. **121**, 15–25
Sandvig, K. & Olsnes, S. (1982) J. Biol. Chem. **257**, 7504–7513
Sandvig, K., Olsnes, S. & Pihl, A. (1976) J. Biol. Chem. **251**, 3977–3984
Shimazaki, K., Walborg, E. F., Neri, G. & Jirgensons, B. (1975) Arch. Biochem. Biophys. **169**, 731–736
Shimoda, T. & Funatsu, G. (1985) Agric. Biol. Chem. **49**, 2125–2130
Steiner, R. F. & Edelhoch, H. (1963) Biochim. Biophys. Acta **66**, 341–355
Strickland, E. H. (1974) CRC Crit. Rev. Biochem. **2**, 113–175
Uhr, J. W. (1984) J. Immunol. **133**, i–x
van Deurs, B., Pedersen, L. R., Sundan, A., Olsnes, S. & Sandvig, K. (1985) Exp. Cell Res. **159**, 287–304
van Deurs, B., Tonnessen, T. I., Petersen, O. W., Sandvig, K. & Olsnes, S. (1986) J. Cell Biol. **102**, 37–47
Villafranca, J. E. & Robertus, J. D. (1981) J. Biol. Chem. **256**, 554–556
Wileman, T., Harding, C. & Stahl, P. (1985) Biochem. J. **232**, 1–14
Yamasaki, N., Hatakeyama, T. & Funatsu, G. (1985) J. Biochem. (Tokyo) **98**, 1555–1560
Zentz, C. Frénoy, J.-P. & Bourrillon, R. (1977) FEBS Lett. **81**, 23–27
Zentz, C., Frénoy, J.-P. & Bourrillon, R. (1978) Biochim. Biophys. Acta **536**, 18–26

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