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A β-D-Galactoside Binding Protein from Electric Organ Tissue of *Electrophorus electricus*

(agglutination/lectin/skeletal muscle/lactose/affinity chromatography)

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ABSTRACT **Extracts of electric organ tissue of *Electrophorus electricus* contain a saccharide-binding protein, named electrolectin, which agglutinates trypsin-treated rabbit erythrocytes and is specifically inhibited by disaccharides containing nonreducing terminal β-D-galactosyl residues. Electrolectin seems at least partially membrane-bound but is also found in soluble fractions of homogenates from which it can be purified by affinity chromatography on cross-linked and desulfated agarose (ECD-Sepharose) as a protein of molecular weight 33,000. About 400 mg of electrolectin are present per kg of tissue. It has an affinity for lactose of 1.0 mM^{-1} and 5.5 mM^{-1} as estimated, respectively, by hapten inhibition and fluorescence spectroscopy. Studies on the distribution of β-D-galactoside-binding activity in animal tissues reveal particularly high levels in skeletal muscle tissue and in cultures of embryonic skeletal muscle and neuroblastoma cells.**

Neural tissue contains large amounts of glycoproteins and glycolipids which are implicated in the structure and function as well as in the development and pathology of the nervous system (1). The synaptic junction is known to be rich in carbohydrates and it has been suggested that complex carbohydrate-containing molecules may function in synaptic recognition and transmission through establishment of cell–cell contacts and possibly also as mediators of communication between the surface and the interior of the cell. In line with these ideas, the presence in neural tissue of enzymes and proteins capable of interacting with saccharides is to be expected, and indeed glycosyl transferases are found in brain in high concentrations, particularly at nerve endings (2). We wish to report the presence in electric organ tissue of the electric eel (*Electrophorus electricus*) of a saccharide-binding protein with specificity towards disaccharides containing β-D-galactoside moieties. This protein has been isolated and partially characterized. It is named "electrolectin" because of its origin in electrogenic tissue and its lectin-like activity of agglutinating erythrocytes. Agglutinating activity with similar specificity towards β-D-galactosides is also found in mammalian tissues and tissue cultures. The ubiquity and high level of this hemagglutinin in excitable tissues raise a number of questions as to its role.

MATERIALS AND METHODS

Chemicals. Lactose and monosaccharides were purchased from Pfanstiehl Laboratories, D-galactopyranosyl-β-thio-galactopyranoside (TDG) from Schwarz/Mann, and other oligosaccharides from Nutritional Biochemicals. Lysozyme, bovine serum albumin, and trypsin were obtained from Worthington and α-bungarotoxin was prepared according to Clark *et al.* (3). Carbamylcholine and *d*-tubocurarine were products of Mann Research Laboratories, gallamine of K and K., Fluram of Roche Diagnostics, and dithiothreitol of Calbiochem.

Cell Cultures and Tissues. Chick embryo breast muscle cultures from 12-day embryos were obtained from Dr. Joav Prives, mouse L cells (strain CCL 1) from Dr. Jean Content, and N-18 mouse neuroblastoma cells from Dr. Henri Schmitt. Chick embryo pectoral muscle tissue was removed from 12-day embryos. Chick rectus muscle was dissected 1 day after hatching. Rat diaphragm was dissected into innervated and noninnervated areas according to Hall (4). Live electric eels, *Electrophorus electricus*, were purchased from Paramount Aquarium (Ardsley, N.Y.) and frozen electric organs of *Torpedo californica* from Pacific Biomarine (Venice, Calif.).

Agglutinin Assay. The agglutination assay, using trypsinized rabbit erythrocytes, was according to Lis and Sharon (5). The inhibition constant for a given sugar was defined as the concentration required to cause 50% inhibition of agglutination caused by 3–5 hemagglutinating units (5).

Fluorescence Measurements and Determination of Association Constants. Fluorescence measurements were performed with an Aminco-Bowman spectrofluorometer at an excitation wavelength of 285 nm. The association constant was determined by fluorometric titration (6), measuring the emission intensity at 335 nm. The protein [1 ml in Solution I (0.9% NaCl/2 mM dithiothreitol)] was added to two matched fluorescence cells. Lactose (0.005 ml–0.075 ml) (10.7 mM in 0.9% NaCl) was added to one cell and identical volumes of 0.9% NaCl to the other. The relative fluorescence intensity of the protein saturated with sugar, F_∞, was extrapolated by plotting $1/(1 - F/F_0)$ against $1/S$, where F is the fluorescence of the protein at a sugar concentration S, and F_0 of the protein alone. When $\log [(F_0 - F)/(F - F_\infty)]$ is plotted against $\log S$, the value of pK_a equals the value of $\log S$ at $\log [(F_0 - F)/(F - F_\infty)] = 0$.

Protein Determination. Protein was routinely determined by the method of Lowry *et al.* (7). Dilute protein solutions were assayed with Fluram according to Udenfriend *et al.* (8).

Preparation of Tissue Homogenates. Fresh animal tissues were minced in the cold and suspended in 5 volumes per weight tissue of cold Solution I containing 2 mM EDTA. Homogenization was performed at 4° in a 50 ml stainless steel

Abbreviation: TDG, D-galactopyranosyl-β-thiogalactopyranoside.

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† *This paper is dedicated to Gad Resheff who fell on October 9th, 1973, in defense of the State of Israel.*

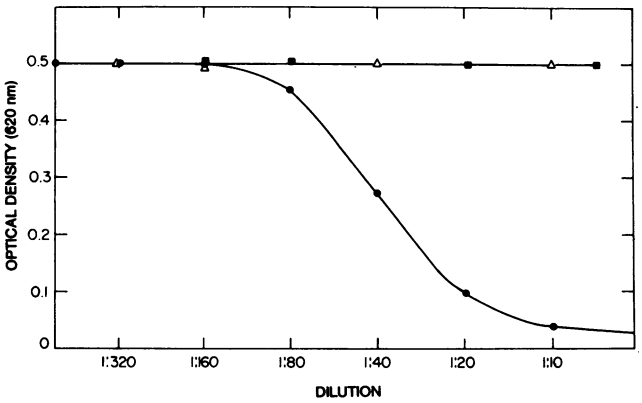

FIG. 1. Agglutination of trypsinized erythrocytes by a homogenate of electric organ tissue from *Electrophorus electricus*. The optical density at 620 nm of the erythrocyte suspension is plotted versus the dilution of the homogenate. ●, Homogenate; △, homogenate + 10 mM lactose; ■, homogenate + 10 mM TDG.

vessel using a Sorvall Omnimixer at full speed for three periods of 1 min with 1 min intervals. The homogenates were filtered, first through cheesecloth and then through a nylon mesh, to remove large particles of connective tissue, and kept in ice until used.

Fractionation of the Electrophorus electricus Electric Organ Homogenate. The filtered homogenate (H) from electric organ tissue of *Electrophorus electricus*, prepared as described above, was centrifuged for 20 min at 1500 × g and 4°. The pellet (P_1) was resuspended in Solution I. The supernatant (S_1) was centrifuged at 4° for 30 min at 30,000 × g. A pellet (P_2) and a supernatant (S_2) were obtained. S_2 was centrifuged at 100,000 × g for 70 min at 4° to give a pellet (P_3) and a supernatant (S_3). The pellets were resuspended in 3.5 volumes of cold Solution I.

Glycerol Gradient Centrifugation. The procedure used was that of Martin and Ames (9), with glycerol instead of sucrose, since the latter, at high concentrations, inhibited the hemagglutinating activity of electrolectin. Linear gradients of 10–35% glycerol in Solution I were employed. Centrifugation was at 2° for 16 hr at 48,000 rpm in a SW50.1 rotor using a Beckman L3-50 centrifuge. Bovine serum albumin and lysozyme, which served as markers, were located by measuring the absorbance at 280 nm; their sedimentation constants were assumed to be 4.4 S and 1.9 S, respectively (10). The sedimentation profile for electrolectin was determined by adding 0.8 ml of Solution I and 1 ml of the trypsinized erythrocyte suspension to 0.2 ml fractions from the gradient.

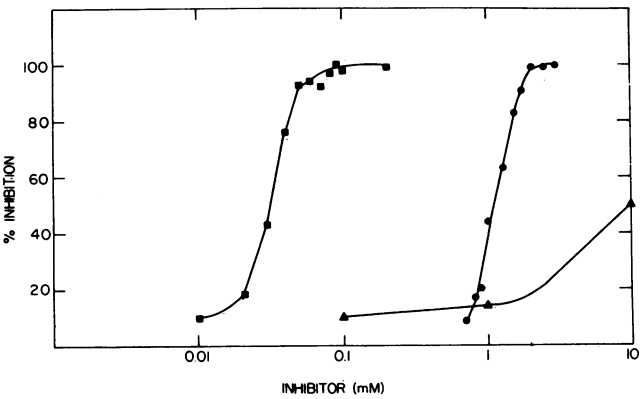

FIG. 2. Inhibition by β-D-galactosides of agglutination of erythrocytes by electric organ homogenate. ■, TDG; ●, lactose; ▲, *o*-nitrophenyl-β-D-galactopyranoside.

TABLE 1. *Subcellular fractionation of electric eel electric organ homogenate*

Fraction	Specific activity, agglutination units/mg of protein	Yield of activity, %	Yield of protein, %	Relative specific activity, % of total activity/% of total protein
H	36	100	100	1
P_1 (3 × 10^4 g min)	43	41	34	1.2
P_2 (9 × 10^5 g min)	22	4.4	6.2	0.7
P_3 (7 × 10^6 g min)	77	11	4.4	2.5
S_3	21	30	47	0.64

Preparation of ECD-Sepharose 6B. Cross-linked and desulfated agarose beads (ECD-Sepharose 6B) were prepared from Sepharose 6B by treatment with epichlorohydrin in alkaline milieu followed by alkaline and acid hydrolysis according to Porath *et al.* (11).

RESULTS

Agglutination of Rabbit Erythrocytes by Eel Electric Organ Homogenate. When aliquots from an eel electric organ homogenate are added to trypsinized rabbit erythrocytes, aggregation occurs (Fig. 1). After 150 min a compact pellet of aggregated erythrocytes and a clear supernatant are obtained, whereas the turbidity of a reference solution of erythrocytes alone stays constant. The pellet cannot be dissociated by shaking or stirring, but dissociation is obtained in the presence of lactose. No agglutination occurs with nontrypsinized erythrocytes. Boiling of the homogenate abolishes agglutinating activity completely. The presence of a reducing agent was found to be essential for maintaining the agglutinating activity of homogenates and of purified electrolectin; 2 mM dithiothreitol was, therefore, used in all experiments.

Inhibition of Hemagglutination by Saccharides. Saccharides are known to inhibit agglutination of cells by lectins. A number of saccharides were, therefore, tested to determine the specificity of the eel agglutinin (electrolectin). The following saccharides inhibit agglutinating activity of electric organ homogenates: lactose: Gal-β(1 → 4)Glc; *N*-acetyllactosamine: Gal β(1 → 4)GlcNAc; D-galactopyranosyl-β(1 → 1)-thiogalactopyranoside (TDG); *o*-nitrophenyl β-D-galactopyranoside (Fig. 2). TDG inhibits 50% of the agglutination at 32 μM, lactose at 1.0 mM and *o*-nitrophenyl-β-D-galactopyranoside at 10 mM. Thus, electrolectin has an affinity for disaccharides containing non-reducing terminal β-D-Gal residues. In support of this conclusion, the following saccharides were found to be ineffective in inhibiting agglutination at concentrations up to 10 mM: *N*-acetyl-D-galactosamine, L-fucose, methyl α-D-glucopyranoside, methyl-α-D-mannopyranoside, β-D-galactose, 2-deoxygalactose, D-galactose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, D-glucose, sucrose, maltose, cellobiose, melibiose,

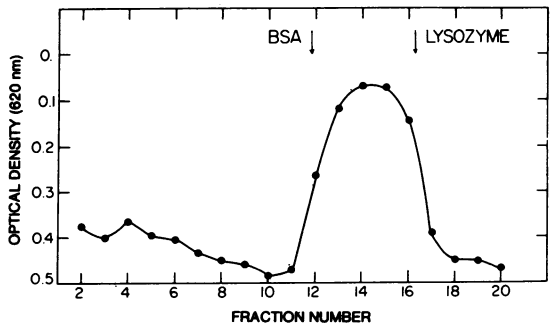

FIG. 3. Glycerol gradient sedimentation of subcellular fraction S_3 of electric organ homogenate. Fractions of 0.2 ml were collected and assayed for agglutinating activity on trypsinized erythrocytes. The optical density at 620 nm of the erythrocyte suspension is plotted versus fraction number. The arrows indicate the positions of the bovine serum albumin (BSA) and lysozyme markers.

turanose, *N*-acetyl-D-glucosaminyl-β(1 → 4)-*N*-acetylmuramic acid, raffinose, chondroitin sulfate, chitotetraose.

Effect of Cholinergic Agents. The effect of cholinergic agents on agglutination of erythrocytes by electric organ homogenates was investigated in order to find a possible connection between the acetylcholine receptor present in electric organ tissue and the electrolectin. *d*-Tubocurarine (10 μM), gallamine (10 μM), carbamylcholine (1 mM), and α-bungarotoxin (0.1 μg/mg of protein in the homogenate), had no effect either on agglutination or on its inhibition by lactose.

Distribution of Electrolectin in Subcellular Fractions of Electric Organ Tissue. Table 1 shows the results of subcellular fractionation of the electric organ homogenate. Clearly most of the agglutinating activity is in the nuclear fraction (P_1), which contains mainly cell debris, membrane fragments, nuclei and mitochondria. Successive washes of this fraction did not affect its specific activity. The microsomal fraction (P_3) has the highest specific activity, although it comprises only 11% of the total activity; 30% of the activity in the homogenate is obtained in the final supernatant (S_3), which contains mainly soluble proteins.

Glycerol Gradient Centrifugation. Fig. 3 shows the agglutinating activity profile of fraction S_3 when centrifuged in a glycerol gradient. A principal peak of agglutinating activity is observed of sedimentation coefficient 2.8 S. If one assumes the formula of Martin and Ames (9) to be valid for glycerol gradients, a molecular weight of 33,000 can be estimated for electrolectin.

Purification of Electrolectin on ECD-Sepharose. ECD-Sepharose (11) is an agarose gel cross-linked to make it more resistant to acid treatment. Since most lectins will not complex with internal galactosyl residues of linear galactan molecules, the number of end groups is increased by splitting the galactan chains with acid at a number of sites without degrading the interlocking network. Although this treatment increases the lectin adsorption capacity of the gel, it decreases drastically its flow rate. A 2 cm × 10 cm column was used. Nineteen milliliters of fraction S_3 containing 6 mg of protein per ml were applied to the column at an initial flow rate of 5 ml/hr, which gradually decreased to about 1 ml/hr due to precipitation within the gel of nonsoluble material. After

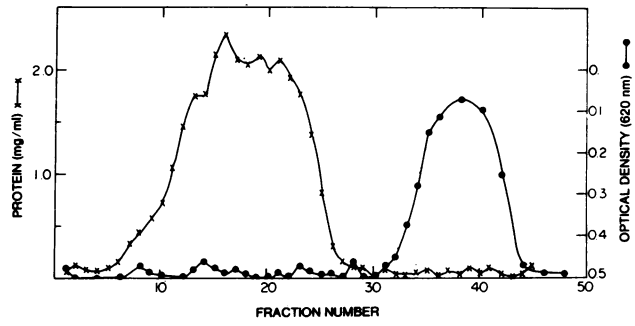

FIG. 4. Chromatographic profile for the purification of electrolectin on ECD-Sepharose 6B. ×, Protein concentration; ●, agglutinating activity.

loading of the column, Solution I was applied. Fractions of 1.8 ml were collected and monitored for protein and for electrolectin. The profile obtained is shown in Fig. 4. An accurate estimate of the protein content in the peak of electrolectin activity (Peak II) could not be obtained by the Lowry method. The fractions in this peak were, therefore, pooled and concentrated by vacuum dialysis, and their protein content was determined by the Fluram method. The recovery of protein in Peak II was 300 μg, whereas Peak I contained about 60 mg of protein. The specific activity of Peak II was 3130 hemagglutination units/mg of protein, i.e., 87-fold purification relative to the homogenate.

Fluorescence Measurements. The fluorescence emission spectrum of the purified electrolectin preparation (Peak II) shows a typical tryptophan emission spectrum with a peak at 350 nm (Fig. 5). Addition of lactose (final concentration 10 mM) produces a shift to the blue of the emission spectrum as well as a 20% enhancement of emission intensity.

The enhancement in fluorescence of purified electrolectin produced by lactose was used to measure the association constant of lactose. A value of 5.5 mM^{-1} was obtained (Fig. 6).

Distribution of Hemagglutinin Activity in Tissues and Cell Cultures. Various tissue and cell culture homogenates were assayed for the presence of an agglutinating activity that could be completely inhibited by TDG (0.69 mM) and by lactose (1.2 mM). As seen in Table 2, a large number of sources do possess such activity. Among the tissue types tested, muscle seems to contain the highest levels of aggluti-

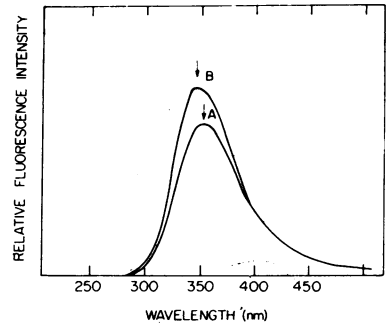

FIG. 5. Fluorescence emission spectrum of purified electrolectin in the absence (A) and the presence (B) of 10 mM lactose. Excitation at 285 nm. The arrows indicate the positions of the wavelengths of maximal emission.

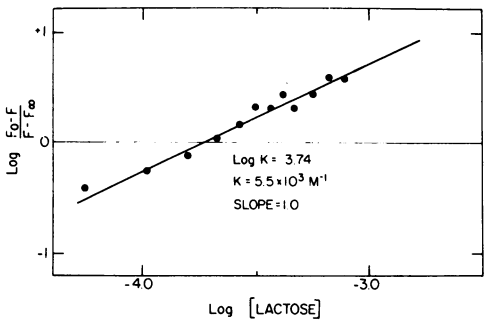

FIG. 6. Spectrofluorometric determination of the binding of lactose to purified electrolectin. F_0, F, and F_∞ are the relative fluorescence intensities at 335 nm of electrolectin alone, electrolectin in the presence of a given concentration of lactose, and of electrolectin saturated with lactose, respectively.

nating activity, and particularly high values were observed for chick embryo pectoral muscle and for tissue cultures of muscle and nerve cells. A series of rabbit muscles (smooth and skeletal) were assayed, but only low levels of activity were detected. Frozen electric organ tissue of *Torpedo californica* was totally devoid of hemagglutinating activity. The inhibition constant for inhibition of agglutination by TDG was measured for homogenates of chick embryo pectoral muscle and chick rectus muscle. Values of K_I = 0.31 mM and K_I = 95 μM, respectively, were obtained.

DISCUSSION

The electric organ of *Electrophorus electricus* possesses considerable amounts of a protein, which we have named electrolectin, which has the ability to agglutinate trypsinized rabbit erythrocytes. Lectin-like activity, although widely distributed in plant tissues, has only been reported in a few cases in lower animals (12). Electrolectin, like many plant lectins, does not agglutinate nontrypsinized erythrocytes. Trypsin is known to remove sialic acid residues from the terminal ends of polysaccharide chains and one may, therefore, assume that electrolectin interacts with polysaccharides that are devoid of such groups. Its specificity seems to be directed to saccharides containing nonreducing-terminal D-galactose linked in a β-configuration, since the oligosaccharides melibiose and raffinose, which contain nonreducing α-D-galactosyl residues, did not inhibit agglutination. The fact that addition of lactose to agglutinated erythrocytes brings about dissociation of the pellet suggests that the interaction of electrolectin with saccharides is noncovalent and reversible. The values of the inhibition constant and dissociation constant of lactose, measured respectively by inhibition of agglutination and by fluorescence spectroscopy, are in good agreement, and fall in the range of sugar dissociation constants of most lectins. The electrolectin in the supernatant of homogenates from electric organ tissue migrates as a single species of sedimentation coefficient about 2.8 S. It can be purified 87-fold by affinity chromatography. The 20% increase in fluorescence intensity observed upon addition of lactose to purified electrolectin indicates a high degree of purification, since such a large effect would not be observed in a crude protein preparation. Moreover, it indicates that at least one tryptophan is present in the vicinity of the lactose-binding site. Since electrolectin must possess at least two lactose-binding sites in order to agglutinate cells, it can be assumed, on the basis of the value of the slope obtained in Fig. 6, that the lactose-binding sites are noninteracting and are presumably independent and identical (13). The requirement of a reducing agent for maintaining agglutinating activity of electric organ homogenates and of purified electrolectin indicates the presence of a sensitive sulfhydryl group or of a metal ion which has to remain in its reduced form. From the total electrolectin activity of electric organ tissue and the specific activity of the purified material (3100 U/mg) it can be estimated that there is about 400 mg of electrolectin per kg of tissue. This is a very high value if one remembers that this tissue contains only about 4% total protein and that such functional proteins as acetylcholinesterase (50–100 mg/kg of tissue) and acetylcholine receptor (5–10 mg/kg of tissue) are present in much smaller amounts.

The large amount of electrolectin in electric organ tissue, and of hemagglutinins with similar specificity in mammalian tissue, clearly raises a number of questions concerning their possible structural, functional or developmental roles. One obvious possibility is that the activity is due to a glycosyl transferase that agglutinates erythrocytes through its binding sites. Various arguments can be raised against this hypothesis. Rabbit erythrocytes do possess, on their surface membrane, a galactosyl transferase (14). This enzyme does not, however, agglutinate rabbit erythrocytes, nor does it agglutinate human erythrocytes in its isolated form. Among the glycosyl transferases that can use lactose as a glycosyl acceptor are fucosyltransferase and the sialyl transferases (15). These enzymes show, however, a relatively low affinity for lactose compared to that of electrolectin. Moreover, the tissue dis-

TABLE 2. *Distribution of β-D-galactoside-specific hemagglutinin activity in tissues and cell cultures*

Origin	β-D-galactose-specific hemagglutinin activity*, U/mg of protein
Rat lung	1.54
Rat heart	2.24
Rat kidney	0.58
Rat thymus	0.50
Rat spleen	2.0
Rat soleus muscle	2.68
Rat brain	0.35
Rat liver	0.12
Rat diaphragm (innervated area)	5.42
Rat diaphragm (noninnervated area)	3.18
Chick embryo (pectoral muscle)	87.5
Chick rectus muscle	2.94
Chick embryo pectoral muscle culture	48
Mouse fibroblast (L cells)	2.68
Mouse N-18 neuroblastoma	14.6
Electrophorus electricus swimming muscle	12.6
Electrophorus electricus electric organ	36
Torpedo californica electric organ	None
Rabbit muscles	<0.1
Escherichia coli	None
Micrococcus luteus	None

*β-D-Galactoside-specific hemagglutinin activity is that agglutinating activity which is completely inhibited by 0.69 mM TDG or 1.2 mM lactose.

tribution of sialyl transferase in the rat clearly indicates high activity in spleen, kidney and lung, and almost no activity in striated muscle (15). Furthermore, the liver is the site of intense polysaccharide synthesis and possesses a battery of glycosyl transferases present in relatively high amounts. The level of β-D-galactoside-specific hemagglutinin activity in rat liver is, however, lower than in any other rat organ tested. Thus, the characteristics of the hemagglutinin do not seem to correspond to those of the known glycosyl transferases. Obviously, electrolectin, like the plant lectins, could possess an as yet unidentified enzymic function.

Subcellular fractionation of electric organ homogenates yields preliminary information as to the location of electrolectin. Much of the activity in the homogenate is recovered in the nuclear fraction which contains, besides the nuclei, large membrane fragments and cell debris. Only small amounts of activity are released from this fraction on repeated washing. However, the fact that electrolectin is also found in a soluble form suggests that it is only loosely bound to the membrane. Since the microsomal fraction (P_3) seems to have the highest relative specific activity, it is possible that electrolectin is associated more specifically with the endoplasmic reticulum or the plasma membrane. Such a conclusion must, however, await further studies.

The tissue distribution of the β-D-galactoside-specific hemagglutinins may shed some light on their possible function. From Table 2 it is clear that activity is widely distributed, but occurs in different tissues in very different amounts. Furthermore, the affinity for TDG and lactose seems variable. Muscle tissue was found to contain the highest concentration of hemagglutinin, but the parameter studied, i.e., the specific activity of agglutinin per mg of tissue protein, may not always be a genuine reflection of the level of the protein in a given tissue. In general, hemagglutinating activity seems to be preferentially associated with tissue containing excitable membranes. The failure of extracts of rabbit smooth and skeletal muscle to agglutinate rabbit erythrocytes may be due to a kind of "autoimmunity". The absence of agglutinating activity from *Torpedo* tissue is more surprising. However, preliminary observations indicate that extracts of *Torpedo marmorata* electrogenic tissue agglutinate goose erythrocytes, but not those of rabbits and several other species. Thus, lectins with different specificity may occur in tissues of different species. It should also be pointed out that β-D-galactoside-specific hemagglutinating activity is present in particularly high amounts in chick embryonic muscle and in tissue cultures of nerve and muscle cells, i.e., in rapidly differentiating systems. In this connection it is worth mentioning the recent observations of Simpson and coworkers (16), who have characterized and purified a galactose-binding protein, discoidin, from the slime mold *Dictyostelium discoideum*, the biosynthesis of which closely parallels the development of cell cohesiveness.

Note Added in Proof. After this paper was submitted for publication a report appeared describing the isolation and properties of a mammalian lectin (17).

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