The effect of zinc-deficiency on activities of OCT, GDH, arginase and AAT

Enzyme	Tissue	Unit*/g fresh wt Pair-fed control	Zn-deficient	Unit*/mg protein Pair-fed control	Zn-deficient
OCT	Liver	210.13±16.80	124.48± 25.82**	1.01±0.03	0.66±0.11**
	Intestine	9.08± 2.52	5.97± 2.73	0.13±0.01	0.08±0.03
GDH	Liver	139.41±22.60	231.50± 29.61**	0.71±0.11	1.17±0.17**
	Kidney	26.68±10.12	28.59± 6.91	0.24±0.08	0.25±0.06
arginase	Liver	572.93±45.75	528.03±118.42	3.00±0.25	2.73±0.37
	Intestine	39.53±13.73	33.22± 14.55	0.60±0.10	0.49±0.20
AAT	Liver	215.34±35.79	280.44± 37.80	1.03±0.11	1.47±0.26
	Kidney	94.53± 8.82	80.00± 11.06	0.86±0.11	0.71±0.08

* Unit is expressed as μmole product liberated per min; ** significantly different from value for pair-fed control ($p<0.01$). The data is presented as the mean ± SD of at least 10 rats.

increased activity. These authors killed their rats after a 2–3 week dietary regimen and a recent report[4] indicated that blood urea nitrogen started to decline sharply and significantly at the 4th week. Obviously, further studies are needed to ascertain the role of zinc in the urea cycle.

1 We wish to thank Professor P.M. Newberne for supplying the plastic cages and ingredients for the zinc-deficient diet.
2 J.M. Hsu and W.L. Anthony, J. Nutr. *105,* 26 (1975).
3 R.E. Burch, R.V. Williams, H.K.J. Hahn, M.M. Jetton and J.F. Sullivan, Clin. Chem. *21,* 568 (1975).
4 P. Rabbani and A.S. Prasad, Am. J. Physiol. *235,* E203 (1978).
5 L.Y.Y. Fong, P.M. Newberne and A. Sivak, J. natl Cancer Inst. *61,* 145 (1978).
6 P.J. Snodgrass, Biochemistry *7,* 3047 (1968).
7 A. Herzfeld and S.M. Raper, Biochem. J. *153,* 469 (1976).
8 Boehringer Mannheim GMBH Instruction Sheet (GOT UV-Method) (1975).
9 G. Ellis and D.M. Goldberg, Clin. Chem. *18,* 523 (1972).
10 J.L. Bollman, F.C. Mann and T.B. Magath, Am. J. Physiol. *69,* 371 (1924).
11 L. Raijman, Biochem. J. *138,* 225 (1974).
12 B.L. Vallee, S.J. Adelstein and J.A. Olson, J. Am. chem. Soc. *77,* 5196 (1955).
13 R.F. Colman and D.S. Foster, J. biol. Chem. *245,* 6190 (1970).
14 G.A. Kfoury, J.G. Reinhold and S.J. Simonian, J. Nutr. *95,* 102 (1969).

Biochemical evidence for two types of noradrenaline storage particles in rabbit iris

W.P. De Potter[1] and F.H. De Smet

Heymans Institute of Pharmacology, University of Ghent Medical School, De Pintelaan 135, B-9000 Ghent (Belgium),
8 February 1980

Summary. Centrifugation techniques were used to determine the subcellular distribution of noradrenaline (NA) and dopamine β-hydroxylase (DβH) in the rabbit iris. By application of isopycnic and differential gradient centrifugation methods 2 types of NA vesicles could be demonstrated. Of the total particle bound NA about 70% is associated with 'light' and about 30% with 'heavy' vesicles. For both types of vesicles the distribution of DβH reflected that of NA.

Since the original observation[2] that some of the NA in homogenates of bovine splenic nerves is present in particles, there have been many subcellular fractionation studies on this tissue as well as on other sympathetically innervated tissues[3–6]. Whereas the initial studies suggested the presence of 1 type of NA storage vesicle, during the last decade evidence has been presented that some of the adrenergically innervated tissues (as a source for adrenergic nerve endings) contain 2 types of NA storage vesicles[7–10].

Using sucrose gradient centrifugation techniques, Roth et al.[7] showed, for the rat heart, 1 peak of NA accumulating at 0.47 M sucrose whereas a 2nd peak of NA was present at the interface of 1.0 M and 2.0 M sucrose. A bimodal distribution of NA (1 peak at a density 1.130, the other at 1.178) has also been demonstrated for the dog spleen[8,9] as well as for the cat spleen[10]. These 2 populations of NA vesicles have been defined as NA-1.130 and Na-1.178[9], or more generally, as 'light' and 'heavy' NA vesicles[7].

Ultrastructural studies on adrenergic nerve terminals also favour the existence of 2 populations of NA vesicles, since they have shown that there are 'small' and 'large' dense cored vesicles which are thought to correspond with the biochemically demonstrated light and heavy vesicles respectively[10].

In contrast to these few biochemical studies on the rat heart and dog and cat spleen, for which a bimodal distribution of NA was clearly demonstrated, other gradient centrifugation experiments on the same (rat heart) as well as on other tissues such as e.g. rat submaxillary gland, rat vas deferens and rat iris failed to reveal a bimodal distribution of NA[4]. In the latter studies only 1 peak of NA (light NA vesicles) was found, despite the electron microscopic observations that the adrenergic nerve endings of such tissues contain, in addition to small, also large dense cored vesicles[10,11]. A likely explanation for this apparent discrepancy could be that the latter tissues contain also heavy NA vesicles but in a relatively low proportion so that the peak of heavy NA vesicles is masked in the usual isopycnic sucrose gradients. In order to test this hypothesis the rabbit iris was studied using different types of gradient centrifugation procedures. This tissue had been shown to contain both small and large dense cored vesicles[12]. Part of the results presented in this paper appeared in a preliminary report[13].

Materials and methods. Homogenization and differential centrifugation. Rabbits of both sexes (1–1.5 kg) were killed by an air embolism. The irises were dissected out and the tissue was rinsed 3 times in ice-cold 0.25 M sucrose (buffered with 5 mM Tris-HCl pH 7.3), chopped, suspend-

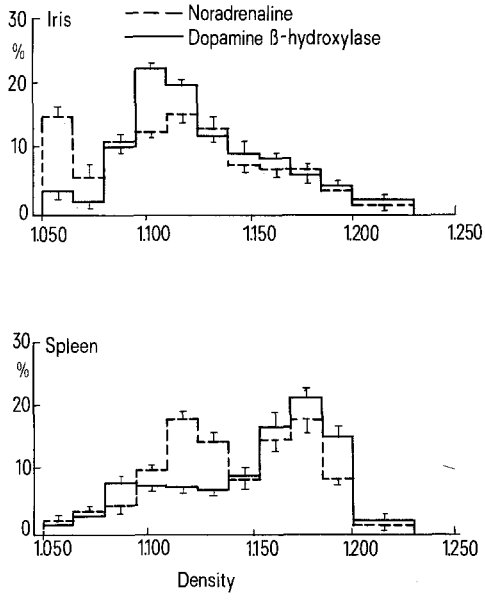


Fig. 1. Distribution of NA and DβH as obtained by isopycnic gradient centrifugation in a sucrose gradient (type A) of a NA enriched pellet from rabbit iris and dog spleen. Diagrams show average of results with SEM (n = 3). The distribution of NA[9] and DβH (unpublished results) from the dog spleen are given for comparative purposes.

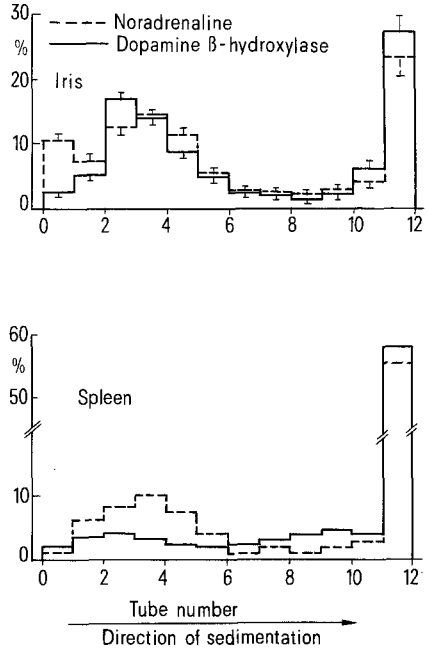


Fig. 2. Distribution of NA and DβH as obtained by differential sucrose gradient centrifugation (type B) of a NA enriched pellet from rabbit iris and dog spleen (n = 3). The distribution of NA[9] and DβH (unpublished results) from the dog spleen are given for comparative purposes.

ed in 50 vol. of ice-cold buffered sucrose per g of original tissue and homogenized in a motor driven Potter-Elvehjem homogenizer (Kontes, Vineland, New Jersey, USA) using a Teflon pestle (clearance 0.12 mm). The homogenization was continued until the pestle had been passed up and down 2 times. During the fractionation, materials and tissue were kept at ca +4 °C.

The homogenate was submitted to differential centrifugation in 3 subsequent steps; $480 \times g_{av}$ for 10 min (Sorvall) to yield a sediment P_1 - mainly containing unbroken material and nuclei - which was washed in 30 vol. buffered sucrose, homogenized and centrifuged as before. The pooled supernatant S_1 was centrifuged at $9750 \times g_{av}$ for 10 min (Sorvall). The pellet P_2 was discarded and the supernatant S_2 submitted to $88{,}000 \times g_{av}$ for 45 min in a T 40 rotor (Beckman L 2-65 B) to yield sediment P_3 (microsomal fraction) and a supernatant. Sediment P_3 was further subfractionated using different gradient centrifugation techniques.

Isopycnic gradient centrifugation. Sediment P_3 was resuspended in 0.45 M buffered sucrose (3 ml/g of original tissue) and 0.75 ml of the suspension layered on top of a buffered sucrose gradient from 0.5 M to 1.7 M, with a 0.5-ml 2 M sucrose cushion (gradient type A). The total volume (sample + gradient + cushion) was 12.85 ml. The gradient was spun at $190{,}000 \times g_{av}$ for 150 min.

Differential gradient centrifugation. a) Sucrose-H_2O gradient (gradient type B). Sediment P_3 was resuspended in 0.25 M buffered sucrose (3 ml/g of original tissue) and 0.75 ml applied on a 0.3-0.8 M linear sucrose gradient. The bottom of the tube contained a 1.0-ml cushion of 2 M sucrose (total volume 12.85 ml). The gradient was centrifuged for 40 min at $190{,}000 \times g_{av}$.

b) Sucrose-D_2O gradient (gradient type C). With this technique[6], 6 ml of supernatant S_2 (see before) was directly layered on a gradient. This gradient was linear from 0.25 M sucrose (50% D_2O) to 0.60 M sucrose (D_2O) on top of 1 ml of a 1.2 M sucrose (D_2O) cushion. The total volume of the gradient (sample + gradient + cushion) was 13 ml.

After centrifugation, 12 fractions of 1 ml were obtained by pumping a 2.5 M sucrose solution through the bottom of the tube and collecting by overflow using an Isco-model 640 density gradient fractionator. Aliquots of these fractions were used for the determination of NA[14] and DβH[15].

Results. Isopycnic centrifugation of a particulate fraction (sediment P_3) from rabbit iris (figure 1) results in the distribution of NA in 3 parts of the gradient: 1. between 15 and 20% of the total NA remained at the top where the particulate fraction was initially layered on the gradient, 2. between 50 and 60% of the total amount accumulated in a density region of the gradient where light NA vesicles were usually found, and 3. the remainder was recovered from the more dense region of the gradient.

The DβH activity peaks in the region of the light vesicles but parallels also the NA distribution in the more dense region of the gradient. Upon differential gradient centrifugation (figure 2) the NA distribution can also be divided into 3 parts: a) between 10 and 15% of the NA which remained at the top of the gradient, b) between 50 and 60% which was found in the upper fractions of the gradient, and c) about 25% of the NA which was concentrated at the interface of the gradient with the cushion.

Apart from a lower amount of DβH activity at the top of the gradient there appears to be no significant difference in the distribution of NA and DβH.

Upon application of the gradient centrifugation procedure of Lagercrantz[6] (gradient C, figure 3) about 60% of the total NA entered the gradient; 60-70% of this was found in the upper region of the gradient and 30% at the interface with the heavy sucrose solution.

Apart from about 25% of the DβH, which did not enter the gradient, the remainder of the enzyme shows a distribution similar to that of NA. The ratio of DβH/NA appears to be higher in the most dense region of the gradient.

In order to assure that the NA which accumulated at the interface with the heavy sucrose solution (gradient B,

figure 2) was truly contained in heavy vesicles, this fraction was diluted to isotonicity with 0.1 M buffered sucrose, resedimented by centrifugation for 1 h at 88,000 × g_{av} in the T 40 rotor (Beckman L 2-65 B), resuspended and subjected to isopycnic gradient centrifugation. From figure 4 it can be seen that NA and DβH activity now accumulate in the position where heavy vesicles of the dog spleen are found and that the distribution of DβH activity almost completely coincides with the NA distribution.

Discussion. From the results of the isopycnic gradient centrifugation experiments it was clear that the major part of the NA accumulated in the gradient in a position where light vesicles from the spleen[16] (a tissue for which light and heavy NA vesicles have been well demonstrated and are given here for comparative purposes) as well as from other tissues[3–5] including the rat iris[17] are found. In fact, the distribution of NA was similar to that obtained for the rat iris[17], although the skewness of the NA distribution on the more dense side of the gradient (figure 1) was rather pronounced and possibly indicative for the occurrence of heavy NA vesicles. In this connection the results obtained by Bisby et al.[18] and by Aberer et al.[19] for the rat vas deferens are worth mentioning since these authors also found a skewness of the NA distribution. In fact, the latter authors even reported a small peak of NA in the more dense region of the gradient.

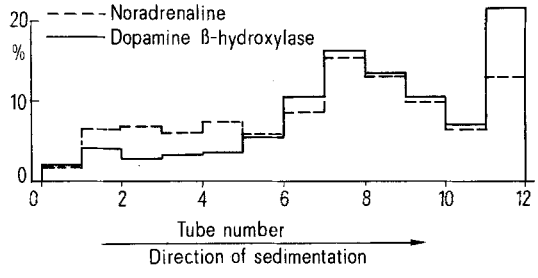


Fig. 3. Distribution of NA and DβH as obtained by subjecting 6 ml of the postnuclear fraction S_2 to a Lagercrantz gradient (type C) centrifugation. The results are from 1 experiment but they are representative for the 2 experiments carried out.

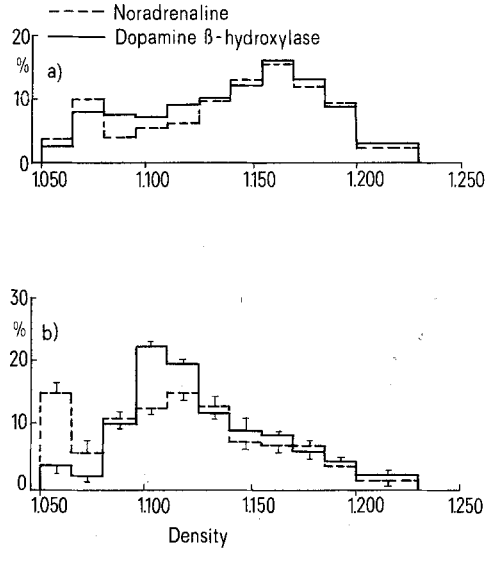


Fig. 4. Distribution of NA and DβH as obtained by isopycnic gradient centrifugation of the particulate fraction resting on top of the cushion after differential sucrose gradient centrifugation (type B) of a NA enriched pellet from rabbit iris (a). Results are compared with those obtained for the isopycnic centrifugation (figure 1) of a total NA enriched pellet (b).

Clearer evidence, however, for the existence of heavy NA vesicles was obtained from the experiments in which the differential gradient centrifugation method was applied, a procedure for which the separation is based on the size rather than on the density of the subcellular particles. Indeed, upon differential gradient centrifugation (gradient B, figure 2), a bimodal distribution of NA was obtained, 1 peak corresponding to the position of the light NA vesicles, the other – at the interface of the gradient and the 2 M sucrose cushion – with that of the heavy NA vesicles of the spleen. The relative amounts of NA associated with each of the presumed vesicles (between 50 and 60% is associated with light NA vesicles and about 25% with heavy NA vesicles) correspond fairly well with the proportion estimated from the results obtained by isopycnic gradient centrifugation. It has to be noted that for both types of gradients a small peak of NA (10–15%) was found at the top of the gradient; this is considered to be derived from vesicles broken during the resuspension of the microsomal fraction by subsequent gradient centrifugation. The NA peaks were not accompanied by a significant peak of DβH although this could hardly be expected since – in contrast to NA – only about 20% of the enzyme appears to be present in a soluble state within NA vesicles[4].

Additional evidence for the occurrence of heavy NA vesicles was obtained by using gradient C, a method which has successfully been used to purify heavy NA vesicles from bovine splenic nerves[6]. Upon application of this method (figure 3) a peak of NA was found at the interface of the gradient with the 1.2 M sucrose cushion where heavy NA vesicles from bovine splenic nerves also accumulate. Furthermore, when the amount of NA accumulating at the interface is expressed as a percentage of the total vesicle bound NA – the amount of NA which entered the gradient – again a value of 25–30% was obtained. Whereas these results suggested the presence of heavy as well as of light NA vesicles, it might still be argued that NA found at the interface of the gradient and the sucrose cushion (gradients B and C) is the result of a centrifugation artifact. This possibility, however, seems rather unlikely since for both types of gradients the amounts of NA accumulating at the interface are highly reproducible and in accordance with the amounts of NA expected to be associated with heavy NA vesicles on the basis of the results obtained by isopycnic gradient centrifugation (figure 1).

More direct evidence against a centrifugation artifact was obtained from the experiment in which the particles accumulating at the interface, upon differential gradient centrifugation (gradient B, figure 2), were sedimented, rehomogenized, recentrifuged and layered on gradient A (figure 4). Under these centrifugation conditions, the distribution of NA corresponded to the distribution of heavy NA vesicles from dog spleen (compare with figure 1) and was completely reflected by the distribution of DβH.

On the basis of these data we conclude that, in the rabbit iris, heavy NA vesicles are present in addition to light NA vesicles. These vesicles most probably possess DβH activity and contain at least 25–30% of the total bound NA particles. It therefore seems possible that other tissues with an apparently unimodal distribution of light NA vesicles in isopycnic gradients might also contain a considerable amount of heavy NA vesicles when studied by differential gradient centrifugation methods.

In previous studies on the spleen, evidence has been provided that NA and DβH are released from heavy NA vesicles by a mechanism of exocytosis[20] and that thereby light NA vesicles might be formed from heavy NA vesi-

cles[16]. The fact that heavy NA vesicles are also present in other tissues suggests that these vesicles may play a similar role in adrenergic nerve endings in general.

1 Present address: Universitaire Instelling Antwerpen, Department of Medicine, Universiteitsplein 1, B-2610 Wilrijk (Belgium).
2 U.S. von Euler and N.Å. Hillarp, Nature, Lond. *177,* 44 (1956).
3 L.B. Geffen and B.G. Livett, Physiol. Rev. *51,* 98 (1971).
4 A.D. Smith, Pharmac. Rev. *24,* 435 (1972).
5 W.P. De Potter, I.W. Chubb and A.F. De Schaepdryver, Archs int. Pharmacodyn. Ther. *193,* 191 (1972).
6 H. Lagercrantz, Neuroscience *1,* 81 (1976).
7 R.H. Roth, L. Stjärne, F.E. Bloom and N.J. Giarman, J. Pharmac. exp. Ther. *162,* 203 (1968).
8 W.P. De Potter, Thesis, University of Ghent, Belgium 1968.
9 I.W. Chubb, W.P. De Potter and A.F. De Schaepdryver, Nature, Lond. *228,* 1203 (1970).
10 M.A. Bisby and M. Fillenz, J. Physiol., Lond. *215,* 163 (1971).
11 T. Hökfelt, Acta physiol. scand. *76,* 427 (1969).
12 S. Blumcke and H.J. Dengler, Virchows Arch. path. Anat. Physiol. B *6,* 281 (1970).
13 W.P. De Potter, F. De Smet and E. Cambie, Archs int. Pharmacodyn. Ther. *227,* 157 (1977).
14 D.P. Henry, B.J. Starman, D.G. Johnson and R.H. Williams, Life Sci. *16,* 375 (1975).
15 L. Cubeddu and N. Weiner, J. Pharmac. exp. Ther. *192,* 1 (1975).
16 W.P. De Potter and I.W. Chubb, Neuroscience *2,* 167 (1977).
17 M. Israel and P. L'Hermite, C. R. Séanc. Acad. Sci., Paris D *268,* 1445 (1969).
18 M.A. Bisby, M. Fillenz and A.D. Smith, J. Neurochem. *20,* 245 (1973).
19 W. Aberer, R. Stitzel, H. Winkler and E. Huber, J. Neurochem. *33,* 797 (1979).
20 W.P. De Potter, Peripheral Sympathetic Neurotransmission. Arscia, Brussels 1976.

The lectins from *Agaricus edulis.* Isolation and characterization

R. Eifler and P. Ziska

Staatliches Institut für Immunpräparate und Nährmedien, Forschungsabteilung, DDR-1120 Berlin-Weissensee (German Democratic Republic), 1 February 1980

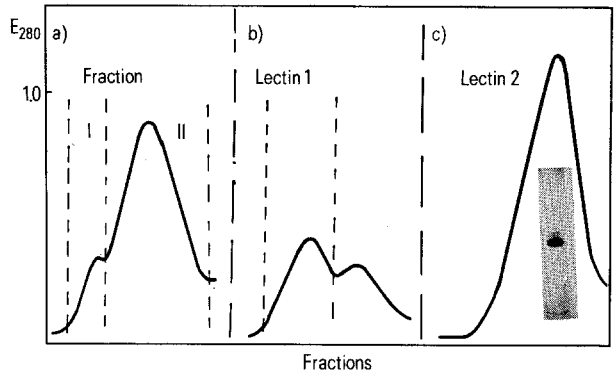
Summary. 2 lectins from the mushroom *Agaricus edulis* were isolated, after heating the crude extract at 75 °C, by ion exchange chromatography and gel chromatography using QAE-Sephadex A-50 and Sephadex G_{75}. Some hemagglutinating and physicochemical properties of the agglutinins are reported.

Lectins used to be commonly referred to as plant-seed, carbohydrate-binding proteins. However, they have also been found in bacteria, fish roes, snails, vertebrates and mushrooms[1,2]. Hitherto, lectins from the genus *Agaricus* have been isolated from *A. bisporus*[3] and *A. campestris*[4]. In the present paper we describe the lectins from *A. edulis.*

Materials and methods. Fresh mushrooms of the species *A. edulis,* collected in the streets of Berlin, were cleaned and dried at 50 °C. 50 g of the ground powder was suspended in 800 ml 0.9% NaCl-solution and heated for 10 min at 75 °C. The suspension was then cooled with water and centrifuged at 5000 × g for 10 min. The clear supernatant was treated with 59 g ammonium sulphate per 100 ml and stored overnight in the refrigerator. The precipitate was centrifuged at 6000 × g for 15 min and the sediment was suspended in 15 ml of distilled water. This solution was centrifuged at 15000 × g for 15 min to remove the last contamination. The clear dark brown solution was dialyzed against 0.3% NaCl solution. To remove the brown colored components, the solution was then applied to a column packed with 80 ml QAE-Sephadex A-50 equilibrated against 0.3% NaCl solution. All of the colored material remained on the column and the nearly colorless lectin fraction came through after the hold-up volume. The purified fraction was concentrated by ultrafiltration to a final concentration of 1% protein. Chromatography and rechromatography on a column of Sephadex G_{75} (90 × 1.5 cm) equilibrated with 0.9% NaCl solution revelaed 2 hemagglutinating lectin fractions. Both were desalted and freeze dried.

For polyacrylamide gel disc electrophoresis the method of Maurer[5] was used, which involved electrophoresis in columns of 7.5% gel in buffers of pH 4.3 and 8.9 respectively. Estimation of the subunits of the lectins was carried out in 10% polyacrylamide gel in the presence of sodium dodecylsulphate by comparison with marker proteins.

Hemagglutination assays were performed using a Takatsy microtitrator. Tests were performed with a 2% suspension of human erythrocytes type A and incubated for 1 h at room temperature. A hemagglutination inhibition test was carried out as described[6]. TCL for determination of the mol.wt of the lectins was performed with Sephadex superfine G_{200} and Sephadex G_{75} superfine at pH 7.8 and 4.8 as described by Andrew[7]. Carbohydrate content of the lectins was determined by the phenol-H_2SO_4 method with reference to glucose[8]. N-terminal amino acids were determined with the DNP-method of Fraenkel-Conrat[9]. Determination of sulfhydryl and disulfide groups was carried out with the reagent of Ellman[10]. Antisera were produced by immunizing rabbits with pure lectins. The animals were injected in



a Gel chromatography of the crude lectins from *A. edulis* on Sephadex G_{75} (1.5 × 90 cm). *b* Rechromatography of fraction 1. *c* Rechromatography of fraction 2 and the result of the disc electrophoresis of the purified lectin 2 (200 µg).