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# Multiple proteins related to the soluble galactose-binding animal lectin revealed by a monoclonal anti-lectin antibody

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A monoclonal antibody (NIBy 142-36/8) raised against the soluble galactose-binding lectin of bovine heart muscle has been tested by solid-phase vinyl-plate radiobinding and nitrocellulose immunoblotting with homogenates of various bovine tissues, and the muscle tissues of pig, rabbit, chicken and rat. Muscle lectins of chicken, rabbit and rat differed from those of man and pig in their lack of reactivity with the 36/8 antibody. There was a good correlation of haemagglutinating activities and immunoreactivities of the bovine tissue homogenates, suggesting that the soluble galactose-binding protein is a major haemagglutinin in various tissues. Immunoblotting experiments revealed an array of antigenically active components in the homogenates in addition to the 13 and 26kDa proteins that were previously detected in preparations of purified lectin. These were in the range 36kDa to more than 200kDa, and a different spectrum of immunoreactive components was found in various cell types. Galactose-binding activity was demonstrable in 13, 26 and 36kDa components in certain bovine tissues, suggesting that the immunoreactive components of higher $M_r$ may be inactive precursor forms of the lectin.

A class of soluble lectins with galactose-binding specificity has been isolated from tissues of several animal species, including the electric eel, cow, chicken, rat, rabbit and man (reviewed by Barondes, 1984). The amount of extractable lectin varies from tissue to tissue, and is developmentally regulated in certain animal species. These proteins have usually been isolated in the mono-, di- or oligo-meric state with subunits in the range of 13–17kDa (de Waard *et al.*, 1976; Childs & Feizi, 1979; Beyer *et al.*, 1980; Oda & Kasai, 1983). In preparations of lectin isolated from bovine and primate tissues by ligand affinity chromatography, three minor protein components of approx. 27, 54 and 68kDa were detected by SDS/polyacrylamide-gel electrophoresis under dissociating conditions (Childs & Feizi, 1979), although their relationship with the predominant 13kDa protein was not investigated. From bovine lung, a 29kDa lectin was recently isolated (Cerra *et al.*, 1984) in addition to the major 14.5kDa lectin; these proteins were found to be antigenically related and to have partially overlapping peptide maps.

We have raised two rat hybridoma antibodies that recognize the 13kDa lectin of bovine heart muscle and have shown that they also react with the muscle lectins of man and rhesus monkey (Carding *et al.*, 1984). These antibodies are apparently directed at epitopes that are distinct from the carbohydrate-binding site of the lectin and thus would not be masked in the presence of lectin receptors. The antigenic determinants are unaffected by boiling the lectin in SDS and 2-mercaptoethanol before electrophoresis in polyacrylamide gels. These antibodies are therefore ideal reagents for detecting and quantifying the protein in unfractionated tissue homogenates and their electrophoretically separated components. In the present studies we have used one of these hybridoma antibodies, NIBy 142-36/8, in immunoblotting experiments and solid-phase radiobinding assays with several bovine tissues and the cardiac and skeletal-muscle tissues of pig, rabbit, rat and the chicken. We report here the occurrence of different levels of the 13kDa component in the

Abbreviations used: SDS, sodium dodecyl sulphate; PMSF, phenylmethanesulphonyl fluoride.

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tissues examined and the presence of several antigenically cross-reactive components of high apparent $M_r$ (in the range of 26000 to more than 200000) that are represented in various amounts in the different tissues.

## Experimental

### *Chemicals*

$Na^{125}I$ was purchased from Amersham International, Amersham, Bucks., U.K. Microtitre U-well plates were from Dynatech Laboratories, Billingshurst, West Sussex, U.K. Nitrocellulose membrane filters (0.2 $\mu$m pore size) were from Schleicher and Schüll, Dassal, Germany.

### *Tissue homogenates*

Bovine and pig tissues were obtained from a local abattoir and skeletal and cardiac muscles of rabbit, chicken and rat were provided by the Division of Comparative Medicine at the Clinical Research Centre. The tissues were stored at −70°C immediately after removal until used (up to 4 weeks). Frozen tissues (1 g) were homogenized with four parts (w/v) of 150mM-NaCl/0.02% (w/v) $NaN_3$/10mM-$NH_4HCO_3$/10mM-2-mercaptoethanol/2mM-EDTA/0.2M-lactose/2mM-PMSF at maximum speed for 45s in an MSE Atomix homogenizer. The pancreas tissue homogenate was prepared with additional proteinase inhibitors, benzamidine (25mM) and aprotinin (0.055 trypsin-inhibitory units·$ml^{-1}$). The homogenates were filtered through a single thickness of gauze and centrifuged at 2000*g* for 60min at 4°C, and the supernatants were assayed for protein content (Lowry *et al.*, 1951) and stored at −70°C until used.

Lectin haemagglutinating activity in the supernatants of the tissue homogenates was determined after dialysing against 150mM-NaCl/10mM-$NH_4CO_3$/1mM-dithiothreitol/0.02% (w/v) $NaN_3$ (to remove lactose), trypsin-treated rabbit erythrocytes being used as described previously by Childs & Feizi (1979) and summarized in legend to Fig. 1. Haemagglutination was inhibited by 0.2M-lactose in every case. The lectin of bovine heart muscle was that isolated previously (Carding *et al.*, 1984).

### *Monoclonal antibodies*

The rat hybridoma IgM antibody, designated 'NIBy 142-36/8' (abbreviated to '36/8'), which recognizes the $\beta$-galactoside-binding lectin of bovine heart muscle, has been described previously (Carding *et al.*, 1984). IgM-rich fractions of immune ascites and of 5-fold-concentrated (by Amicon cell filtration) tissue-culture supernatant from 36/8 hybridoma cells were prepared by chromatography on a column of Sephadex G-200 equilibrated with phosphate-buffered saline (150mM-NaCl/0.02% $NaN_3$/22mM-sodium phosphate buffer, pH7.0), containing 0.1M-glycine and 0.02% (w/v) $NaN_3$, as described previously (Carding *et al.*, 1984). As controls, IgM-rich fraction of rat ascites containing the IgM immunocytoma IR-202 (Lou rat origin), kindly provided by Dr. H. Bazin, Faculty of Medicine, Experimental Immunology Unit, University of Louvain, Brussels, Belgium, was used as well as the 'IgM-rich' fraction of tissue-culture medium containing 10% (v/v) foetal-calf serum.

### *Solid-phase radiobinding assay for lectin content of tissue homogenate*

Serial dilutions of tissue homogenate supernatants were used to coat the wells of vinyl microtitre plates in a solid-phase radiobinding assay performed as described previously (Carding *et al.*, 1984). After incubation with the IgM-rich fraction of 36/8 or control immunocytoma IR-202 ascites, used at a concentration of 1 $\mu$g/ml, bound antibody was detected by using $^{125}I$-labelled (Greenwood *et al.*, 1963) sheep antibodies against $F(ab')_2$ fragments of rat immunoglobulins (sp. radioactivity $10^6$ c.p.m./$\mu$g).

### *SDS/polyacrylamide-gel electrophoresis and immunoblotting*

SDS/polyacrylamide-gel electrophoresis of purified lectin and supernatants of tissue homogenates was performed in 4–16%-(w/v)-polyacrylamide gradient slab gels with the buffer systems described by Laemmli (1970). Electrotransfer of proteins from gels on to nitrocellulose sheets was performed at 4°C for 18h at 30V, and immunostaining, with IgM-rich fraction of 36/8 ascites and tissue-culture supernatant and, as controls, IgM-rich fractions of immunocytoma IR-202 and of a 1mg/ml concentration of tissue-culture medium containing 10% (w/v) foetal-calf serum, was carried out by using the conditions described previously (Carding *et al.*, 1984).

### *Overlay with $^{125}I$-labelled glycoproteins*

Purified lectin and tissue homogenate supernatants were alkylated and electrophoresed on SDS/polyacrylamide gradient slab gels and then electrotransferred on to nitrocellulose paper as described previously (Carding *et al.*, 1984). The nitrocellulose sheets were incubated in 0.5M-NaCl/20mM-sodium phosphate buffer, pH7.5, containing 3% (w/v) bovine serum albumin at 37°C for 60min. The sheets were then overlayed at 20°C for 6h with $^{125}I$-labelled sheep gastric glycoproteins (Wood *et al.*, 1979) containing $6 \times 10^6$ c.p.m. of $^{125}I/\mu g$, enriched for terminal galactose residues

by adsorption to (at 4°C), and elution from (at 37°C), an immunoadsorbent column made with anti-I [(designated 'Step') (Feizi & Kabat, 1974)]. For each sample track (12cm × 0.5cm), $10^6$ c.p.m. of labelled glycoprotein was included in 100ml of 20mM-sodium phosphate buffer, pH7.5, containing 0.5M-NaCl and 5% (w/v) bovine serum albumin (Solution A). In duplicate experiments the radiolabelled glycoproteins were added in the presence of 0.2M-lactose. The nitrocellulose sheets were washed eight times with Solution A and then subjected to autoradiography as described previously (Carding *et al.*, 1984).

## Results

### *Haemagglutinating and immunological reactivities of bovine tissue homogenate supernatants*

Preliminary experiments showed that lectin haemagglutinating activity in muscle homogenate supernatants was 2–8-fold higher and immunoreactivities with 36/8 antibody were stronger when the homogenates were obtained in the presence of 0.2M-lactose (results not shown). Therefore all subsequent experiments were performed with homogenates prepared in the presence of lactose and dialysed to remove lactose before haemagglutination assays and immunoblotting.

There were considerable differences in the lactose-inhibitable haemagglutinating activities of the supernatants of the bovine tissues tested (Fig. 1*a*). The highest activities per unit weight of protein were in skeletal muscle and omasum; intermediate levels were detected in the heart, lung, abomasum and spleen, and low levels were detected in the kidney, small intestine, pancreas and liver. There was a good correlation between the haemagglutinating activities of the tissue supernatants, their reactivities in solid-phase radiobinding assays (Fig. 1) and their detection by immunoblotting (Fig. 2 below).

### *Species differences in the haemagglutinating activities and immunoreactivities in supernatants of muscle homogenates*

Fig. 1(*b*) shows that there were differences not only in the haemagglutinating activities in the skeletal- and heart-muscle homogenates in the animals tested, but also that the relative activities of the two types of muscle differed in each animal. Thus, with the bovine muscles, which contained the highest haemagglutinating activities, the skeletal-muscle homogenate was approximately six times more active per unit weight of protein than the heart homogenate (0.3 μg and 2 μg of supernatant protein/ml gave haemagglutination). The pig muscle homogenates contained intermediate haemagglutinating activities and the levels were comparable in the two muscles. In contrast, the chicken and rabbit skeletal-muscle homogenates had substantially lower haemagglutinating levels than had the heart homogenates.

Among the muscle homogenates tested for immunoreactivity in solid-phase radiobinding assays, only those of the cow and pig showed reactivity with 36/8 antibody that corresponded to lectin and related proteins (see below). Despite the presence of considerable haemagglutinating activities in the heart muscle preparations of the chicken and rabbit, there was negligible immunoreactivity in the solid-phase assays. The immunoreactivity detected in the solid-phase radiobinding assay with the rat skeletal-muscle homogenate was found, in separate experiments (results not shown), to represent a reaction of the $^{125}$I-labelled anti-[rat(Fab′)$_2$] with immunoglobulins in the homogenate.

### *Multiple immunoreactive components revealed by immunoblotting of tissue homogenates with anti-lectin antibody 36/8*

Fig. 2 shows an autoradiograph of homogenates of various bovine tissues, and the muscle homogenates of cow, rabbit, pig and chicken, after immunostaining with 36/8 antibody. Almost identical results were obtained whenever the IgM-rich fraction of ascites or tissue-culture supernatant containing this antibody was used. In addition to the 13 and 26kDa components previously identified, multiple immunoreactive bands of varying intensities were revealed in the range 13 to more than 200kDa. A differing spectrum of immunoreactive bands was detected in various cell types, and these are summarized in Table 1.

Among the preparations tested, all except the muscle homogenates of the rabbit, rat and chicken showed an immunoreactive component of approx. 13kDa. This gave a very strongly stained band in the bovine tissues examined, except in the kidney, pancreas and liver, where it was only faintly immunostained. In bovine brain and spinal-cord homogenates, a prominent 18kDa component was revealed. Either a 26 or 36kDa component was prominent in small intestine, omasum, abomasum, skeletal muscle and spleen, suggesting that the two components may be alternative forms. Two bands, one of 180kDa and another of more than 200kDa, were prominent in the brain. The other immunoreactive bands (shown as dotted lines on Fig. 2) were minor components revealed with the 36/8 culture supernatant and ascites, but not with control monoclonal antibody.

Of these *additional* immunoreactive bands, only the 80kDa and 130kDa components were weakly

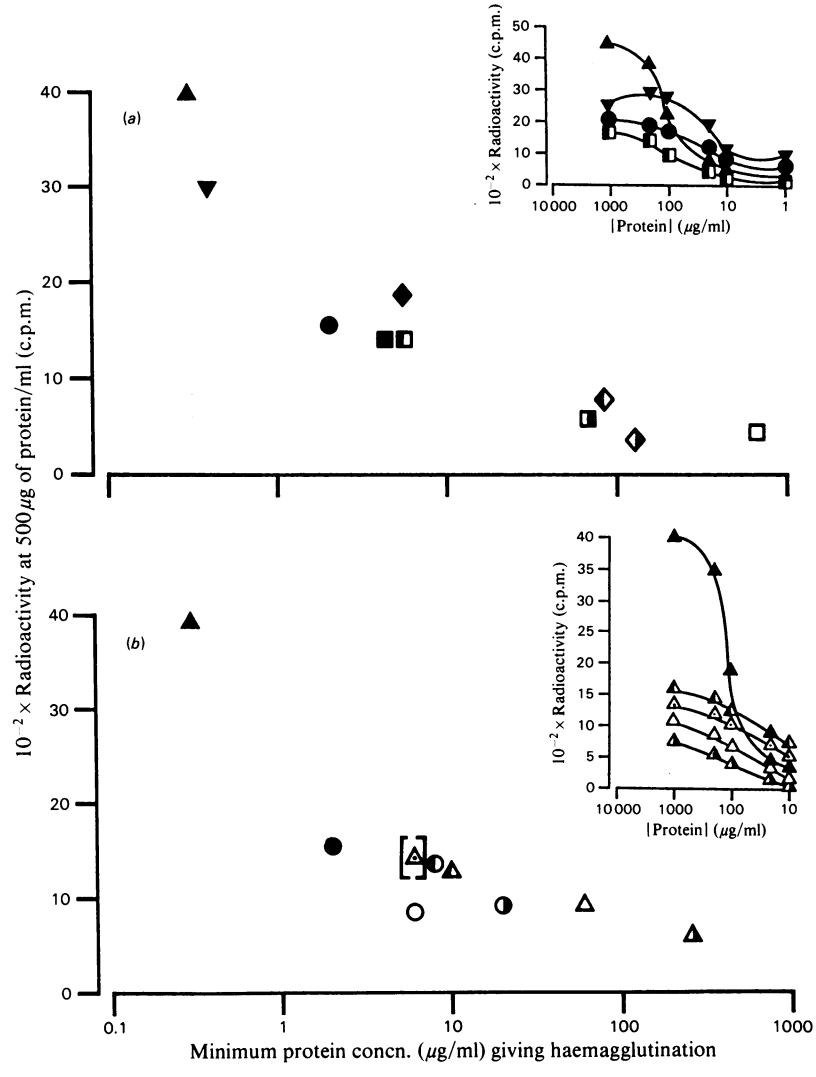


Fig. 1. *Lectin haemagglutinating activities and immunoreactivity with monoclonal antibody 36/8 detected in the supernatants of homogenates of various bovine tissues (a) and muscle tissues of several animal species (b)*

Serial dilutions of the supernatants of homogenates of the various tissues were tested for haemagglutination of trypsin-treated rabbit erythrocytes or for binding to the IgM-rich fraction of 36/8 ascites in solid-phase radiobinding immunoassays as described in the Experimental section. The results are expressed as the minimum concentration of supernatant protein giving haemagglutination and radioactivity bound when 500 μg of protein/ml was applied to plastic wells. Insets show the binding curves of selected supernatants. Symbols in (*a*) for bovine tissues: ▲, skeletal muscle; ●, heart; ▼, omasum; ◆, abomasum; ◇, pancreas; ◈, small intestine; ◧, spleen; ◨, kidney; □, liver; ■, lung. Symbols in (*b*): skeletal-muscle and heart homogenates respectively of the following sources: ▲, ●, bovine; ◭, ◐, pig; △, ◑, rabbit; △, ○, chicken; ◬, rat skeletal muscle (the square brackets indicate that immunoreactivity in the rat skeletal muscle is unrelated to lectin but due to the reaction of the $^{125}$I-labelled anti-[rat F(ab')$_2$] antibody with the rat immunoglobulins).

immunostained in affinity-purified preparations of bovine heart lectin (Fig. 2).

*Reactivities of tissue homogenates with $^{125}$I-labelled sheep glycoprotein*

In order to investigate whether the various immunoreactive components represent proteins with galactose-binding specificity, $^{125}$I-labelled sheep gastric glycoproteins were applied to nitrocellulose sheets containing electroblotted purified lectins of bovine heart and skeletal muscles, or various tissue homogenate supernatants (as indicated in Table 1). Initial experiments showed that all the samples tested strongly bound the radiola-

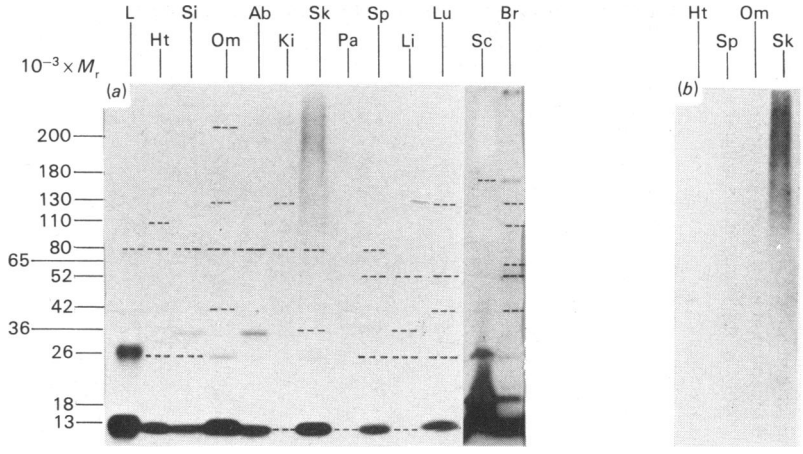


Fig. 2. *Autoradiograph showing immunostaining of purified lectin, and homogenates of bovine tissues*
Isolated lectin (1 μg) from bovine heart muscle and supernatants of homogenates (100 μg of protein) of various bovine tissues were electrophoresed on SDS/polyacrylamide-gradient gels, electrophoretically transferred on to nitrocellulose sheets and immunostained with either the IgM-rich fraction of 36/8 antibody (*a*) or, as a control, with the IgM-rich fraction of rat ascites containing the immunocytoma protein IR-202 (*b*) followed by $^{125}$I-labelled anti-[rat F(ab′)$_2$] (as described in the Experimental section). Faintly immunostained bands are indicated by broken lines. Non-specific interaction of $^{125}$I-labelled anti-[rat F(ab′)$_2$] with skeletal-muscle homogenate seen as 'smear' with 36/8 and control antibody. Symbols for tissues: L, lectin; Ht, heart; Si, small intestine; Om, omasum; Ab, abomasum; Ki, kidney; Sk, skeletal muscle; Pa, pancreas; Sp, spleen; Li, liver; Lu, lung; Sc, spinal cord; Br, brain.

belled glycoproteins when they were applied on to nitrocellulose after alkylation, boiling in SDS and incubation in the electrotransfer buffer (without electrophoresis) as spots containing up to 10 μg of protein (results not shown). However, if the samples were electrophoresed and electrotransferred on to nitrocellulose, only some weak bands of reactivity were revealed with some of the tissue homogenates, and none with the purified lectins. Fig. 3 shows examples of the radioactive bands revealed after 28 days of autoradiography. Such bands of radioactivity were obtained with the bovine skeletal muscle and pig heart homogenates at 13 kDa, with the bovine and pig skeletal muscle homogenates at 26 kDa, and with the bovine skeletal-muscle homogenate at 36 kDa. These corresponded to the immunoreactive bands obtained in these tissues when the anti-lectin monoclonal antibody, 36/8, was used (Table 1). The third glycoprotein-binding band of 18 kDa in the skeletal-muscle homogenates did not correspond to an immunostained band. Faint radioactive bands in the region of 12–13 kDa were obtained when the labelled glycoproteins were tested with bovine abomasum and small intestine. Additional bands of 18, 36, 42 and 65 kDa were revealed in bovine abomasum, pig skeletal muscle, pig heart and in bovine liver respectively, that did not correspond to immunoreactive components. All these bands were decreased in intensity or abolished in the presence of 0.2 M-lactose.

## Discussion

The immunoblotting experiments with the tissue homogenates have revealed an array of antigenically active components in addition to the 13 kDa and 26 kDa components detected in the previous study (Carding *et al.*, 1984). The relationship of these multiple components to each other remains to be elucidated. They could represent degrees of post-translational modification of a high-$M_r$ precursor form of the 13 kDa lectin. There may be processing differences of this protein in different tissues, since for example, several of the bovine tissues contained predominantly the 26 kDa or the 36 kDa component (Fig. 2). However, in certain related tissues, such as the small intestine and abomasum, where the same pattern of bands is seen, the processing may be identical. The lack of detection of immunoreactive bands in certain tissues does not necessarily imply their absence, since the monoclonal antibody recognizes a single epitope of only a few amino acids which may be 'cleaved' from certain of the processed components, or antigen expression may be masked, possibly through aggregation of subunit forms. Alternatively, the multiple immunoreactive forms may be due to the 13 kDa subunit being a part of high-$M_r$ complexes containing similar subunits or other components, possibly receptors. Biosynthetic labelling and pulse–chase experiments should resolve these questions.

Table 1. *Radioactive bands detected with 36/8 antibody and $^{125}I$-labelled glycoproteins in preparations of lectin and tissue homogenates*

Isolated lectins and supernatants of tissue homogenates were electrophoresed on SDS/polyacrylamide gels, electrophoretically transferred on to nitrocellulose sheets and immunostained with 36/8 antibody or, where indicated, overlayed with $^{125}I$-labelled sheep glycoprotein (as described in the Experimental section). Immunoreactive components are identified as: +, strongly staining; (+) weakly staining. Preparations tested for binding of the radiolabelled sheep glycoprotein are indicated with asterisks; radioactive bands obtained with the glycoprotein are indicated by the letter 'g' under the appropriate molecular-mass columns.

| Preparation | Species | Tissue | Approx. molecular mass (kDa) ... | Immunoreactive bands | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 13 | 18 | 26 | 36 | 42 | 52 | 65 | 80 | 110 | 130 | 180 | >200 |
| Isolated lectins | Bovine | Heart* | | + | | + | | | | | (+) | | (+) | | |
| | | Skeletal muscle* | | + | | + | | | | | | | | | |
| | Human | Foetal heart | | + | | (+) | | | | | | | | | |
| | | Adult heart | | + | | (+) | | | | | | | | | |
| | Rhesus monkey | Skeletal muscle | | + | | (+) | | | | | | | | | |
| Muscle homogenates | Bovine | Heart* | | + | | (+) | | | | | (+) | (+) | (+) | | |
| | | Skeletal muscle* | | +g | g | +g | (+)g | | | | (+) | | | | |
| | Pig | Heart* | | +g | | + | | g | | | + | | | | |
| | | Skeletal muscle* | | + | g | (+)g | g | | | | | | | | |
| | Rabbit | Heart | | | | | | | | | | | | | |
| | | Skeletal muscle | | | | | | | | | (+) | | | | |
| | Chicken | Heart | | | | | | | | | | | | | |
| | | Skeletal muscle | | | | | | | | | | | | | |
| | Human | Skeletal muscle | | + | | + | | | | | | | | | |
| | Rat | Skeletal muscle | | | | | | | | | | | | | |
| Tissue homogenates | Bovine | Brain | | + | + | + | | (+) | (+) | (+) | | (+) | (+) | + | + |
| | | Spinal cord | | + | + | + | | | | | | | | (+) | |
| | | Small intestine* | | +g | | (+) | + | | | | (+) | | | | |
| | | Abomasum* | | +g | g | (+) | + | | | | (+) | | | | |
| | | Omasum* | | + | | + | | (+) | | | (+) | | (+) | | (+) |
| | | Kidney | | (+) | | | | | | | (+) | | (+) | | |
| | | Pancreas | | (+) | | | | | | | | | | | |
| | | Spleen* | | + | | (+) | + | | (+) | | (+) | | | | |
| | | Liver* | | (+) | | (+) | (+) | | (+) | g | | | | | |
| | | Lung* | | + | | (+) | | (+) | (+) | | | | (+) | | |
| | Pig | Thymus | | + | | | | | | (+) | | | (+) | | |

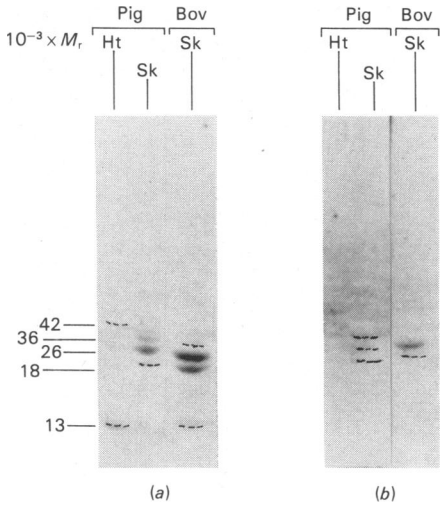


Fig. 3. *Autoradiograph showing reactivities of tissue homogenates with $^{125}$I-labelled sheep glycoprotein*
Bovine (Bov) and pig muscle homogenate supernatants (100 μg of protein) were electrophoresed on SDS/polyacrylamide gradient gels, electrophoretically transferred on to nitrocellulose and overlaid with $^{125}$I-labelled sheep glycoprotein alone (*a*) and in the presence of 0.2M-lactose (*b*) (as described in the Experimental section). Symbols used for tissues: Sk, skeletal muscle; Ht, heart. Faintly stained bands are indicated by broken lines.

In an attempt to ascertain which of the immunoreactive bands have β-galactose-binding activity, $^{125}$I-labelled sheep glycoprotein, rich in terminal galactose residues, was added to nitrocellulose strips of electrophoresed bovine and pig tissue homogenates. The finding that the predominant binding activity was with the 26kDa immunoreactive component suggests this may be the major haemagglutinating component in tissue homogenates. The lack of carbohydrate-binding activity in the higher-$M_r$ components is consistent with their being inactive precursor forms of the lectin or stable complexes of lectin associated with receptors. Alternatively, the immunoreactive forms that did not bind the glycoproteins may be antigenically cross-reactive proteins that are functionally distinct from the lectin. These possibilities also require investigation. The marked decrease in carbohydrate-binding activity in the homogenates, and the almost undetectable binding of the 13kDa subunit of the lectin in the majority of tissues examined, were not related to denaturation in SDS or the buffers used for electrophoresis and electrotransfer. Taken together, these observations suggest that the lectin may need to be in di- or oligomeric form for carbohydrate binding.

The antigenically conserved nature and the ubiquitous distribution of the lectin in diverse cell types, which is supported by immunocytochemical studies (S. Thorpe, S. Carding, T. Feizi, unpublished work) are hallmarks of a 'household protein' with an important function. The availability of monoclonal antibodies should greatly facilitate studies of the biosynthesis and processing of the lectin and provide an opportunity of isolating the various antigenically cross-reactive components in order to establish their structural relationships to the 13kDa subunit.

Since the original submission of this paper we have observed that human lymphocytes contain substantial amounts of 36, 65 and 130kDa proteins reactive with monoclonal antibody 36/8, and that the levels and patterns of expression of these proteins change markedly in transformed and mitogen-stimulated lymphocytes (Carding *et al.*, 1985).

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