

Correspondence of gradual developmental increases of expression of galectin-reactive glycoconjugates with alterations of the total contents of the two differentially regulated galectins in chicken intestine and liver as indication for overlapping functions

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Summary. The duplication of genes for recognition molecules and the ensuing diversification of the members of such families generate complex groups of homologous proteins. One example are galactoside-specific lectins whose sequences display constant features related to sugar binding, the galectins. Based on the inverse abundance of the chicken galectins CG-14 and CG-16 in adult intestine and liver, these two lectins represent a model to comparatively study expression of the related proteins and the galectin-reactive sites (glycoproteins and glycolipids) biochemically and histochemically. Functional overlap and/or acquisition of distinct functions would be reflected in qualitative and/or quantitative aspects of ligand display. Using five different stages of embryogenesis, differential regulation of the two galectins was detected in liver and intestine. The clear preference for one galectin (CG-14) was observed in intestine already at rather early stages, whereas equivalence for both proteins was noted in liver from day 12 to day 18 prior to hatching, as seen by ELISA assays and Western blot analysis. Presentation of galectin-reactive glycoproteins showed a tendency for gradual increase in both organs. Galectin-blotting analysis revealed primarily very similar patterns of positive bands at the different stages of development and only few quantitative and qualitative changes. The reactivity of glycolipids in a solid-phase assay was more variable, even surpassing the response of extracts of the adult organ at several embryonic stages. While the localization patterns of the galectins and galectin-reactive sites were nearly indistinguishable in the liver, intestinal tissue differed with respect to the placement

and accessibility of binding sites. Thus, the results suggest a differential regulation of galectin activities in the two organs. As a sum they resemble the course of development of availability of glycoprotein ligands *in vitro*. These findings support the notion for a partial functional redundancy in this family. The described approach to employ galectin-specific antibodies and the labeled galectins as tools to assess presentation of ligands is suggested to be of general relevance to address the question of distinct vs. overlapping functions of related recognition molecules.

Key words: Agglutinin, Embryogenesis, Galectin, Glycoprotein, Intestine, Lectin, Liver

Introduction

Starting with the duplication of an ancestral gene, the ensuing diversification can produce an array of related proteins. Ideally, they can acquire special properties in the course of this process which are tailored to fulfill distinct functions. Under these circumstances, it is readily apparent that a separation into partially redundant activities will confer a selective advantage with evolutionary stability (Thomas, 1993). This process entails the requirement for devising control mechanisms to accurately tune on gene expression of the specialized products in response to the demand. Although proteins are definitely precious commodities, cells may tolerate to continue to synthesize a member of a gene family, if the expenses for providing an accurate organ-specific control mechanism surpass that of producing an innocuous protein (Erickson, 1993). We are thus faced with the problems to define the overlap of biological significance and to distinguish truly functional from

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evidently superfluous expression in the different organs. To clarify these issues, it is a reasonable starting point to precisely monitor the expression of the different receptor proteins in different organs. When they exert their functions by recognitive processes, the next step is the quantitative assessment of the presence of ligands to reveal any measurable adaptation between a certain receptor and its binding partners. These two questions will be addressed for a model of two homologous receptor proteins in the development of two chicken organs.

Owing to their placement in the non-reducing terminal section of glycan chains galactosides are spatially accessible docking points for sugar receptors. Glycosylation imparts glycoproteins the ability to be engaged in recognitive interactions on the basis of the sugar code for biological information (for a collection of recent reviews illuminating the field, see Gabius and Gabius, 1997; Gabius and Sinowatz, 1998). Indeed, this assumed function has actually been verified in the interplay with C-type lectins and galectins. The members of the latter family of animal lectins share a conserved motif for the carbohydrate recognition domain, which is the basis for their classification (Kasai and Hirabayashi, 1996; Gabius, 1997a; Hirabayashi, 1997; Kaltner and Stierstorfer, 1998). Following the initial descriptions of a chicken galectin (CG-16) activity, its developmental regulation and biochemical characteristics were determined (Nowak et al., 1976, 1977; Den and Malinzak, 1977; Kobiler and Barondes, 1977; Eisenbarth et al., 1978; Kobiler et al., 1978; Beyer et al., 1979; Cook et al., 1979; Yamagata and Nishiwaki, 1979). Systematic studies were pertinent to assess whether the capacity for galectin expression was confined to this protein. In the course of this work a second activity was subsequently purified, termed CG-14 on the basis of its monomer molecular weight (Beyer et al., 1980). Relative to CG-16 it has a rather weak activity for hemagglutination, what is explained by a more pronounced tendency to be present as monomer in the native state relative to CG-16 (Beyer et al., 1980; Hirabayashi et al., 1987; Kakita et al., 1991; Castagna and Landa, 1994; Schneller et al., 1995). Remarkably, initial measurements of the developmental regulation indicated independent control mechanisms to be operative for the two galectins (Beyer and Barondes, 1982a; Levi and Teichberg, 1989; Akimoto et al., 1993, 1995; Guay and Zalik, 1994). Since the time of origin of the two genes after duplication of the ancestral genetic coding unit had been assigned to a period close to the divergence of birds and mammals approximately 3×10^8 years ago (Sakakura et al., 1990), the length of the subsequent period clearly should have been sufficient to enable the galectins to acquire distinct features. As an indicator for disparate properties the mapping of the combining sites with engineered ligands had recently unraveled differences in their architecture (Solís et al., 1996). Since neither the extent of ligand presentation for the two galectins nor the developmental regulation of galectin-reactive sites

are known, we addressed these questions by biochemical and histochemical studies at various stages of development pre and post hatching for two organs with widely different abundance for the two galectins in adult chicken. These investigations were rendered feasible by purification of the endogenous lectins, their labeling under activity-preserving conditions and their introduction as analytical tools for systematic ligand monitoring, as exemplarily documented previously for mammalian galectin-1 and heparin-binding lectin (Gabius et al., 1991a,b, 1993; Joubert-Caron, 1993). Since apparently differential regulation has also been inferred for mammalian galectins in normal and transformed cells (Cerra et al., 1985; Gabius et al., 1986a,b; Fowles et al., 1995; Xu et al., 1995; Colnot et al., 1996, 1997; Gillenwater et al., 1996; Gabius, 1997b; Ohannesian and Lotan, 1997; van den Brule et al., 1997) the results of this study can have relevance beyond avian development.

Materials and methods

Lectin isolation, antibody preparation and probe labeling

Following tissue extraction (liver and intestine) with 75 mM phosphate-buffered saline (pH 7.2) containing 4 mM β -mercaptoethanol, 2 mM EDTA, 0.1 mM benzyloxycarbonyl fluoride and 0.1M lactose, centrifugation and dialysis of the supernatant to remove lactose, the chicken galectins were purified by successive steps of affinity chromatography on lactosylated Sepharose 4B, obtained after divinyl sulfone activation, and anion-exchange chromatography on a Mono Q column (1 ml resin; Pharmacia, Freiburg, FRG), as described previously (Gabius, 1990; Schneller et al., 1995). To stabilize their activities which are sensitive to oxidation of sulfhydryl groups, carboxyamidomethylation of the galectins was routinely carried out with 50 mM iodoacetamide during elution in the first chromatographic step. The galactoside-specific lectin from mistletoe (*Viscum album* agglutinin, VAA) was purified by affinity chromatography, as described by Gabius (1990). Analysis of lectin purity by one- and two-dimensional gel electrophoresis, silver staining of gels, determination of protein concentration and of lectin activity by hemagglutination was performed, as described in detail previously (Gabius et al., 1984; Schneller et al., 1995). Polyclonal antibodies against the chicken galectins were raised in rabbits, the lack of cross-reactivity was ascertained by solid-phase assays in microplates and by Western blotting and affinity purification on lectin-containing Affi-gel 10/15 (3 mg/ml; Bio-Rad, Munich, FRG) was performed, as described (Bardosi et al., 1989; Zeng and Gabius, 1991). The purified fractions of antibodies which were free of cross-reactive activity were labeled by covalent conjugation with periodate-treated horseradish peroxidase (Nakane and Kawaoi, 1974). Chemical modification with biotinyl-N-hydroxy-succinimide ester (Sigma, Munich, FRG) under mild,

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activity-preserving conditions yielded labeled lectins, which were routinely checked for the integrity of the activity and of the native molecular weight after label introduction and after storage to exclude fractions with aberrant properties (Gabijs et al., 1991a; Kaltner et al., 1997).

Quantitation of galectins and of galectin-reactive glycoproteins in tissue extracts

Fertilized eggs were incubated at 37 °C and 63-67% humidity for distinct periods of time. Tissue samples of liver and intestine were collected at defined stages of embryogenesis. Following extractions of the specimen in detergent-free and in 1% Triton X-100 and 0.1% sodium deoxycholate-containing buffers to which 0.1M lactose was added to reduce binding of soluble galectin to insoluble cell material, a sandwich ELISA assay in microtiter plates (Greiner, Nürtingen, FRG) was performed using affinity-purified antibody fractions in the first step (0.5 µg/ml) and the peroxidase-labeled fractions in the detection step (5 µg/ml), as described by Kaltner et al. (1997). Using calibration curves with the purified galectins, the quantity of lectin present in each extract sample was determined routinely in the linear range of the calibration curve, the detection limit being approximately 2-2.5 ng/ml. In order to similarly quantitate the amount of galectin-reactive glycoconjugates in a solid-phase assay, a constant quantity of extract protein (2.5 µg) was exposed to the surface of microtiter plate wells for adsorption over 12 h at 37 °C. Further assay steps including blocking, washing, probing with biotinylated lectin, washing, further probing with streptavidin/peroxidase conjugate (0.5 µg/ml; Sigma, Munich, FRG), washing and addition of the chromogenic substrates o-phenylenediamine (1mg/ml)/H₂O₂ (0.004%; v/v) were carried out, as described previously (Gabijs et al., 1991a; Kaltner et al., 1997). The level of carbohydrate-dependent binding was assessed by assays, in which each biotinylated lectin, which had been preincubated with a solution containing 50 µg asialofetuin/ml and 50 µM lactose to saturate the binding sites, and the noted potent glyco-inhibitors were present to preclude any interaction between glycoproteins on the surface of the plate wells and the agglutinins by protein-carbohydrate interaction. The OD-values of the assays are expressed as ng asialofetuin based on standard curves with this model glycoprotein.

Visualization of galectins and galectin-reactive glycoproteins on blots

Aliquots of tissue extracts with constant quantities of either 20 µg protein/lane for Western blot analysis after one extraction step with detergent-containing buffer or 5 µg/lane after fractionated extraction for lectin-binding analysis of lectin-reactive glycoproteins were subjected to electrophoresis under denaturing and reductive conditions either on a 15 % polyacrylamide gel (Western

blotting) or a 12% polyacrylamide gel (lectin blotting). The proteins were thereafter transferred by tank blotting to nitrocellulose (Schleicher und Schuell, Dassel, FRG; 0.2 µm). Probing was performed either with affinity-purified immunoglobulin G fractions or biotinylated lectins in conjunction with commercial peroxidase-labeled goat anti-rabbit immunoglobulin G or a streptavidin/alkaline phosphatase conjugate, as described previously (Gabijs et al., 1986b, 1991a; Kaltner et al., 1997). Addition of a mixture of 0.3M lactose and 0.25 mg asialofetuin/ml was employed to determine carbohydrate-independent binding, and addition of an excess of galectin to the antibody-containing solution to exclude antigen-independent binding. The incubation steps with primary antibody or labeled lectin were deliberately omitted in independent experiments to visualize staining, for which indicator reagents (second antibody, kit components) are responsible.

Quantitation of galectin-binding neutral glycolipids and gangliosides in tissue extracts

Tissue samples of embryonic and adult chicken liver or intestine (approximately 1 g wet weight) were homogenized in 4 ml chloroform/methanol (2:1; v/v), as described previously (Kaltner et al., 1997). Following centrifugation each supernatant was passed over a Sephadex A-25 column (bed volume: 18 ml; Pharmacia, Freiburg, FRG) to fractionate neutral glycolipids, obtained by a washing step with 50 ml chloroform/methanol/water (40:60:8, by volume), and gangliosides, obtained by subsequent elution with chloroform/methanol/0.8M sodium acetate (40:60:8, by volume). After cautious evaporation of the solvent below 40 °C waterbath temperature each sample of dried glycolipid was thoroughly resuspended in 5 ml methanol and aliquots of this solution diluted with methanol were used to coat the surface of the microtiter plate wells with glycolipids over a period of 2 h at 37 °C. Further processing has been described in detail elsewhere (Kaltner et al., 1997). The concentrations of the biotinylated lectins had to be adjusted to different values to maintain a constant ligand display in the assay, i.e. 0.1 µg mistletoe lectin/100 µl, 0.2 µg CG-14/100 µl and 5 µg CG-16/100 µl. Each series of triplicates was accompanied by the assessment of the extent of sugar-inhibitable binding and the background values due to the interaction of kit reagents with the matrix. Purified lactosylceramide and ganglioside GM₁ were also tested. For comparison between the individual samples the OD-values for carbohydrate-mediated binding were normalized to 1 mg wet weight of each tissue sample.

Visualization of lectin-reactive sites and of the galectins in tissue sections

Fresh tissue specimens were immediately fixed in Bouin's solution or 4% buffered paraformaldehyde. In addition to the different time points of embryogenesis,

explicitly given in Table 1, livers and intestines were removed from adult chicken and from chicken 1, 2, 3 or 6 weeks post hatching. Further processing of the samples by embedding in paraffin, preparation of 5 μm sections and probing with biotinylated lectins at a concentration of 1 $\mu\text{g}/\text{ml}$ or with galectin-specific immunoglobulin G fractions at a concentration of 2 $\mu\text{g}/\text{ml}$ overnight at 4 °C followed the optimized protocol (Kaltner et al., 1997). Control reactions included preadsorption of the antibody preparations with an excess of corresponding galectin, deliberate omission of the incubation step with the probe (biotinylated lectins or antibodies) and inhibition of sugar-dependent binding by coinubation of the lectins with 0.1M lactose (mistletoe agglutinin) or with a mixture of 0.2M lactose and 0.65 mg asialofetuin/ml (galectins).

Statistical analysis

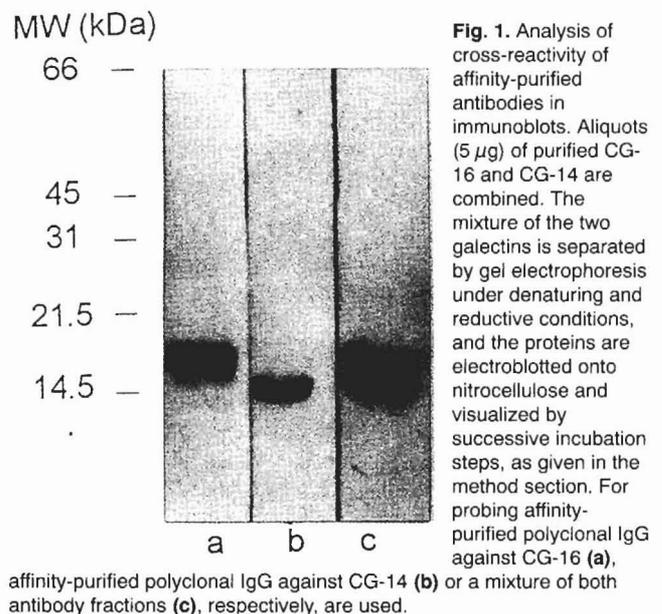
Based on the commercial Sigma Stat 1.0 package (Jandel Scientific, Erkrath, Germany) the one-way analysis of variance on ranks, Dunn's multiple comparison test for calculation of significance of sets of values and the Mann-Whitney rank sum test were used.

Results

To monitor galectin expression in the course of development, antibody fractions are convenient tools. Due to the expression of two galectins in chicken liver and intestine contamination of a preparation by the less abundant protein can be the source for undesirable cross-reactivity of antibody fractions. Following affinity chromatography anion-exchange chromatography was crucial to completely weed out minor traces of the other galectin owing to the difference in isoelectric points between CG-14 and CG-16 of more than two units (Schneller et al., 1995). Using preparations which were free of any contamination according to silver staining after slab gel electrophoresis as starting material for immunization, polyclonal immunoglobulin G fractions against the two galectins were raised in rabbits. Their analysis by solid-phase assays in microtiter plates and on nitrocellulose ascertained that these fractions were specific for the galectin, which had been employed as antigen, as shown for a Western blot in Fig. 1. Having gained access to the required monospecific probes technical details of the methods could be addressed. To exclude an assay-inherent bias two independent methods were used to determine the expression of the two galectins in the course of development, i.e. blotting analysis and a sandwich ELISA technique. Its detection limit was approximately 2-2.5 ng galectin/ml and the recovery ratio of each galectin, which had been added to extracts in known quantities, was consistently above 85%. To exclude masking of galectins by association to insoluble material, 0.1M lactose was added to the detergent-containing extraction buffer. Moreover, treatment of the residual pellets with the standard sample

buffer for gel electrophoresis under denaturing conditions and boiling rendered no measurable amount of galectin assessable to the analytical tools. To rule out a lack of immunodetection of ligand-bound galectins in the supernatants, this situation was simulated with asialofetuin as model ligand, revealing unimpaired concentration-dependent signal development. Having thus documented the validity of the reagents and assay conditions, the questions could be addressed as to what extent the expression of the two galectins is developmentally regulated and whether the course of regulation is different in liver and intestine, which harbor a predominant type of galectin at the adult stage.

While Table 1 presents the summary of the individual data sets of the sandwich ELISA assay, an exemplary documentation of the course of galectin expression for a single case per time point as monitored by Western blotting is presented in Figs. 2, 3. The contents of the two galectins in liver remained rather constant from day 12 of embryogenesis and the preference for CG-16 was established after hatching, CG-14 only reaching a relative amount of below 20% of CG-16 (Table 1, Fig. 2). The course of galectin expression was significantly different in intestine. Evidently, the relative abundance of the two galectins was not kept constant in the two types of organ. An obvious preference of CG-14 expression in the intestine was apparent even at early stages of embryogenesis (Table 1, Fig. 3). Whereas the CG-16 content remained nearly unchanged during embryogenesis from day 10 to day 18 and further dropped for the adult stage, the expression level of CG-14, which already surpassed that of CG-16 at day 10, continuously increased and dominated by far at the adult stage (Table 1, Fig. 3). It is thus suggestive to assume a preferential role of CG-14 in



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intestine from late stages of pre-hatching maturation onwards and at the adult stage. Having addressed the question of regulation of two galectins in development of liver and intestine, it is imperative to determine whether the ligand availability is also subject to developmental regulation. It may be controlled by completely different regulatory constraints in development. It may, too, be dissimilar with respect to the two proteins owing to fine-specificity differences of the galectins for carbohydrate ligands. The monitoring of

ligand sites was exclusively based on carbohydrate recognition, using OD-values of the solid-phase assay and the staining profile of lectin blots only after adequate controls to prove inhibition by glyco-inhibitors. Two independent methods were applied to assess the total amount of accessible glycoprotein ligands in extracts and the profile of glycoproteins which are reactive in lectin blots.

Prior to the presentation of the results of the microtiter plate assay for lectin-reactive glycoproteins a

Table 1. Determination of the CG-14 and CG-16 content (ng/mg protein) in extracts of embryonic and adult chicken liver and intestine.

TISSUE TYPE	STAGE OF DEVELOPMENT (days)					
	10	12	14	16	18	adult
<i>CG-14</i>						
Liver	182.3±40.00** ^a (5)	104.8±33.6 (5)	102.0±29.2 (4)	107±20.9 (4)	103.4±22.3 (5)	64.3±20.9 ^b (5)
Intestine	216.0±86.3*** ^c (5)	650.8±279.5** ^c (5)	830.9±217.5*** ^c (5)	1108.2±318.8*** ^c (6)	1444.3±22.5*** ^c (6)	4651.3±874.1 ^c (6)
<i>CG-16</i>						
Liver	52.5±14.9*** (5)	97.8±14.9*** (5)	88.4±20.08*** (6)	106.8±26.2*** (5)	93.5±13.0** (5)	370.4±93.7 (5)
Intestine	50.1±11.3*** (5)	57.6±14.6*** (5)	45.3±21.8*** (5)	34.5±10.6* (5)	36.8±8.2** (5)	6.6±4.6 (6)

*0.05 ≥ p > 0.01, **0.01 ≥ p > 0.001, ***p ≤ 0.001: significant difference of result for embryonic stages relative to that of the adult organ; ^a 0.05 ≥ p > 0.01, ^b 0.01 ≥ p > 0.01, ^c p ≤ 0.001: significant difference of result for CG-14 relative to that for CG-16. Number in brackets denotes the number of individual animals for each stage of development.

MW (kDa)

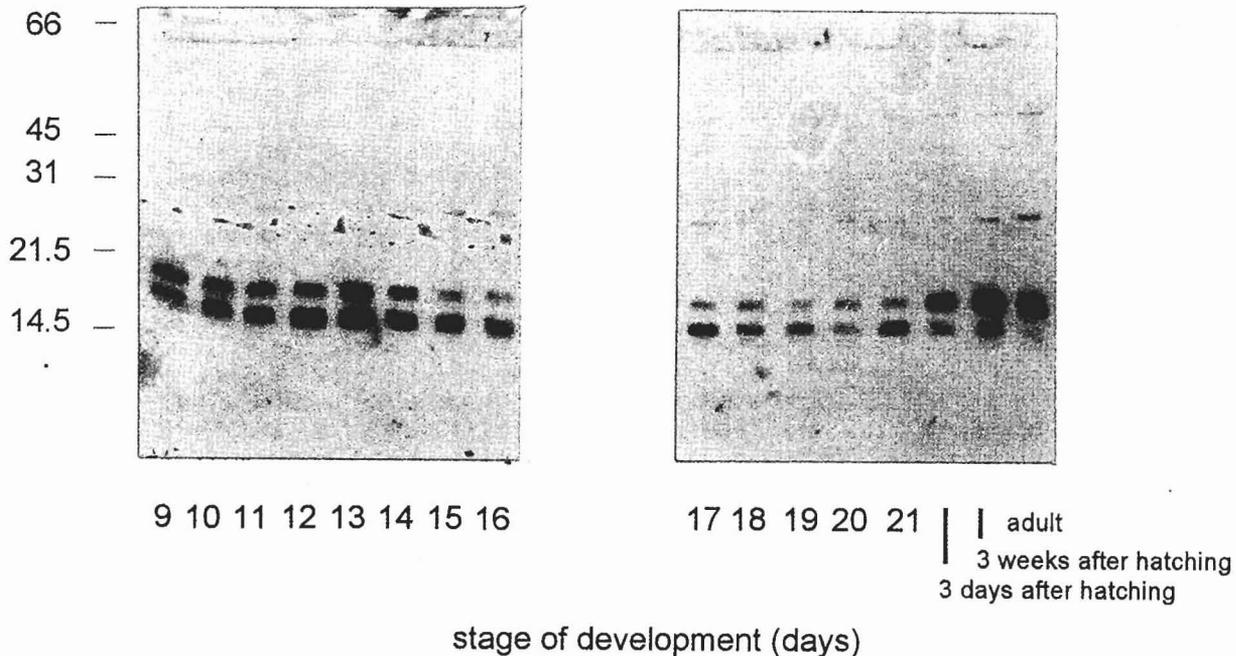


Fig. 2. Immunoblotting analysis of extract proteins (10 µg per lane) from embryonic, post-hatching and adult chicken liver. A mixture of affinity-purified polyclonal immunoglobulin G preparations against CG-14 and CG-16 is used for probing.

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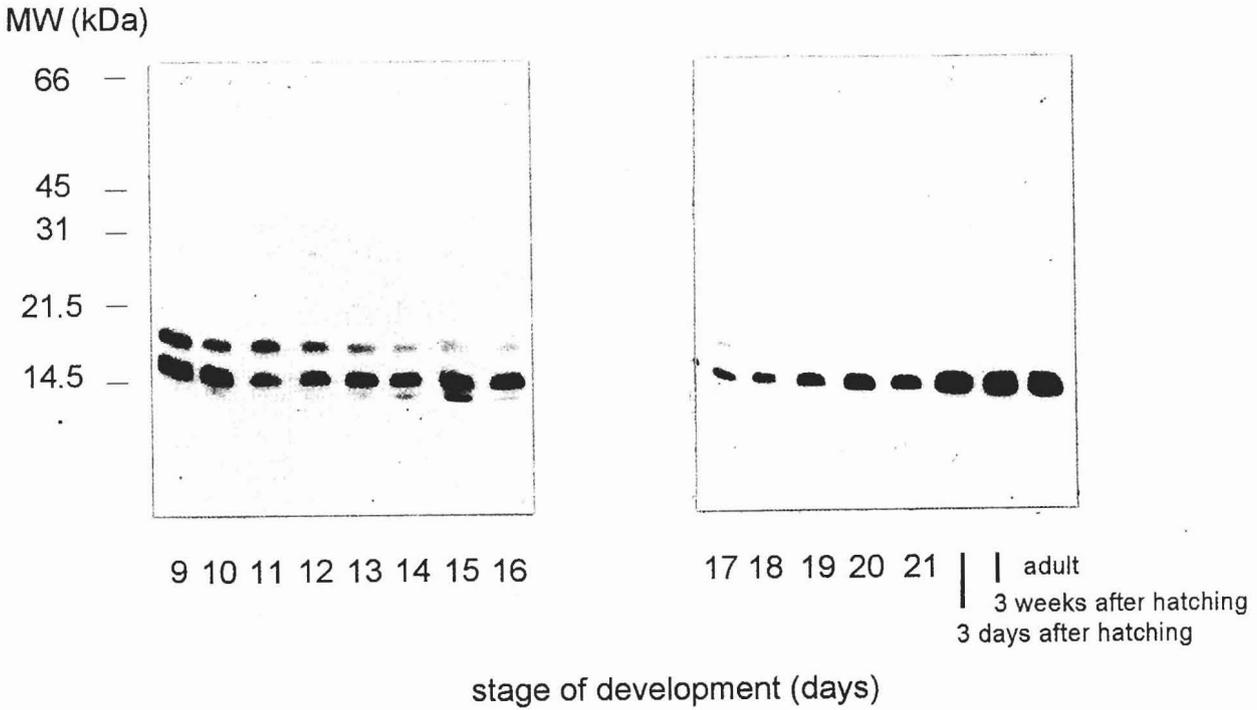


Fig. 3. Immunoblotting analysis of extract proteins (10 μ g per lane) from embryonic, post-hatching and adult chicken intestine. A mixture of affinity-purified polyclonal immunoglobulin G preparations against CG-14 and CG-16 is used for probing.

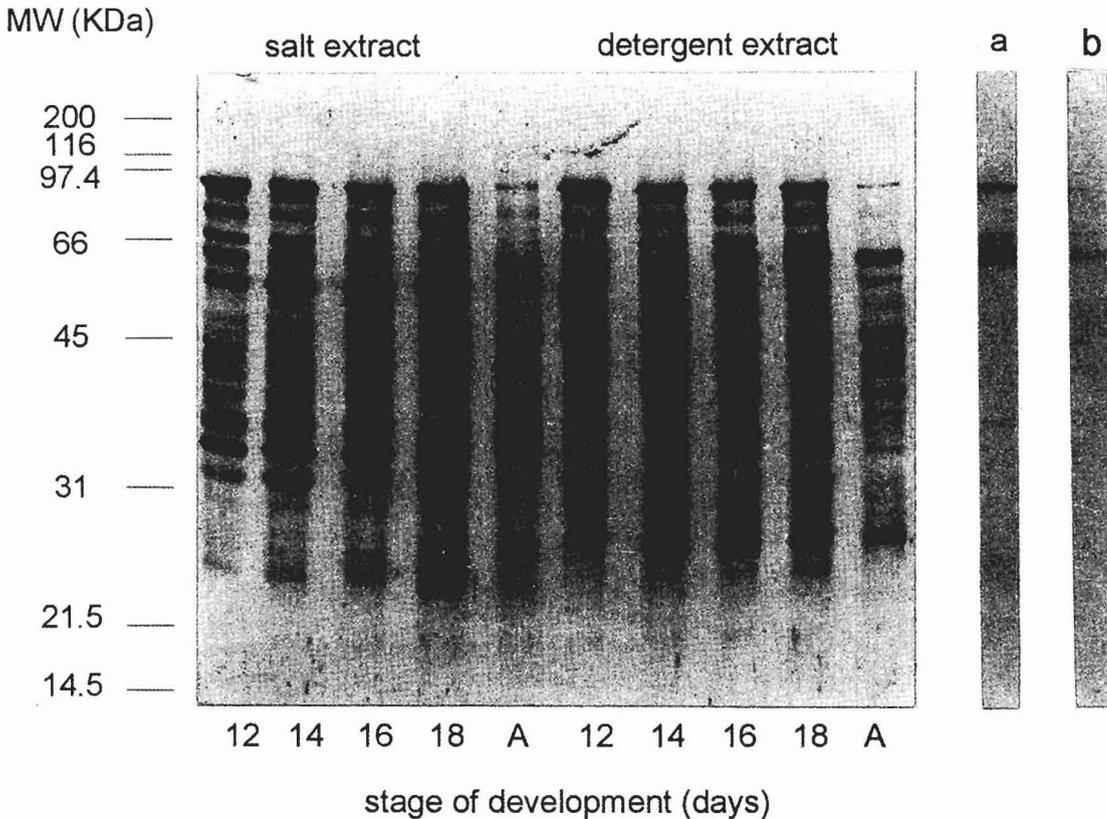


Fig. 4. Detection of CG-16-binding glycoproteins from extracts of embryonic and adult chicken liver. Protein aliquots of identical quantity (5 μ g per lane) are separated by gel electrophoresis under denaturing and reductive conditions. The proteins are electroblotted onto nitrocellulose and visualized by successive incubation steps with biotinylated CG-16 and signal-generating reagents. The effect of the presence of 0.3M lactose and 0.25 mg/ml asialofetuin to block carbohydrate-dependent binding is documented for extracts from adult chicken liver (**lane a**). Staining of proteins by the streptavidin/alkaline phosphatase-conjugate and chromogenic substances in the absence of the

biotinylated lectin is shown for an aliquot of this specimen (**lane b**). The blot is calibrated with colored standard molecular weight markers.

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Table 2. Determination of the content of lectin-binding glycoproteins in salt and detergent extracts of embryonic and adult chicken liver and intestine, expressed as ng model ligand (asialofetuin)/mg protein.

ORGAN	EXTRACT	STAGE OF DEVELOPMENT (days)					
		10	12	14	16	18	adult
<i>CG-14</i>							
Liver	S	2061.2±482.5** (6)	2861.7±289.2** (6)	2969.8±749.1 (6)	3110.2±1461.2 (6)	4078.5±1069.8 (6)	4309.3±689.3 (6)
	D	1630.5±203.8*** (6)	1719.9±309.1*** (6)	1091.5±189.5*** (6)	1371.3±280.5*** (6)	1879.3±289.3*** (6)	4261.3±1029.3 (6)
Intestine	S	1240.3±362.4*** (6)	1271.3±280.1** (6)	1999.2±174.2 (4)	2164.7±1332.3 (4)	2501.3±761.2 (5)	3290.5±1003.2 (6)
	D	954.2±200.2*** (6)	980.7±201.3*** (6)	1871.2±83.2** (4)	2067.1±587.1* (4)	3126.0±1011.3 (5)	3824.0±1204.3 (5)
<i>CG-16</i>							
Liver	S	145.3±36.9*** (4)	191.0±59.9*** (5)	330.6±119.9*** (6)	581.2±149.8 (6)	562.3±130.7 (7)	669.9±80.6 (8)
	D	160.5±73.4*** (7)	171.2±59.8*** (6)	200.3±71.2*** (6)	449.8±170.6 (6)	420.3±120.9 (7)	569.7±110.3 (6)
Intestine	S	186.2±73.3* (5)	267.2±51.3 (6)	353.4±50.7 (5)	285.2±121.6 (6)	261.3±21.3 (6)	340.7±90.7 (6)
	D	121.7±61.2** (5)	111.7±41.1*** (6)	121.6±22.2** (5)	131.3±41.2** (5)	180.4±40.7 (5)	243.9±42.1 (5)
<i>VAA</i>							
Liver	S	149.8±49.3 (6)	151.3±31.7 (6)	110.7±9.8 (6)	109.2±20.5 (7)	178.5±89.4 (7)	191.4±59.5 (6)
	D	104.3±10.2*** (6)	108.9±40.5*** (6)	149.3±10.1 (6)	119.5±8.7** (6)	121.1±11.2** (6)	182.7±21.0 (6)
Intestine	S	59.8±12.3*** (6)	63.5±11.4*** (6)	60.5±20.3*** (5)	71.7±9.9*** (6)	111.3±29.8 (4)	142.8±19.7 (4)
	D	39.7±17.7*** (7)	49.9±11.2*** (6)	70.6±9.8** (5)	82.3±10.7* (6)	103.1±21.2 (5)	119.8±33.1 (4)

S: salt extract; D:detergent extract'; *0.05 ≥ p > 0.01, **0.01 ≥ p > 0.001, ***p ≤ 0.001: significant difference of result for a distinct embryonic stage relative to that of the adult organ.

note of caveat is appropriate. Direct quantitative comparisons of results of solid-phase assays are only valid if the spatial parameters of probes are identical. Presentation of ligands on a surface entails differential accessibility to proteins which differ in size and arrangement of ligand-binding sites. Therefore, the developmental course of ligand expression for an individual lectin can only be correlated to that of another sugar receptor with caution, taking the size of the proteins into account. In this respect, the small size of monomeric CG-14 relative to dimeric CG-16 and tetrameric mistletoe agglutinin is an important factor to be reckoned with.

When carbohydrate-inhibitable binding to extract compounds was quantitated for CG-14, the relation of abundance of reactive sites in salt and detergent extracts differed for liver and intestine (Table 2). Salt extracts of embryonic liver from day 10 to day 18 contained more ligands than respective detergent extracts. In most cases, the alterations with stage of development were rather similar for both fractions obtained by the two different solutions for extraction. There was a general tendency for increased presence of CG-14-binding sites with

maturation, which was reflected on the level of the receptor for this galectin only in intestine (Tables 1, 2). A similar course of increase for ligand presentation was seen, when CG-16 was used as marker (Table 2). It is remarkable that a clear-cut correlation between galectin presence and expression of galectin-reactive glycoproteins only existed in both organs, when the contents of both galectins were added up. To examine whether this enhancement was restricted to distinct galectin-binding components of extracts or could be due to elevated presentation of galactose-containing glycans, a plant agglutinin with selectivity to α - and β -galactosides was similarly assayed. These experiments revealed an increase in binding partners at most time points for this agglutinin also (Table 2). It is inherent in the assay design that the measurement furnishes an insight into the quantitative aspect of ligand presentation on the surface of the microtiter plate wells. It is not possible to infer the characteristics of the glycan-carrying proteins under these conditions. To provide information on this issue, lectin blotting was performed with extract aliquots of samples which were analyzed quantitatively.

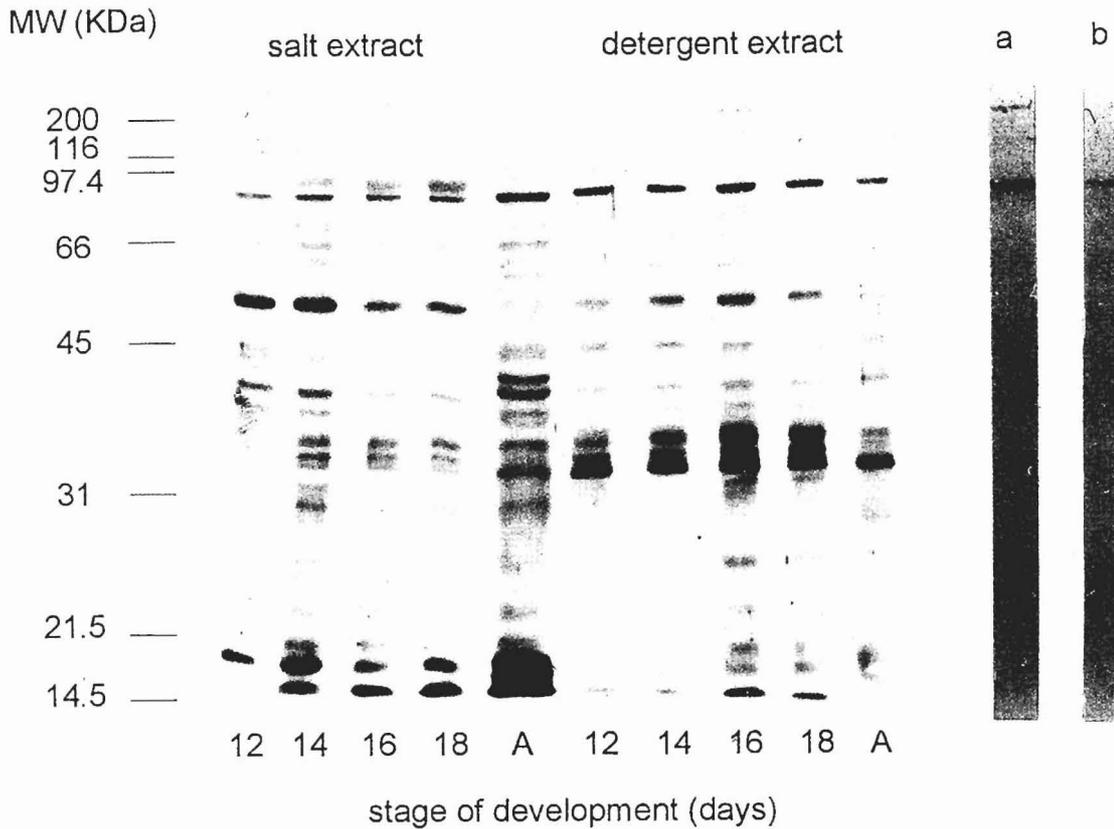


Fig. 5. Detection of CG-14-binding glycoproteins from extracts of embryonic and adult chicken intestine. Protein aliquots of identical quantity (5 µg per lane) are separated by gel electrophoresis under denaturing and reductive conditions. The proteins are electroblotted onto nitrocellulose and visualized by successive incubation steps with biotinylated CG-14 and signal-generating reagents. The effect of the presence of 0.3M lactose and 0.25 mg/ml asialofetuin to block carbohydrate-dependent binding is documented for extracts from adult chicken intestine (**lane a**). Staining of proteins by the streptavidin/alkaline phosphatase

conjugate and chromogenic substances in the absence of the biotinylated probe is shown for an aliquot of this specimen (**lane b**). The blot is calibrated with colored standard molecular weight markers.

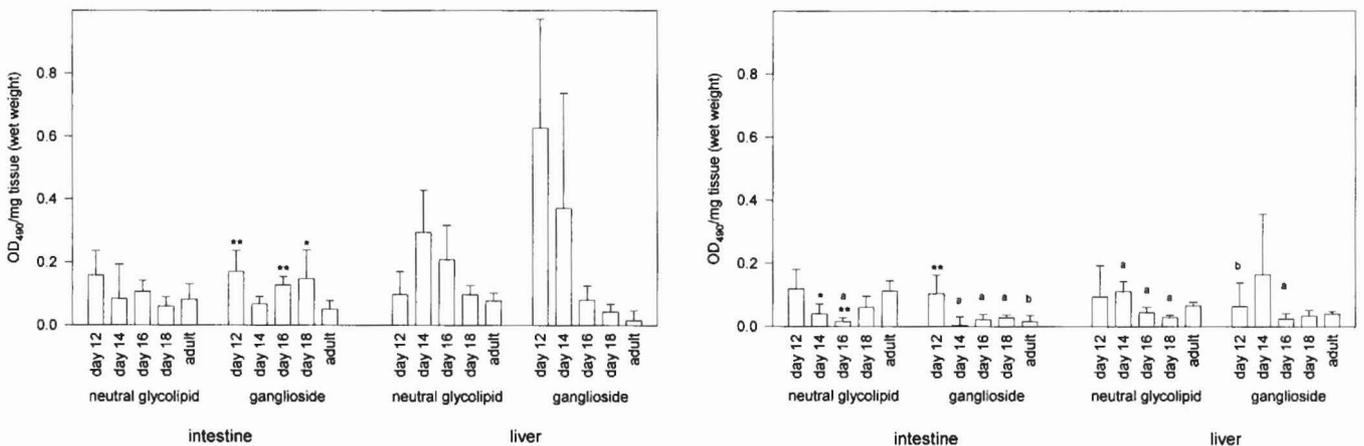


Fig. 6. A. Extent of expression of neutral glycolipids and gangliosides with reactivity to CG-14 from extracts of embryonic and adult chicken intestine and liver from 4-8 different animals in a solid-phase assay. The given OD-value for each developmental stage is normalized to the response of an aliquot of each fraction which is equivalent to 1 mg tissue (wet weight). Statistical significance of differences of the OD-values between the embryonic stages and the adult organ is indicated by *0.05≥p≥0.01, and **0.01≥p≥0.001. **B.** Extent of expression of neutral glycolipids and gangliosides with reactivity to CG-16 from extracts of embryonic and adult chicken intestine and liver from 4-8 different animals in a solid-phase assay. The given OD-value for each developmental stage is normalized to the response of an aliquot of each fraction which is equivalent to 1 mg tissue (wet weight). Statistical significance of differences of the OD-values between the embryonic stages and the adult organ is indicated by *0.05≥p≥0.01, and **0.01≥p≥0.001. Additionally, statistical significance of differences of the OD-values for the two galectins with tissue samples of the same organ and at the same developmental stage is indicated by ^a0.05≥p≥0.01; and ^b0.01≥p≥0.001.

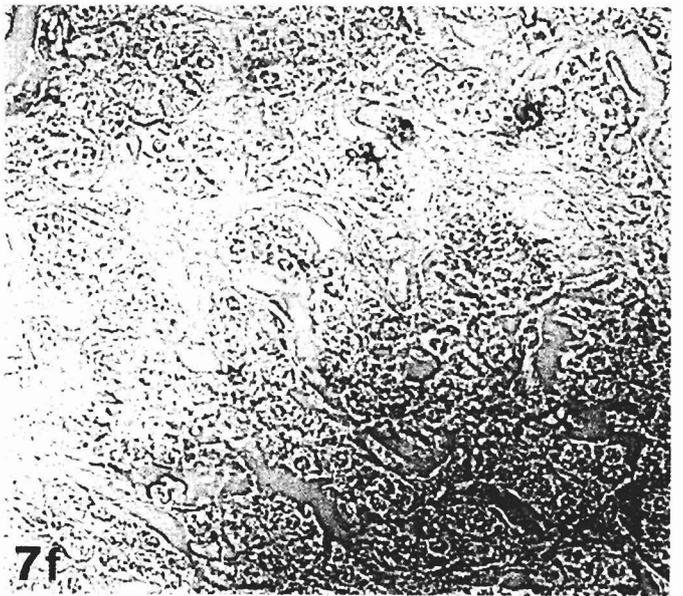
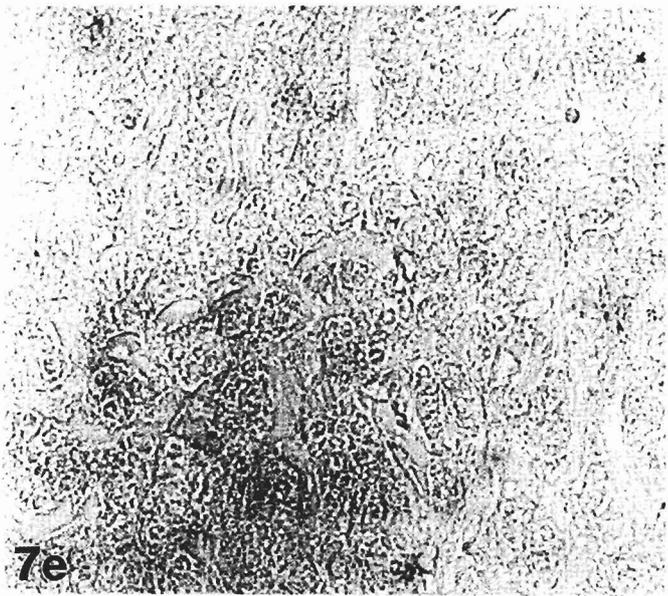
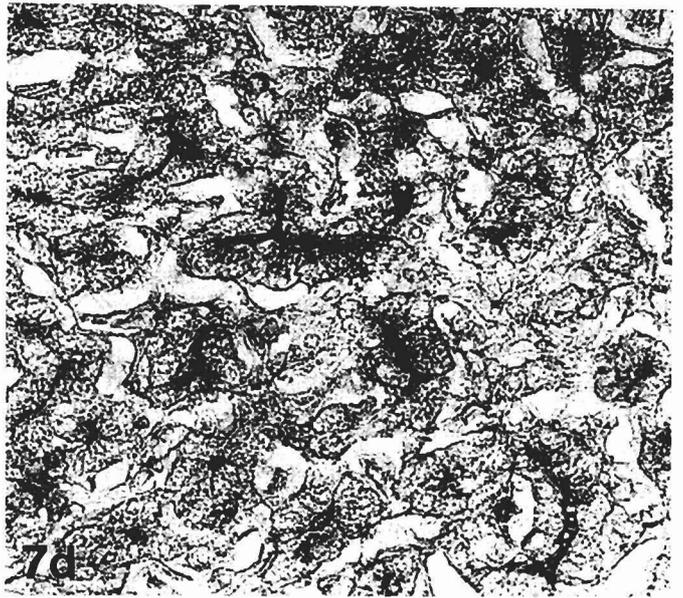
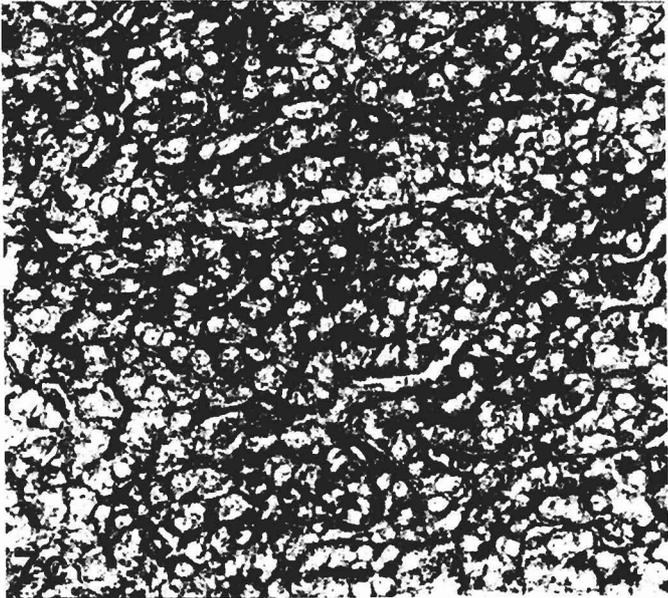
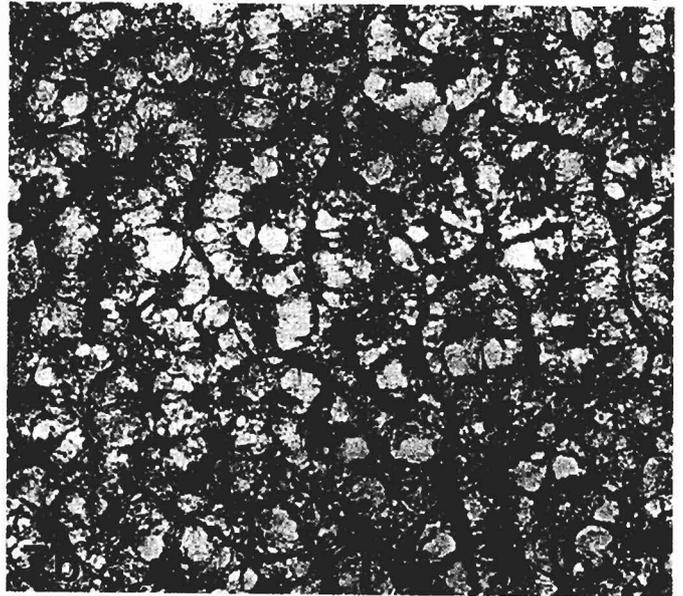
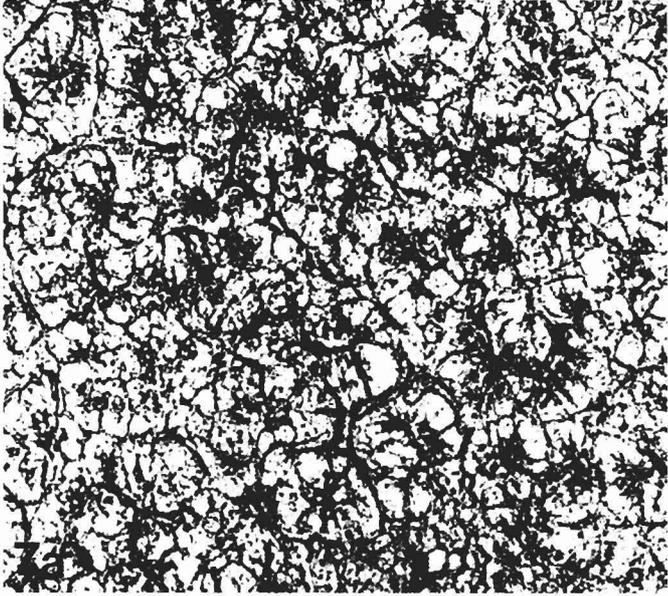


Fig. 7. Localization of mistletoe lectin-specific binding sites with characteristic staining of the plasma membrane of hepatocytes (a), especially the membrane regions forming the bile canaliculi (b). Only quantitative changes occur with maximal staining intensity measurable on day 14 post hatching (c), which is reduced in the adult organ (d). Omission of the incubation step with the biotinylated lectin (e) and presence of 0.1 M lactose during the incubation step with the lectin (f) provide the essential specificity controls. x 320

At first, it was notable that carbohydrate-independent and kit reagent-dependent binding reactions were visible besides the glyco-inhibitor-sensitive staining. These side reactions had to be taken into

consideration to preclude erroneous interpretation (Figs. 4, 5). Primarily quantitative alterations were assessable in the course of development. However, a CG-16-positive band at 70 kDa was present only during the

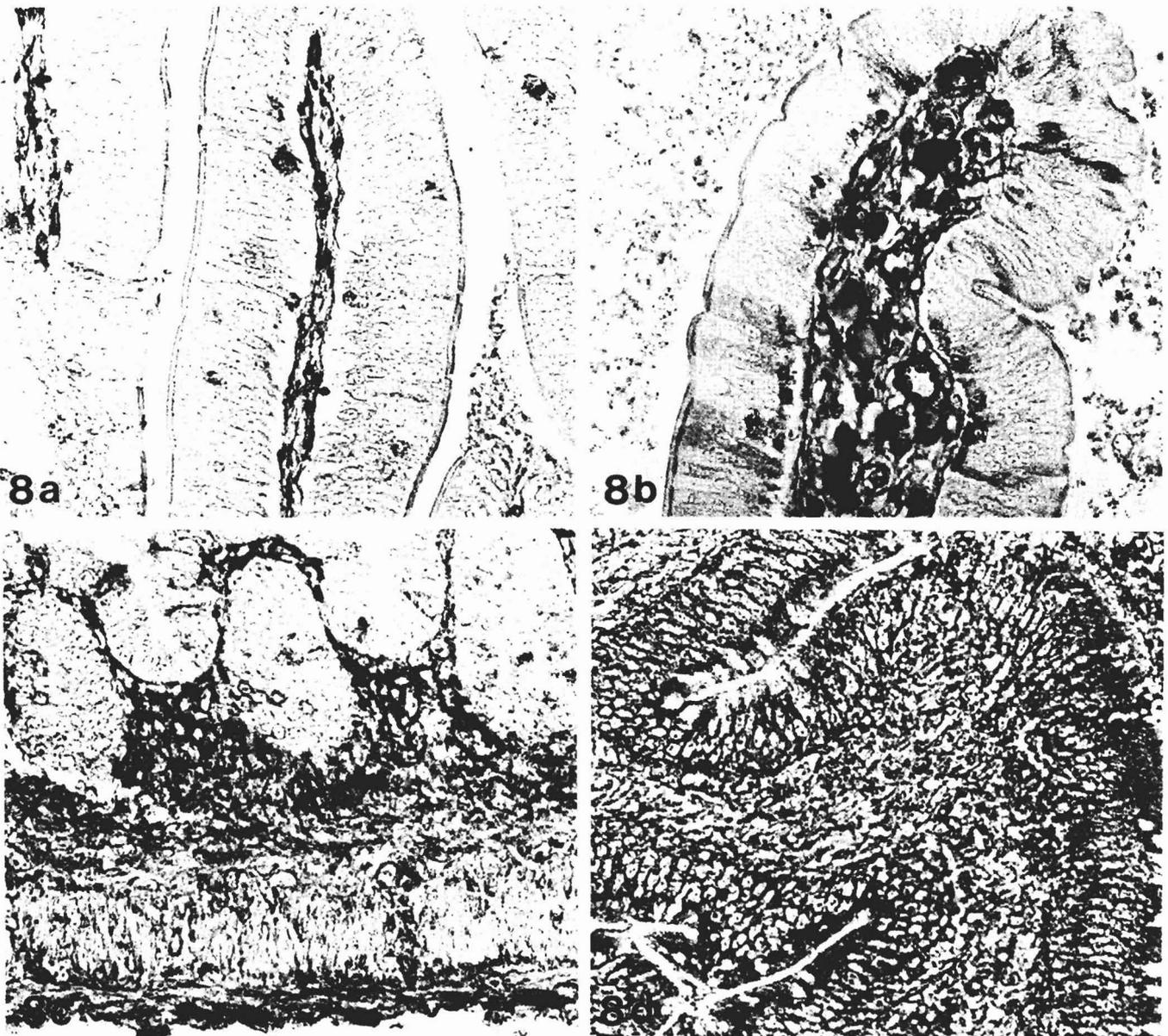


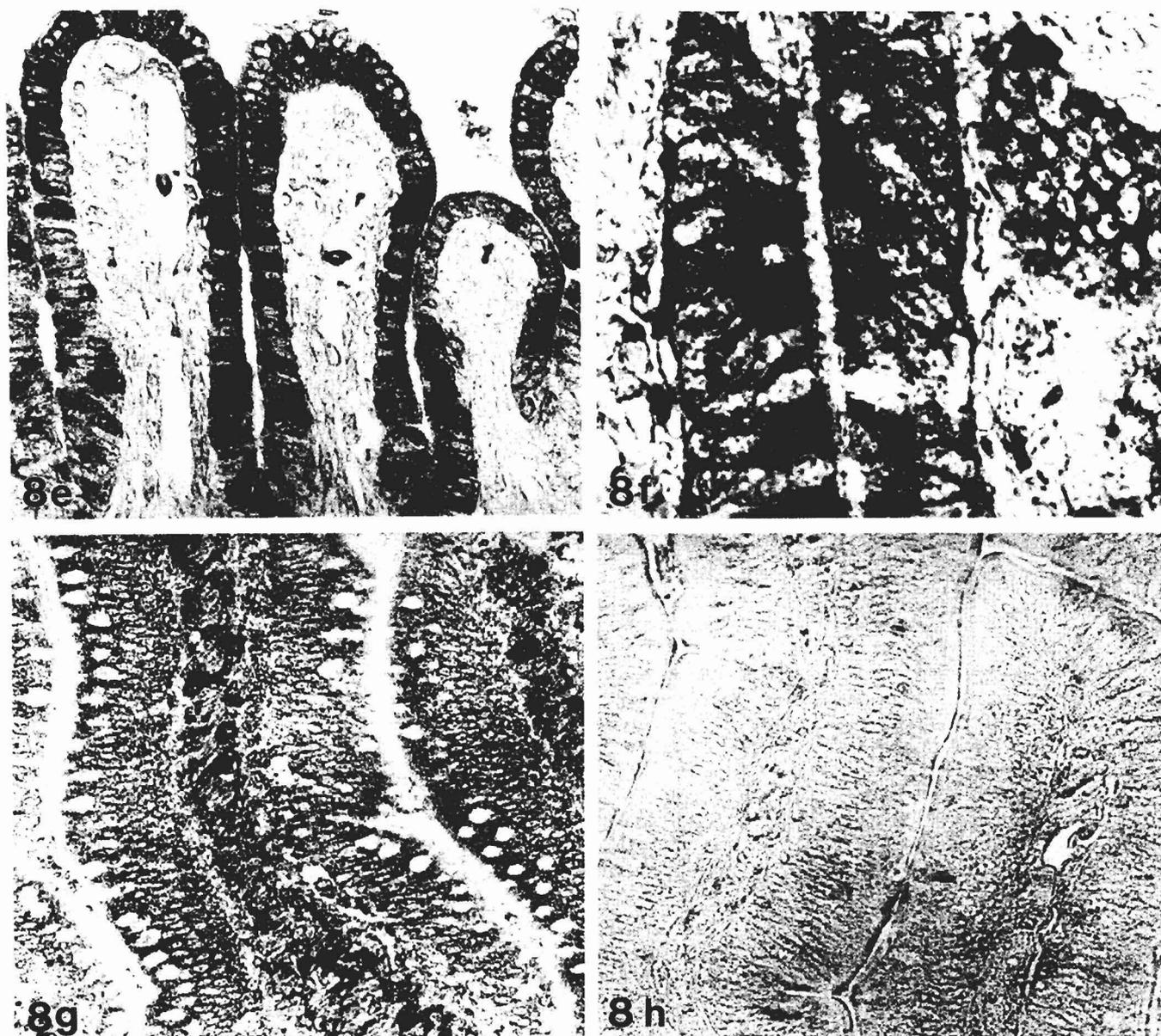
Fig. 8. Localization of mistletoe lectin-specific binding sites in stromal cells in the lamina propria of the intestinal mucosa in sections from embryos (day 5: a) and adult chicken (b), weakly positive cells (possibly lymphocytes) being present within the columnar epithelial lining, of CG-14-specific binding sites in stromal cells in the lamina propria in sections from embryos (day 16: c) and adult chicken (d), of CG-14 in intestinal epithelial cells of embryonic (day 20: e) and adult chicken (f), and of CG-16 in columnar epithelial cells of the adult intestine, positive cells being present in the stroma of the villi (possibly fibroblasts) (g). Preadsorption of the antibody fraction by an excess of purified antigen (CG-16) blocks the reactivity completely (h). a-c, f-h, x 1,280; d, x 640; e, 320

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given embryonic stages in extracts of liver (Fig. 4), whereas a CG-14-positive band at 42 kDa was only detectable in extracts of adult intestine (Fig. 5). Interestingly, the staining intensity of a band at 53 kDa decreased with maturation (Fig. 5), although the overall ligand abundance progressively increased (Table 2). Since not only proteins, but also lipids are carrier backbones of glycan chains, the potential of neutral glycolipids and gangliosides to serve as galectin ligands warranted further study. Again, the inherent limitations of the assay are to be considered.

In accordance with previous observations (Solomon et al., 1991), the immobilization of glycolipids with short sugar chains to the surface of microtiter plate wells reduced their accessibility. Thus, e.g. N-linked glycans

of asialofetuin were readily bound by the galectins, whereas lactosylceramide or ganglioside GM₁ failed to be reactive even at a quantity of 10 μ g glycolipid per assay. Similar to the assays with glycoproteins, the disparate size of the galectins can further influence the actual result, the tetrameric mistletoe lectin only producing very weak responses. The measurements with the galectins provided an indication for an increased level of accessible *in vitro* ligands at certain embryonic stages for both galectins relative to the adult organ (Fig. 6A,B). Moreover, differential binding preferences towards the tested fractions could be inferred, when comparing results of determinations with aliquots of extracts using CG-14 and CG-16, as indicated by letters in Fig. 6A,B. Similar to the quantitative evaluation of



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glycoprotein *in vitro* ligands (Table 2) reduction in content for one galectin would not translate into a concomitant decrease of ligands, supporting the notion for overlapping ligand populations. In that case, the patterns of localization of the individual galectins and of galectin-reactive sites in tissue sections are expected to display similarities. As a control marker, the mistletoe lectin with defined differences in ligand recognition was tested.

Paraffin-embedded sections of the two organs at different stages of development were systematically monitored for lectin-binding properties. Staining of plant agglutinin-specific sites in the liver yielded a characteristic binding pattern which only changed in intensity during pre- and post-hatching development. The plasma membrane of hepatocytes, especially those membrane regions forming the bile canaliculi, was prominent with peak intensity on day 14 post-hatching (Fig. 7a-d). Kit reagents failed to contribute to this reaction (Fig. 7e). Lectin binding was abolished in the presence of 0.1M lactose (Fig. 7f), as was likewise noted in controls for the positivity of stromal cells in the lamina propria of the intestinal mucosa, which is illustrated in Fig. 8a,b. Additionally, connective tissue elements and dispersed lymphocytes in the epithelium were positive. Apical staining of the epithelium was confined to developmental stages around the time of hatching. Intestinal tissue was primarily positive for CG-14 under the given conditions. Biotinylated CG-14 bound to the epithelium at early stages of embryogenesis with a marked labeling of the Golgi area. Stromal cells of the lamina propria were distinctly reactive (Fig. 8c). From day 16 onwards the staining intensity of this compartment, which was also positive immunohistochemically, leveled off. For stromal cells and muscle cells of the lamina muscularis mucosae and tunica muscularis a tendency for increased staining intensity was detectable (Fig. 8d). Intestinal epithelial cells reacted immunohistochemically with the antibody fractions against both galectins, as illustrated for CG-14 presence in intestinal epithelial cells (Fig. 8e,f) and for CG-16, which was only detectable in small amounts in tissue extracts of adult intestine (Fig. 8g). Preadsorption of each antibody fraction with purified galectin completely abolished the immunoreactivity (Fig. 8h). Compared to CG-14 and in line with the determined tissue contents staining intensity was generally rather weak including the tunica muscularis and the lamina muscularis mucosae.

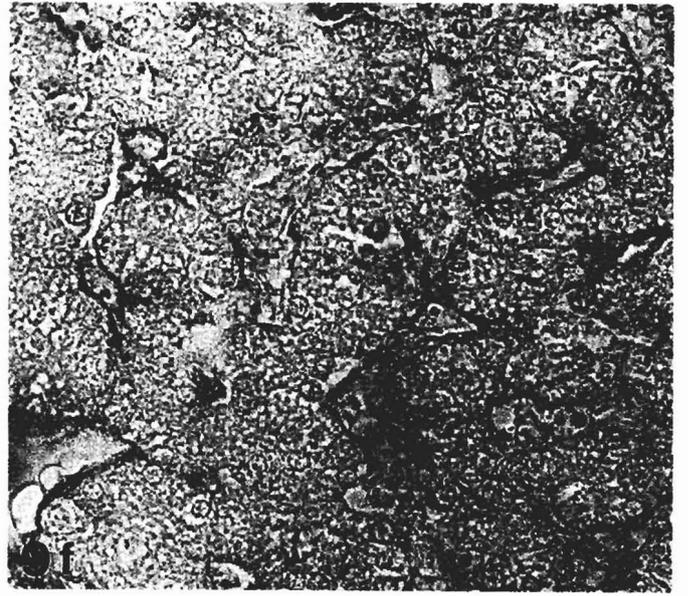
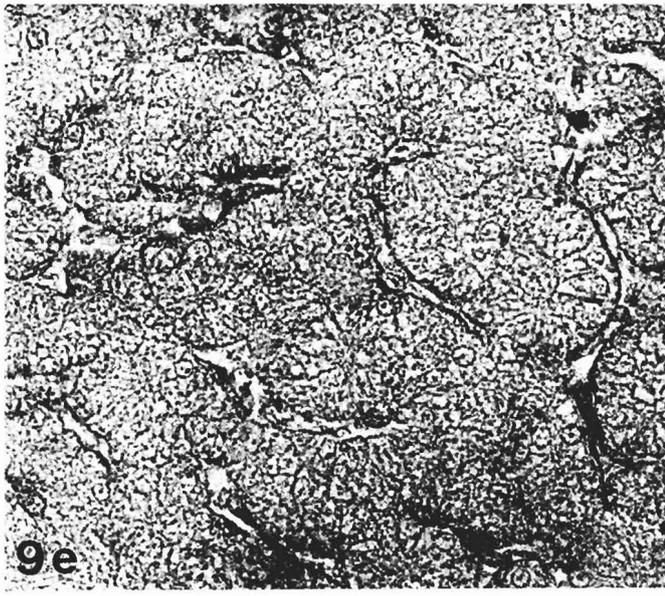
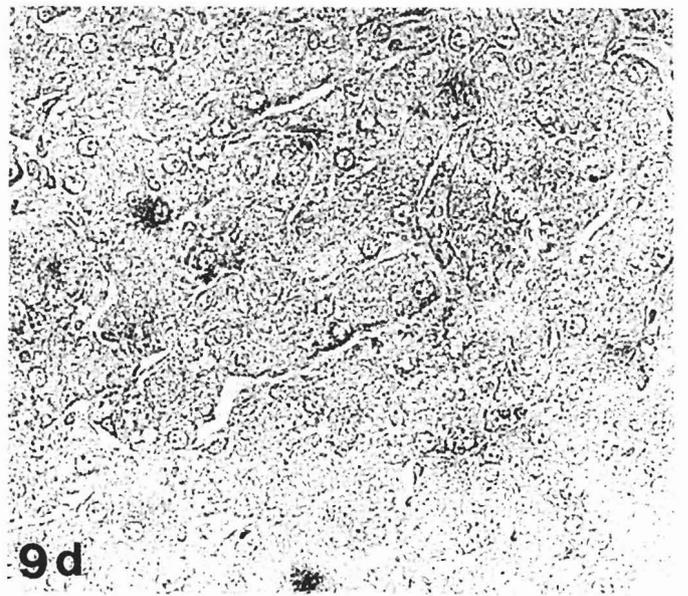
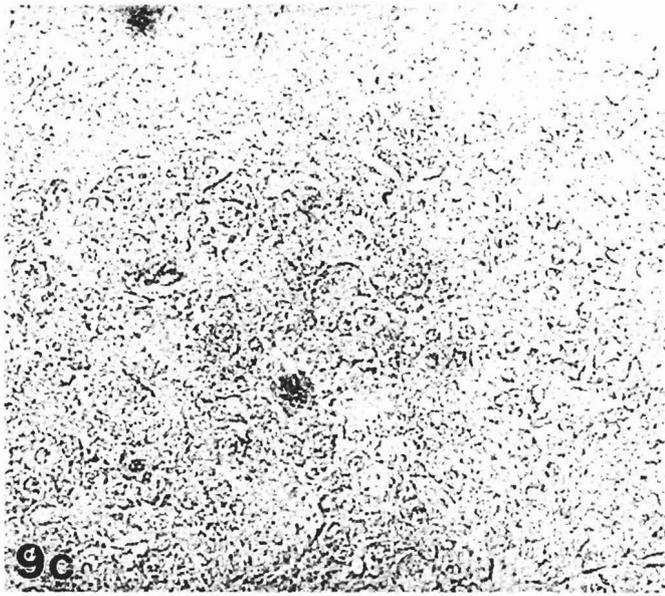
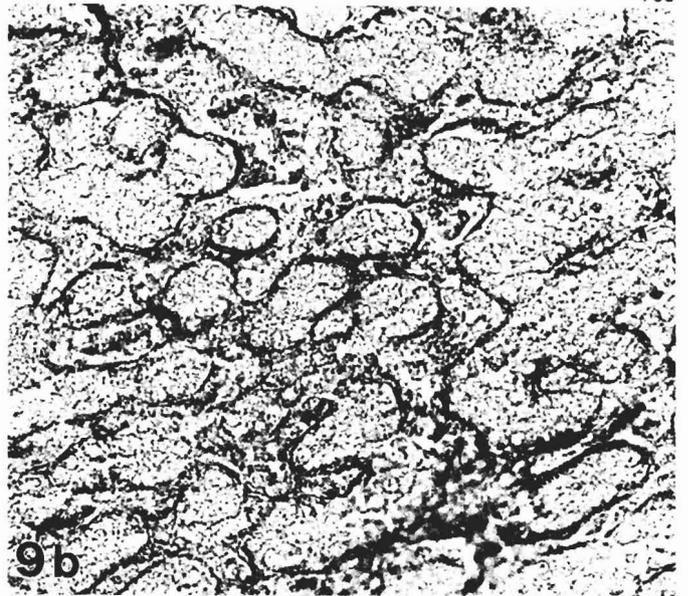
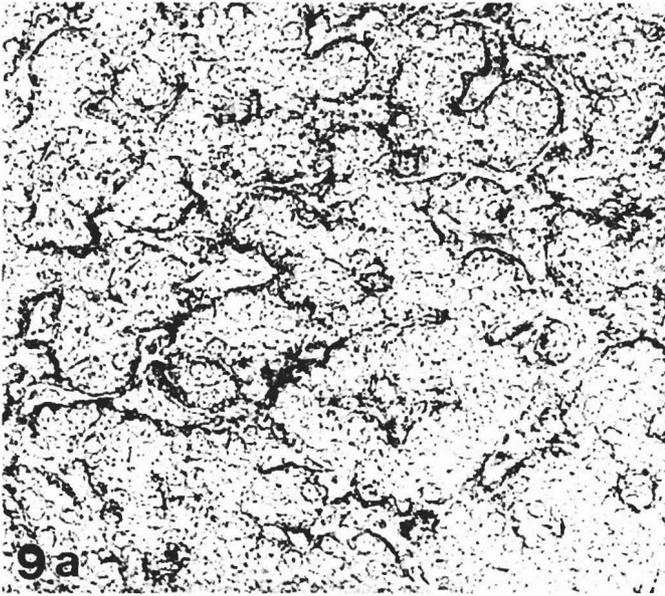
The changes of ELISA-detectable galectin contents in liver were not as pronounced as those seen in intestine (Table 1). The course of expression of *in vitro* ligands was comparable in liver (Table 2). The binding patterns

of the two galectins in liver were nearly indistinguishable with marked labeling of endothelial cells (Fig. 9a,b). Similar to the plant agglutinin, staining was due to carbohydrate-mediated binding (Fig. 9c,d). The extent of binding increased during embryonic development from day 10 (negative) to day 21 (markedly positive; Fig. 9e,f). The differences in fine specificity towards carbohydrate structures between the plant agglutinin and the two galectins, respectively, translated into different tissue-binding profiles. When comparing galectins, not only their ligand sites were similar in liver. The patterns of immunoreactivity for both galectins were comparable with positive reactions in endothelium, parenchymal cells and also erythrocytes (Fig. 10a-f). Since extensive preadsorptions of the immunoglobulin G preparations with the respective galectin failed to abrogate the positivity of erythrocytes, this staining cannot reliably be considered to be indicative of galectin presence.

Discussion

A key to the individual monitoring of related proteins is the availability of non-cross-reactive antibodies. As similarly reported by independent groups (Beyer and Barondes, 1982a; Hirabayashi et al., 1987; Levi and Teichberg, 1989; Castagna and Landa, 1994), immunoglobulin fractions with target specificity in intergalectin analysis could be obtained. Due to the presence of more than one galectin in the extracts, which could be fractionated by two-step chromatography, ELISA measurements with tools devoid of cross-reactivity are indispensable for quantitation. Hemagglutination assays will inevitably only provide a global result for all galectin activities present. In addition to presenting a detailed developmental course of the expression of the two galectins in intestine and liver our results substantiated that CG-14 can be more abundantly expressed in chicken liver, as originally reported (Beyer and Barondes, 1982a). Remarkably, their profiles of immunohistochemical localization were nearly indistinguishable in the liver. While an overlap of galectin expression, albeit less pronounced than in liver, was seen for intestine despite the low level of CG-16 expression in adult chicken in agreement with previous reports (Beyer et al., 1979; Beyer and Barondes, 1982b), a veritable preference was notable for CG-14 with respect to localization of galectin-reactive sites in intestine. Corroborating the results of the two types of solid-phase assays, it can be assumed that the size difference of the galectins can have an impact on the extent of ligand localization. Monomeric proteins will be more suitable to gain access to a binding partner despite the presence of spatial impediments compared to dimeric

Fig. 9. Localization of specific binding sites for CG-14 (a) and CG-16 (b) in endothelial cells of sections of adult liver. Presence of a mixture of glyco-inhibitors (0.2M lactose and 0.65 mg/ml asialofetuin) abolishes the binding reaction (c) and omission of the incubation step with a biotinylated galectin yields no staining reaction (d). Localization of reactivity for biotinylated CG-14 during development from day 18 (e) to day 21 (f). a-d, x 320; e, f, x 640



receptors.

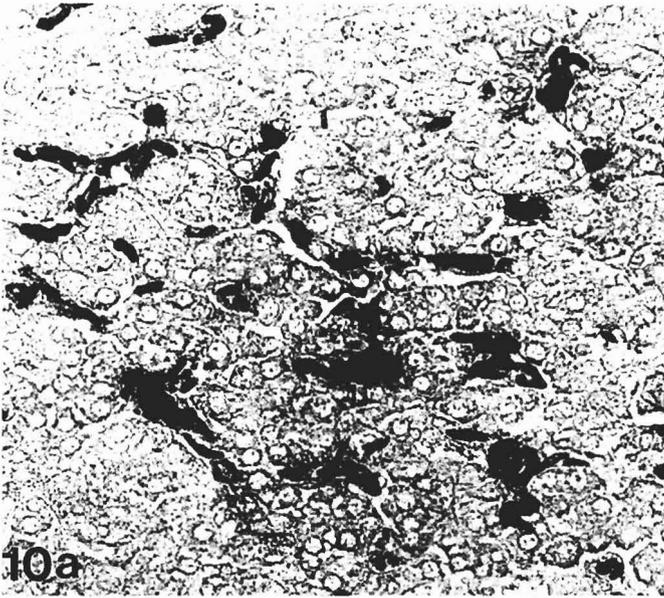
The arrangement of the carbohydrate recognition domains on opposite sites of non-covalently linked dimers, however, is the prerequisite for a crosslinking activity, which is probably indispensable for signal elicitation (Villalobo and Gabius, 1998). Participation of human galectin-1 in growth control of cultured neuroblastoma cells is a graphic example of how a cross-linking galectin can partake in regulation of proliferation (Kopitz et al., 1998). Besides, a ganglioside glycans of glycoproteins can serve as ligands. If their synthesis is impaired, serious consequences such as early death and dysregulation in epithelial cells can ensue, detectable in experimental models with ablated β 1,4-galactosyl-transferase activity (Asano et al., 1997; Lu et al., 1997). It can thus be presumed that the two galectins will differ in their signal-inducing capacity. In line with reduced capacity for hemagglutination, CG-14 lacked the inhibitory effectiveness of CG-16 on stimulated T cells (Schneller et al., 1995). On the other hand, CG-16, which is known as a potent cross-linking agent, was capable of triggering various cellular responses, i.e. promotion of cartilage differentiation *in vitro*, inhibition of lymphocyte proliferation and induction of apoptosis (Matsutani and Yamagata, 1982; Schneller et al., 1995; Gupta et al., 1996; Rabinovich et al., 1997). Histochemical scrutiny of early avian embryogenesis further supported this notion, CG-16 presence being transiently measurable in connection with proliferative activity and/or a phase of differentiation (Levi and Teichberg, 1989). Since these parameters are reflected in morphometric features and derivatives such as structural entropy, which can be readily calculated (Kayser and Gabius, 1997; Kayser et al., 1997), detailed combined studies will be conducive to delineate the actual extent of functional correlation. Moreover, bivalency of a lectin is a crucial factor for bridging mechanisms, which guide e.g. ordered cell migration in discoidin I-expressing slime molds (Gabius et al., 1985). CG-16 presence in most cells of the epiblast and hypoblast in the region of the primitive streak and in migrating cells during gastrulation and the inhibition of primitive streak formation by antibody or glycosubstances implicated this galectin in early organogenesis (Levi and Teichberg, 1989; Jeeva and Zalik, 1996). As attested by this report, later developmental stages were characterized by a coordinated expression of the two galectins. The inherent complexity of established organ-specific control mechanisms for the two galectins is also striking in dermis and epidermis, where CG-14 and CG-16 expression patterns are obviously regulated non-uniformly (Akimoto et al., 1995).

When viewing ligand abundance *in vitro* and galectin quantity, it is remarkable that the total amount

of binding sites nearly dovetailed with the sum of presence of both galectins. The gradual increases with organ maturation intimate a role in structural organization of the two compound classes. In mammalian development, only data for the course of expression of galectin-1-reactive sites are available, pointing to an organ-specific pattern (Kaltner et al., 1997). To obtain a complete picture on mammalian glycoconjugate synthesis comprising any galectin-reactive populations, the presence of further galectins will need to be assessed. To substantiate the concept of assumed functional redundancy by various galectins, as implied by only very restricted aberrations in the olfactory bulb in otherwise normally developing mice carrying a null mutation in the galectin-1 gene (Poirier and Robertson, 1993; Colnot et al., 1997; Key and Puche, 1997), this line of research employing the array of tissue galectins as tools is a promising approach. In this setting, the possibility of compensating functionality involving upregulation of individual galectins or functionally equivalent proteins in the experimental models should be considered, alluding to the thoughtful discussion on the caveats within interpretation of data from experiments with KO mutants (Hynes, 1996). Since tight association with *in vivo* ligands may hamper or even preclude purification of certain galectins by standard procedures presupposing solubility, these factors should not be neglected to avoid a flawed conclusion. It is thus pertinent to refer to the recent description of a cDNA with a galectin-3-like sequence which is upregulated in chondrocyte hypertrophy, raising the possibility for a further extension of the avian galectin family at least in this cell type (Nurminskaya and Linsenmayer, 1996).

Conceptually, the foregoing discussion about distinct binding partners for galactoside-presenting glycan chains underscores a salient aspect. Detailed biochemical analyses with a panel of ligand derivatives have indicated that the identity in nominal monosaccharide specificity for galectins and plant agglutinins will not necessarily entail an identical fine specificity (Lee et al., 1992; Solís et al., 1996; Galanina et al., 1997). The differences in the binding site architecture can extend or even lead to the selection of distinct conformers of an oligosaccharide, further separating the *in vivo* ligand profiles of plant and animal lectins (Siebert et al., 1996; Gabius, 1998; Gilleron et al., 1998; von der Lieth et al., 1998). It is expedient to appreciate that the biochemical disparities will automatically translate into distinct histochemical results (Gabius et al., 1992; Brinck et al., 1995, 1996; Kaltner et al., 1997). Under these circumstances, the inevitable limitations of the monitoring of glycoconjugate expression with exogenous lectins are readily apparent. The employment

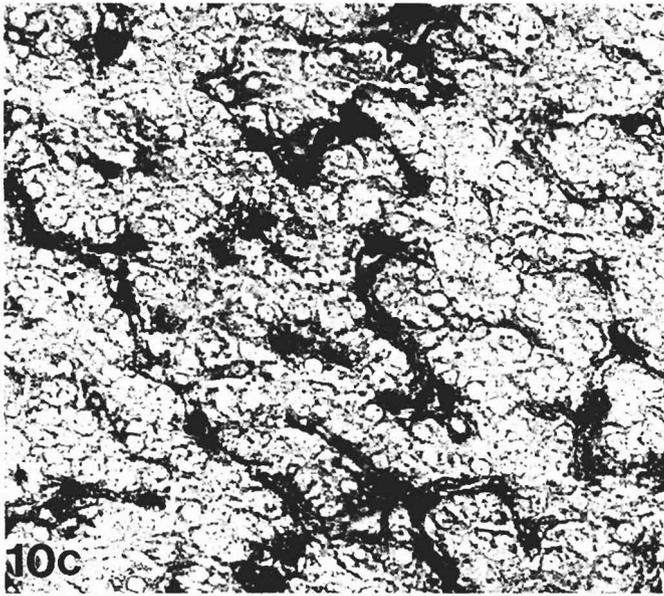
Fig. 10. Immunohistochemical localization of CG-14 in sections of livers 14 days (a), 12 days (b), and 18 days post hatching (c) and from adult chicken (d) with occurrence of distinct staining of the endothelium, and immunohistochemical localization of CG-16 in sections of livers 7 days post hatching (e) and from adult chicken (f), yielding a similar pattern relative to that of CG-14 presence. a, c-f, x 320; b, x 640



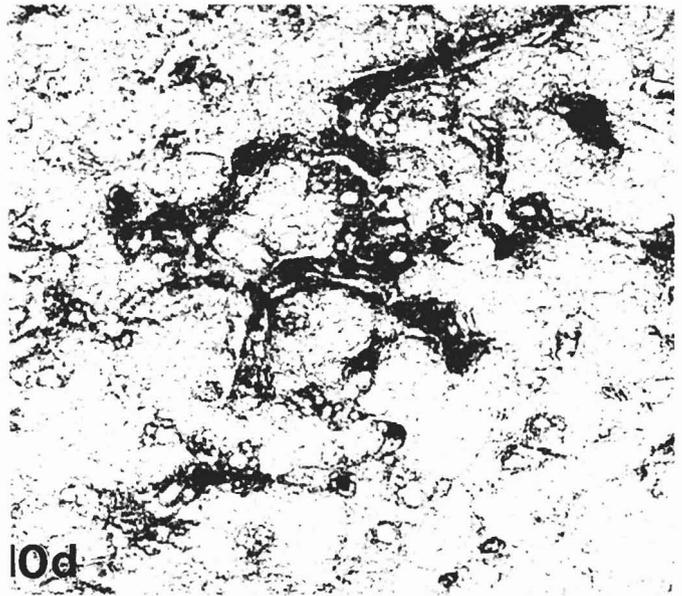
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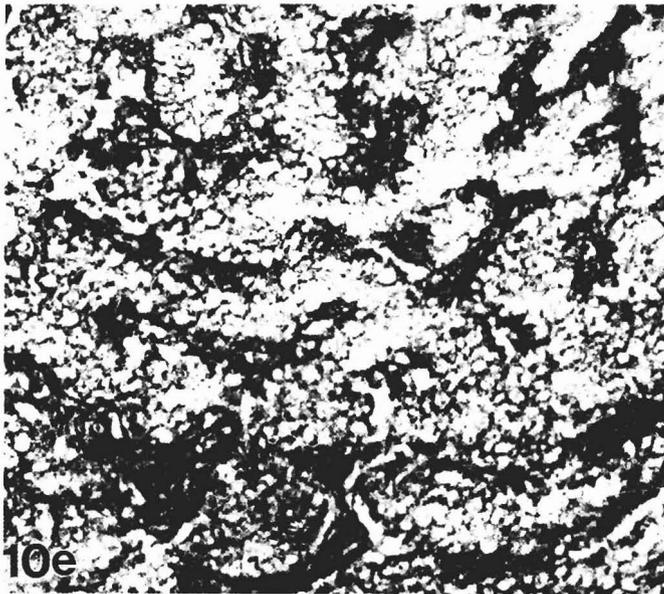
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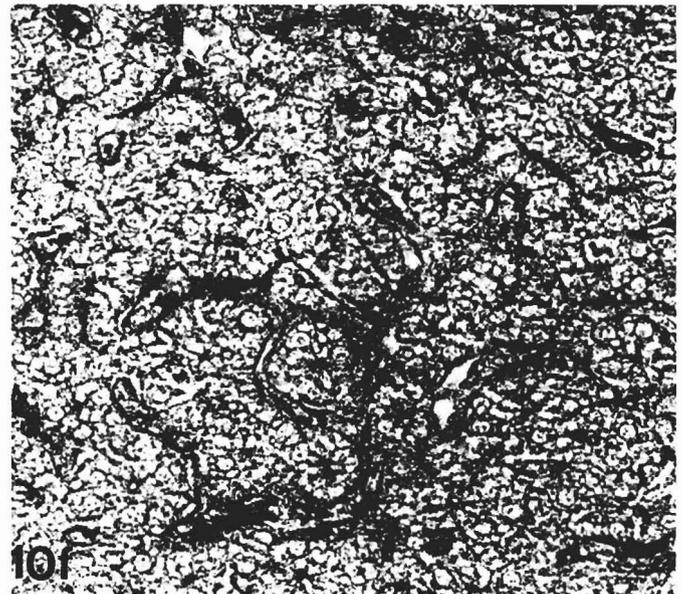
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10f

of tissue lectins in efforts to delineate functionally relevant alterations by monitoring of glycoconjugate expression is strongly advocated.

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