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COMPARISON OF EARLY EMBRYONIC AND DIFFERENTIATING CELL SURFACES

INTERACTION OF LECTINS WITH PLASMA MEMBRANE COMPONENTS

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SUMMARY

1. Cells of the unincubated as well as those of primitive streak chick blastoderm, which are preparing for or are involved in morphogenetic movements, are agglutinated by wheat germ agglutinin, *Ricinus communis* agglutinin and concanavalin A, but not by fucose-binding protein.

2. Agglutination of these cells with soybean agglutinin occurs only after neuraminidase treatment, while that induced by concanavalin A, wheat germ and *Ricinus communis* agglutinins is not affected.

3. Trypsin treatment of blastoderm cells had no effect on lectin-mediated agglutination.

4. In contrast, cells derived from 10- and 12-day differentiating chick liver were agglutinated by wheat germ agglutinin only after trypsinization.

5. Mechanically dissociated embryonic liver cells, which are not agglutinated, bind more ^{3}H-labelled wheat germ agglutinin per cell than trypsinized cells, suggesting that during differentiation there may be a spatial reorganization of wheat germ agglutinin receptors within the plasma membrane.

6. Membranes isolated from the above cell types were examined by analytical polyacrylamide gel isoelectric focusing and, in combination with affinity chromatography using wheat germ agglutinin conjugated to agarose, membrane material in the differentiating liver membrane, which binds to this lectin, was identified.

INTRODUCTION

Lectins have been used to detect cell surface changes undergone during differentiation [1–3] and neoplastic transformation [4, 5]. In this paper we have used a range of these substances as probes in a comparative study of early chick blastoderm cells and those from differentiating embryonic tissue. The purpose of these studies was to attempt to characterize changes taking place at the cell surface during the

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initial stages of morphogenesis. We report on the differential agglutinability by wheat germ agglutinin of cells from unincubated and primitive streak chick blastoderms, as compared to those from differentiating liver. In addition, we describe the isolation of plasma membranes from early embryonic and differentiating cells and an examination of these membranes by analytical isoelectric focusing. Using the latter technique in conjunction with affinity chromatography, we have attempted to identify those membrane glycoproteins in the differentiating cell membrane which bind to wheat germ agglutinin. The results obtained so far suggest that the inability of wheat germ agglutinin to agglutinate 12-day liver cells is not due to the absence of appropriate receptors in the differentiating cell surface. Rather, a reorganization of membrane material may be taking place during subsequent stages of embryonic development.

MATERIALS AND METHODS

Chemicals

All the solutions were prepared in water which had been distilled once in a metal still followed by two distillations in glassware; all distilled water was used within 24 h of the final distillation. Chemicals were "AnalaR" grade obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., or analytical reagent grade from Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire, U.K., unless otherwise stated. Hydroxyapatite (Bio-Gel HT Control No. 10255) and agarose beads (Bio-Gel A 0.5 m 200–400 mesh Control Nos. 11607 and 11990) were purchased from Bio-Rad Laboratories Ltd., Bromley, Kent, U.K. Superbrite 150 glass beads were obtained from 3M Company, St. Paul, Minnesota, U.S.A. Bovine serum albumin prepared as a protein standard was obtained from Armour Pharmaceutical Company Ltd., Eastbourne, Sussex, U.K., and Carbowax (M_r = 15 000–20 000) from Searle Diagnostic, High Wycombe, Bucks, U.K. For the purposes of polyacrylamide gel isoelectric focusing, acrylamide (BDH Chemicals Ltd.) was recrystallized once from chloroform before use. Triton X-100 obtained from BDH Chemicals Ltd. or Sigma London Chemical Company Ltd., Surrey, U.K., suitably diluted (10–15 %, v/v) with distilled water, was passed through a mixed bed of Amberlite analytical grade resins IR-120 (H form), IR-45 (OH) to give a stock solution with a conductivity 8 μmho $\cdot$ cm^{-1} at 20 °C, pH 6.5. The actual concentration of detergent present in this solution (usually 7–10 %, v/v) following resin treatment was determined optically at 278 nm as well as chemically by a modification of the method of Stevenson [6] by Wright and Plummer [7]; the two methods were in excellent agreement.

Cells and enzyme treatments

Eggs of either white Leghorn or Rhodes light Sussex strain chickens were used. Blastoderms were obtained from unincubated as well as from 22–23 h incubated eggs. For the agglutination experiments blastoderms were staged according to Hamburger and Hamilton [8]. For experiments for plasma membrane isolation, 22–23 h blastoderms were used; previous experience showed that most blastoderms ranged between stages 4 and 5.

Early chick blastoderms were removed under sterile conditions as reported previously [9], placed in Panet and Compton saline, and either used immediately or left overnight at 15–18 °C in Panet and Compton saline with 200 μg/ml of Crystamycin

(Glaxo Laboratories, Greenford, U.K.). For experiments of membrane isolation as well as for binding of radioactive lectin, blastoderms were kept overnight in L-15 Medium (BioCult Laboratories, Glasgow, U.K.) with 200 μg/ml of Crystamycin. Blastoderms were placed in Ca^{2+}- and Mg^{2+}-free Panet and Compton Saline, pH 7.8, with 2 mM EDTA and dissociated by gentle pipetting at room temperature. Alternatively they were dissociated by gentle pipetting in the Ca^{2+}- and Mg^{2+}-free Panet and Compton saline for 10 min at the temperature of melting ice. Cells (epiblast and hypoblast cells in the case of Stage 1, while in Stage 4–5 primitive streak mesodermal cells will also be present) were then washed twice and suspended in Panet and Compton saline. Blastoderm cells dissociated in this manner aggregate and sort out [10] and readily incorporate glucosamine into glycoprotein when incubated at 37 °C (Zalik, S. E. and Cook, G. M. W., unpublished).

For the preparation of embryonic liver cells, 10- and 12-day embryos were removed in ice-cold Tyrode saline and the liver was perfused with cold Ca^{2+}- and Mg^{2+}-free Tyrode saline. Tissues were then minced in this saline and placed in the same medium containing 2 mM EDTA, pH 7.8, or 0.2 % trypsin (EC 3.4.4.4) (ETAB, CF Boehringer and Soehne, Mannheim, G. F. R.) at 37 °C for 30 min. The tissues were then washed and placed in Tyrode saline with 24 units of DNAase (EC 3.1.4.5) (Sigma DN-EP, an electrophoretically purified preparation substantially free of RNAase). Cell dissociation was accomplished by pipetting; cells (principally hepatocytes, the majority of blood cells being removed by perfusion) were then washed in Tyrode saline and suspended in the latter until used.

For neuraminidase (EC 3.2.1.18) treatment of blastoderm cell suspensions, 5 units of neuraminidase (from *Vibrio comma*, Behringwerke AG, Marburg, G.F.R.) were added to a cell suspension containing $0.5 \cdot 10^6$ cells in 2 ml Panet and Compton saline. The neuraminidase preparation used here is stated by the manufacturer to be free of protease, lecithinase C and aldolase activity. Using Azocoll (Calbiochem) as a general substrate for proteolytic activity, under the incubation conditions described here we were unable to detect proteolytic activity in 50 units of the neuraminidase preparation. Cells were incubated at 37 °C for 30 min and controls consisted of cells kept under the same conditions with the enzyme omitted. After treatment cells were washed twice in Panet and Compton saline and used directly for agglutination experiments. For trypsin treatment of mechanically dissociated blastoderm and embryonic tissue cells, a 0.05 or 0.025 % enzyme concentration in a 2 ml cell suspension was used. Cells were incubated at 37 °C for 20 min, washed in their respective DNAase-containing salines and used for the agglutination experiments.

Preparation of lectins

Concanavalin A, (3 times crystallized, carbohydrate-free, lyophilized, desalinated powder) wheat germ agglutinin (*Triticum vulgaris*) (Lot WG17) and wheat germ agglutinin conjugated to agarose were purchased from Miles Laboratories Ltd., Stoke Poges, Bucks, U.K. Samples of fucose-binding protein from *Lotus tetragonolobus* and wheat germ agglutinin, both as a native lectin and as an agarose conjugate, prepared by Miles-Yeda Ltd., Kiryat Weizmann, Rehovot, Israel, were also kindly provided by Dr. M. Rashi.

Soybean (*Glycine max*) haemagglutinin was prepared by the method of Lis et al. [11] from soybean meal (BDH Chemicals Ltd.). *Ricinus communis* agglutinin was

prepared from castor beans (Thompson and Morgan (Ipswich) Ltd., Ipswich, U.K.) using the combined affinity chromatography and gel filtration procedure of Nicolson and Blaustein [12]; the larger molecular weight fraction (designated RCA_{120}: M_r approx. 120 000) was used exclusively in the present work.

Preparation of ^{3}H-labelled wheat germ agglutinin

Wheat germ agglutinin was labelled with tritium using [^{3}H] acetic anhydride in benzene (Radiochemical Centre, Amersham, U.K., Batch 16, 500 Ci/mol), using a similar technique to that employed by Ostrouski et al. [13] for tritium labelling of proteins. The labelled wheat germ agglutinin ($1.33 \cdot 10^6$ dpm/mg protein) was found by us to be equally effective at agglutinating embryonic cells as equivalent amounts of the native lectin.

Agglutination assays and binding of radioactive wheat germ agglutinin

Agglutination assays were performed at room temperature using a 10×15 cm perspex titration tray with round bottom wells of 15 mm diameter placed in a gyratory shaker at 90–100 rev./min for 30 min at room temperature. Approximately $1 \cdot 10^5$ blastoderm cells or $2 \cdot 10^6$ liver cells were added to the wells in which the desired lectin, with or without the appropriate sugar inhibitors, was present in a total volume varying from 200 to 300 μl. Agglutination was observed under the dissecting microscope and scored from slight (+) to massive (++++) depending on the proportion of single cells present in the suspension. Some additional assays were performed using excavated slides. Cell suspensions were used with the appropriate lectin solution and the slides were gently swirled at 5-min intervals for 30 min. Agglutination scores using the latter method were similar to the ones obtained using the titration tray and were used for photographic purposes.

Attempts were made to quantify agglutination of blastoderm cells with the celloscope 401 particle counter (Lenson Instruments, Stockholm, Sweden). However, due to massive lectin-induced agglutination and the presence of yolk granules of variable sizes released by some cells, presumably as a secondary effect of lectins on the cell surface, results using this method were difficult to interpret; therefore the visual method of scoring was selected.

For the binding assays $1 \cdot 10^5$ blastoderm cells (23 h) were treated with various concentrations (10–100 μg) of ^{3}H-labelled wheat germ agglutinin in 100 μl Panet and Compton saline; to control for non-specific adsorption parallel assays were performed in the presence of 100 mM GlcNAc. Similar experiments were performed with $1 \cdot 10^6$ liver cells (EDTA- and trypsin-dissociated) from 12-day chick embryos in 100 μl Tyrode saline. Tubes were shaken as in the agglutination assays for 30 min at room temperature. 4 ml of ice-cold saline was then added, cells were collected by centrifugation at $500 \times g$ and 40 μl of 3 % (w/v) bovine serum albumin were added to each tube. The mixture was precipitated by the addition of 1 % dodecatungstophosphoric acid in 0.5 % HCl (5 ml) and collected by centrifugation. The precipitates were washed three times with 5 % (w/v) trichloroacetic acid (8 ml), dried and dissolved at 70 °C in 300 μl of 0.1 M NaOH. They were plated onto glass fiber filters and protein-bound radioactivity was determined in a Packard scintillation counter, standardized as reported previously [14]. Care was taken to control for background counts.

Preparation of plasma membranes

Plasma membranes were prepared from cell suspensions of 22–23 h incubated blastoderms and 12-day chick embryo livers (both EDTA-dissociated) by a modification of the method of Warley and Cook [15]. The membrane isolation procedure used differed from the published method [15] in only two respects. A modified harvesting solution containing 101 mM NaCl, 50 mM borate, 2.0 mM $MgCl_2$ and 2.0 mM $CaCl_2$, pH 7.2, which is isotonic for early embryonic cells [16], was used. The membrane-enriched fraction was passed through a column composed of Superbrite 150/Ballotini No. 12 beads (1 : 1, v/v) as a means of economy since the former beads are no longer manufactured.

In a few experiments brain plasma membranes from 12-day chick embryos were prepared. The tissues used included the cerebral hemispheres, the diencephalon with the exception of the optic cups, the mesencephalon and cerebellum. Cell suspensions were obtained and plasma membranes were prepared by the same procedure used for embryonic liver cells.

For comparative purposes plasma membranes were obtained from trypsin-dissociated 12-day liver cells. Dissociation by this method was similar to that reported for the agglutination experiments. After dissociation, cells were washed twice with Tyrode saline containing DNAase (24 units), as well as *N*-α-*p*-tosyl-L-lysine chloromethylketone (Beichen Feinchemikalen Leistat, Switzerland) $1 \cdot 10^{-3}$ M, in order to inhibit further trypsin activity. Cells were further washed with Tyrode saline before being used for membrane preparation.

Estimation of 5′-nucleotidase activity (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) was performed in cell lysates and membrane preparations of blastoderm and 12-day chick embryo livers by the method of Persijn et al. [17]. Protein was determined by the Lowry [18] procedure.

Isoelectric focusing in thin polyacrylamide gels

Analytical isoelectric focusing of membrane preparations solubilized in Triton X-100 was performed in thin layers of polyacrylamide gel over a pH gradient 3.5–8.0 using a Multiphor apparatus (LKB Produkter AB, Sweden). Preparation of the gel layer used here and the construction of the pH gradient using the appropriate pH range ampholines (LKB Produkter AB, Sweden) together with the focusing and development of the gels was as described in detail by Cook [19]. The polyacrylamide slab, characterized according to the definition by Hjertén [20], has $T = 6.8\,\%$ and $C = 2.4\,\%$ and is similar ($T = 7.4\,\%$, $C = 2.6\,\%$) to the gel described by Wrigley [21] for gel isoelectric focusing.

Samples of biological material were mixed at room temperature with Triton X-100 solution (concentrations between 0.5 and 1.0 %, v/v, examined). In some experiments this was followed by centrifugation at $100\,000 \times g$ for 1 h at 4 °C; material present in the supernatant was regarded as representing solubilized membrane constituents. Material thus solubilized was cast into polyacrylamide-ampholine sample gel blocks as described by Cook [19] and then subjected to isoelectric focusing.

Characterization of wheat germ agglutinin receptors

Isolated plasma membranes were suspended in 50 mM Tris · HCl buffer, pH 7.6, containing 1.0 % (v/v) Triton X-100, and incubated for 30 min at room tempera-

ture. This material was then centrifuged at 100 000 × *g* for 1 h at 4 C. The clear supernatant fluid was passed through a small column (0.65 cm internal diameter, Cambridge Bio Lab, Cambridge, England) containing wheat germ agglutinin-agarose packed to a height of at least 1.5 cm. This column was eluted with 50 mM Tris · HCl buffer, pH 7.6, containing 1.0 % (v/v) Triton X-100 collecting 2 ml fractions until the absorbance of the eluate at 260 nm was reduced to the original level obtained when using the eluting buffer. The column was then developed with 50 mM Tris · HCl buffer, pH 7.6, containing 0.1 M GlcNAc and 1.0 % (v/v) Triton X-100. Although insufficient material was eluted to show any appreciable change in absorbance at 260 nm, a wavelength at which Triton X-100 shows minimum absorption, the first six GlcNAc-containing fractions were pooled and dialysed exhaustively at 4 C against 1.0 % (v/v) Triton X-100. The material remaining in the dialysis sac was reduced to a small volume (< 1 ml) and the same sac was packed in Carbowax (a preparation of polyethylene glycol, M_r = 15 000–20 000) followed by dialysis for 4 h against 1 % (v/v) Triton X-100. Finally this fraction (L2) was cast into polyacrylamide-ampholine sample gel blocks and examined in thin gel slabs as described above.

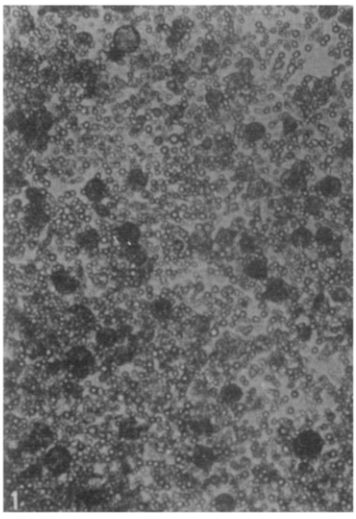
RESULTS

Agglutination of embryonic cells by different lectins

Cells obtained from chick blastoderm at stages 1 and 4–5 readily agglutinated in the presence of concanavalin A, wheat germ agglutinin and *R. communis* agglutinin (Figs 1 and 2). Agglutination was not evident when soybean agglutinin or fucose-binding protein was used (Table I). Under our conditions cells from the unincubated (stage 1) as well as 22–23 h incubated blastoderm agglutinated to the same extent. The degree of lectin induced agglutination was similar when blastoderms were dissociated in Ca^{2+}- and Mg^{2+}-free media or in EDTA containing Ca^{2+}- and Mg^{2+}-free Panet and Compton saline. Agglutination occurred at lectin concentrations as low as 10 μg/ml for concanavalin A, wheat germ agglutinin and *R. communis* agglutinin. Lectin induced agglutination was inhibited by the corresponding sugar haptens with the exception of that induced by wheat germ agglutinin, where a very slight agglutination was still present in 100 mM GlcNAc. Complete inhibition of wheat germ induced agglutination by this sugar occurred after cells were treated with neuraminidase (Table II); these results were similar in the two blastoderm stages examined herein.

The effect of neuraminidase on the agglutination of chick blastoderm cells by soybean and wheat germ agglutinins can be observed in Table II and Fig. 2. While normal dissociated blastoderm cells do not agglutinate with soybean agglutinin, they are readily agglutinated by this lectin after they have been treated with neuraminidase. This suggests that neuraminidase treatment may uncover receptors containing *N*-acetyl galactosaminyl groups. Wheat germ agglutinin-induced agglutination was increased slightly after neuraminidase treatment; in this case agglutination was

Fig. 1. Effect of several lectins on cells obtained from dissociated unincubated (stage 1) blastoderms: (1) saline control; (2) wheat germ agglutinin 125 μg/ml; (3) wheat germ agglutinin 125 μg/ml and GlcNAc 0.1 M; (4) soybean agglutinin 100 μg/ml; (5) neuraminidase-treated cells (30 min, 37 °C) plus soybean agglutinin 100 μg/ml; (6) neuraminidase-treated cells, soybean agglutinin 100 μg/ml and GalNAc 0.1 M. Conditions for agglutination are described in Materials and Methods. Magnification × 120.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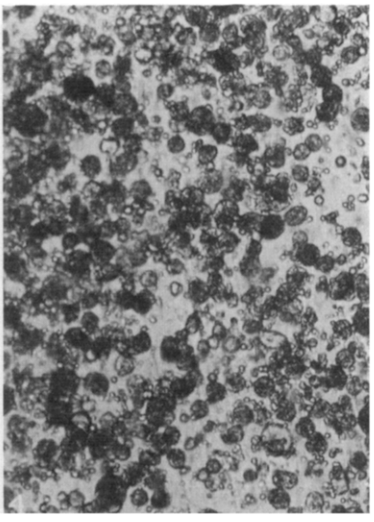


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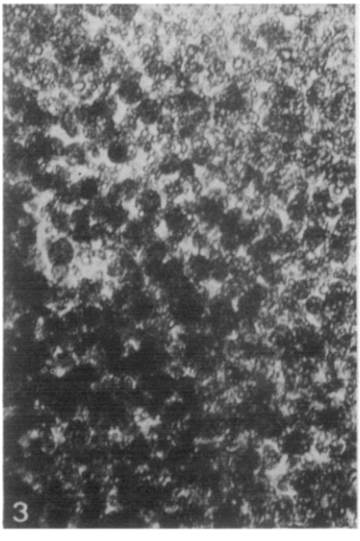
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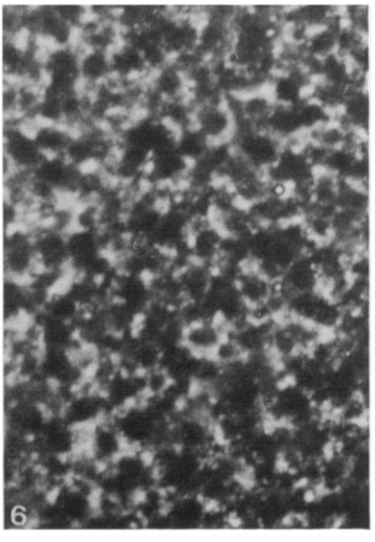
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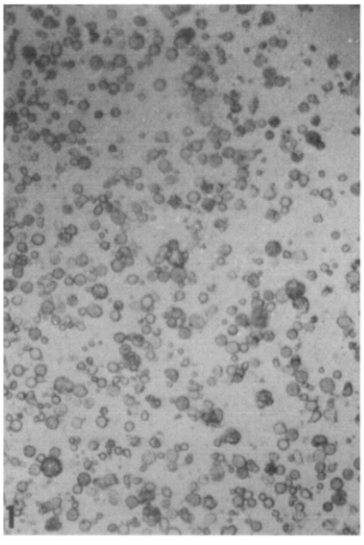
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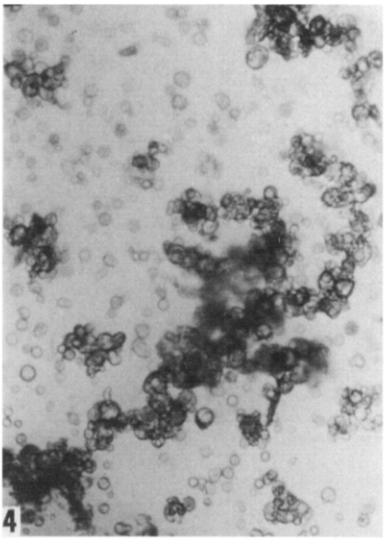
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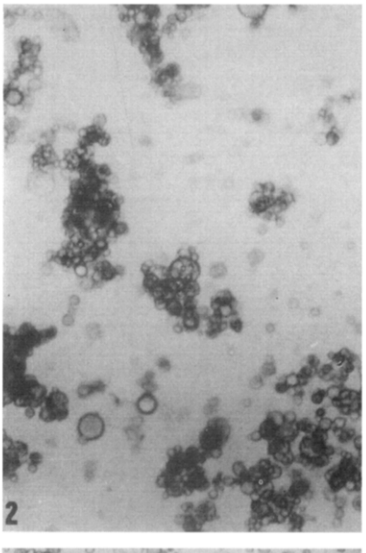
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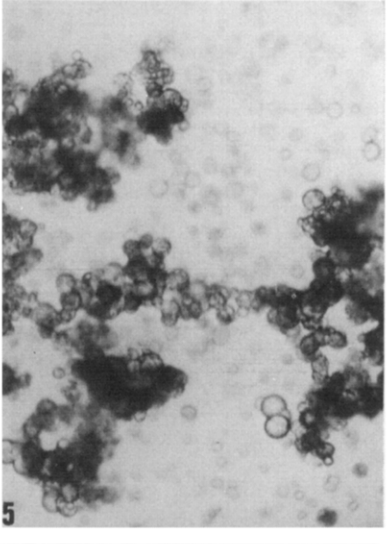
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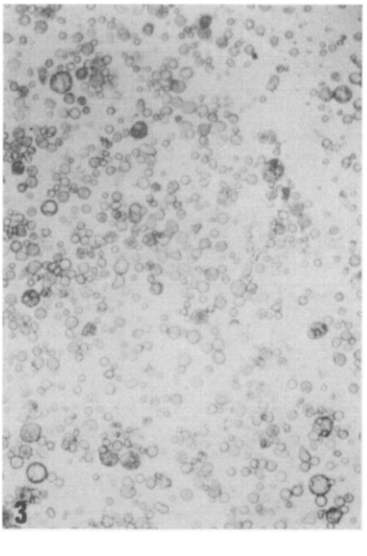
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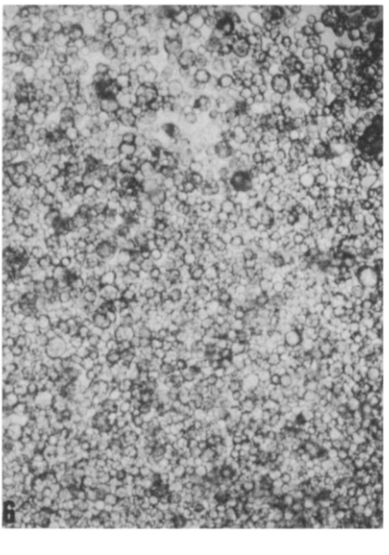
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TABLE I

AGGLUTINATION OF BLASTODERM CELLS BY DIFFERENT LECTINS

The method of assaying agglutination is described in Materials and Methods. Results shown here were obtained with 33 μg/ml of each lectin and the final concentration of haptenic inhibitors present in the assay was 0.1 M. The figures given in parentheses indicate the number of separate experiments performed. In the absence of lectin cells are not agglutinated and have a score = 0 in the assay (see also Fig. 1 (1) and Fig. 2 (1)).

	Concanavalin A	Wheat germ agglutinin	*R. communis* agglutinin	Soybean agglutinin	Fucose-binding protein
Stage 1					
Saline	++++ (4)	++++ (6)	++++ (6)	0 (6)	0 (1)
α-Methyl mannopyranoside	+ (1) 0 (3)				
N-Acetyl-D-glucosamine		+ (5) 0 (1)			
N-Acetyl-D-galactosamine		+++ (1)			
B lactose			0 (6)		
Stages 4–5					
Saline	++++ (2)	+++ (7)	++++ (3)	0 (4)	0 (2)
α-Methyl mannopyranoside	0 (2)				
N-Acetyl-D-glucosamine	+++ (1)	+ (4) 0 (3)			
N-Acetyl-D-galactosamine		+++ (2)			
B lactose		+++ (1)	0 (3)		

completely inhibited by GlcNAc. The latter suggests that some sialic acid residues may be involved directly or indirectly in wheat germ agglutinin-induced agglutination. In neuraminidase-treated cells, soybean agglutinin induced agglutination occurred at lectin concentrations as low as 25 μg/ml and was completely inhibited by 100 mM GalNAc. Similar results were observed in the unincubated as well as 24 h incubated blastoderm cells. Neuraminidase treatment had no effect on concanavalin A and *R. communis* agglutinin-induced agglutination and did not induce agglutination by fucose-binding protein.

Trypsinization has been reported to induce agglutination by wheat germ agglutinin in differentiating embryonic chick liver cells [1]. A series of experiments was performed to corroborate these data and also to determine whether trypsin treatment affected the agglutination of very early embryonic cells. The results from these experiments are presented in Table III. It can be observed that trypsin treatment

Fig. 2. Effect of several lectins on cells from dissociated 24 h incubated chick blastoderms (stages 4–5): (1) saline control; (2) concanavalin A 100 μg/ml and GlcNAc 0.1 M; (3) concanavalin A 100 μg/ml and D-mannose 0.1 M; (4) *R. communis* agglutinin 100 μg/ml; (5) neuraminidase-treated cells (30 min; 37 °C) and wheat germ agglutinin 100 μg/ml; (6) neuraminidase-treated cells, wheat germ agglutinin 100 μg/ml and GlcNAc 0.1 M. Microscope optics are not the same as those in Fig. 1. Conditions for agglutination are described in Materials and Methods. Magnification ×120.

TABLE II

EFFECT OF NEURAMINIDASE TREATMENT ON AGGLUTINATION OF CHICK BLASTODERM CELLS

The method of assaying agglutination is described in Materials and Methods. Results shown in this table were obtained with 33 μg/ml of each lectin and the final concentration of haptenic inhibitors present in the assay was 0.1 M. Cells ($0.5 \cdot 10^6$–$1 \cdot 10^6$) were incubated at 37 C for 30 min with neuraminidase. Further information on enzyme treatment is described in Materials and Methods. Figures quoted in parentheses represent the number of separate experiments performed. In the absence of lectin the cells are not agglutinated and have a score = 0 in the assay (see also Fig. 1 (1) and Fig. 2 (1)).

	Control		Neuraminidase	
	Wheat germ agglutinin	Soybean agglutinin	Wheat germ agglutinin	Soybean agglutinin
Stage 1				
Saline	+ + + + (2)	0 (2)	+ + + + (2)	+ + + + (2)
N-Acetyl-D-glucosamine	+ + + + (2)		0 (2)	
N-Acetyl-D-galactosamine				0 (2)
Stages 4–5				
Saline	+ + + + (2)	0 (3)	+ + + + (2)	+ + + + (3)
N-Acetyl-D-glucosamine	+ + + + (2)		0 (2)	+ + + + (1)
N-Acetyl-D-galactosamine	+ + + + (1)		+ + + + (1)	0 (3)

had no effect on wheat germ agglutinin-induced agglutination of cells from early chick blastoderms, but it drastically increased that undergone by 8-day chick embryo liver and brain and 10- and 12-day chick embryo liver cells. As reported by Moscona [1], results were similar when cells were obtained by trypsinization or when cells were previously dissociated with EDTA and subsequently trypsinized. Trypsin treatment had no effect on concanavalin A- and *R. communis*-induced agglutination of 23 h incubated chick blastoderm cells.

TABLE III

EFFECT OF TRYPSIN TREATMENT ON WHEAT GERM AGGLUTININ-INDUCED AGGLUTINATION OF EMBRYONIC CELLS

The method of assaying agglutination as well as the procedures for enzyme treatment are described in Materials and Methods. In this experiment results obtained with 50 μg/ml of wheat germ agglutinin are portrayed. In the absence of wheat germ agglutinin the cells examined are not agglutinated.

Source	Control	Trypsin	Number of experiments
Chick blastoderm (stages 4–6)	+ + +	+ + + +	4
8-day chick liver	+	+ + + +	2
8-day chick brain	–	+ + + +	2
10-day chick liver	0	+ + +	2
12-day chick liver	0	+ + + +	2

Binding of ^{3}H-labelled wheat germ agglutinin

Both EDTA and trypsinized 12-day chick embryo liver cells bind ^{3}H-labelled wheat germ agglutinin. In the case of the trypsinized cells saturation of the available lectin receptor sites in our assay is achieved with 10 μg wheat germ agglutinin, yielding 200 dpm of protein-bound radioactivity. Interestingly, EDTA-dissociated liver cells which are not agglutinated by wheat germ agglutinin bind more lectin (1240 dpm) when saturating quantities (100 μg) of the agglutinin are used under the same conditions. In both cases, binding is inhibited by the addition of 100 mM GlcNAc. These data suggest that, in addition to the appearance of lectin-induced agglutination, removal of some lectin-binding sites at the cell surface may occur after trypsin treatment. The binding of radioactive wheat germ agglutinin by blastoderm cells was very similar to that observed in trypsinized liver cells.

Preparation of membrane fractions

In an effort to study further the nature of the surface lectin receptors in early embryonic and differentiated liver cells, the isolation of membranes from these cells was attempted. An electron micrograph of a thin section of the membrane fraction obtained from 23 h incubated chick blastoderms is presented in Fig. 3. The membrane

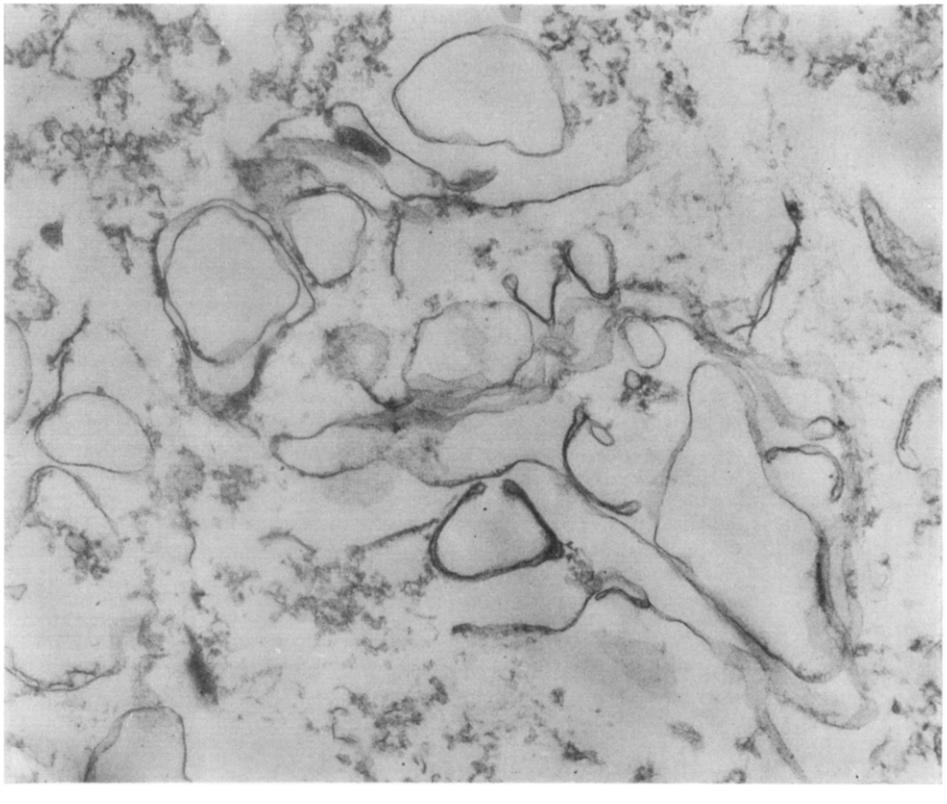

Fig. 3. Electron micrograph of a thin section of material obtained from the plasma membrane fraction of 24 h incubated chick blastoderms. A glass column of Superbrite 150 glass beads was used to purify this fraction. Magnification $\times$ 36 000.

TABLE IV

5′-NUCLEOTIDASE ACTIVITY IN LYSATES AND MEMBRANE PREPARATIONS FROM EMBRYONIC CELLS

Specific activities are reported as nmol · mg protein $^{-1}$ · min^{-1} and total activities as nmol · min^{-1}. Enzyme activity was assayed at 37 °C as described in the Materials and Methods section.

	22 h blastoderm cells		12-day chick liver cells			
	Preparation I		Preparation I		Preparation II	
	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity
Lysate	1.24	9.86	26.24	1133.6	25.66	431.4
Membrane	15.75	2.13	29.17	15.7	27.86	19.3

ghosts from blastoderm cells were heavily concentrated after passage through the glass bead column, although slight contamination with fragments of cytoplasm and mitochondria was also evident. Electron micrographs of thin sections of membrane pellets from EDTA-dissociated liver and brain cells were also examined. Cell membrane preparations from these tissues showed a lower degree of contamination by cytoplasmic fractions and mitochondria, as assessed morphologically.

The relative distribution of 5′-nucleotidase activity in cell lysates as well as in membrane preparations of chick blastoderm and 12-day chick embryo liver is presented in Table IV. Cells were lysed in distilled water and the enzymatic activities of cell lysates were compared with those of the membrane preparation. Under our conditions, between 1.3 % and 4.4 % of the total enzyme activity was recovered in the membrane fractions from 12-day chick embryo livers, while about 21 % of enzyme activity was recovered in those of chick blastoderms. While the specific activity of membrane preparations from chick blastoderms was increased as compared to lysate, which indicates that 5′-nucleotidase may be associated with the plasma membrane at these early developmental stages, similar levels were found in lysates and membrane preparations of embryonic liver cells. This latter result is in agreement with that of Sanford and Rosenberg [22], who found no significant 5′-nucleotidase activity in 15-day embryonic chick liver.

Polyacrylamide gel isoelectric focusing

The components of purified plasma membranes from 22–23 h incubated blastoderms, as well as 12-day liver and brain cells solubilized in 1 % (v/v) Triton X-100, were separated by electrofocusing in thin polyacrylamide ampholine gels and stained for protein with Coomassie brilliant blue. In several experiments in which different plasma membrane preparations derived from blastoderms were employed, it was possible to detect from 10 to 17 Coomassie brilliant blue staining bands (Fig. 4). In each separation the band patterns obtained corresponded to an average concentration of 240 μg of blastoderm cell membrane proteins obtained from 300 blastoderms. The majority of staining components had p*I* values between 4.3 and 5.2, as well as between 5.5 and 5.8. A similar picture of the distribution of the major components was obtained in the case of plasma membranes derived from EDTA-dissoci-

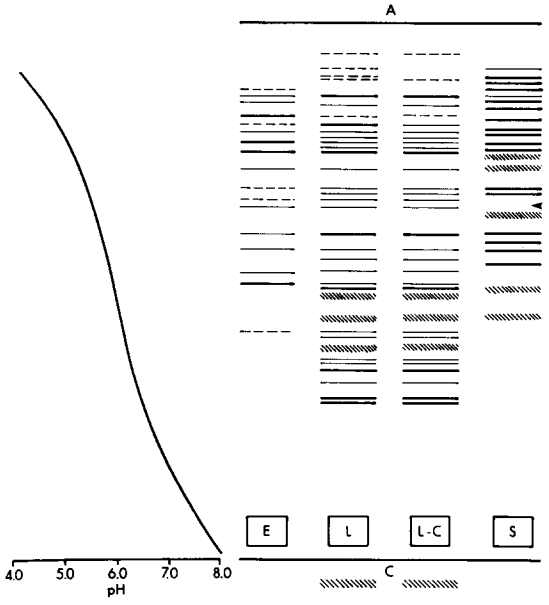

Fig. 4. Isoelectric focusing of membrane fractions from embryonic tissue and embryonic chick serum. Membrane fractions were solubilized in 1.0 % Triton X-100, mixed with the appropriate gel solution and cast in sample gel blocks according to the procedures outlined in Materials and Methods. This figure is a map of the Coomassie brilliant blue staining bands in the analytical gel slab after isoelectric focusing. The rectangles show the location of the sample blocks containing the various solubilized membrane and serum preparations. E, membrane fraction from 24 h incubated blastoderm; L, membrane fraction from 12-day chick embryo liver; L-C, same as L, centrifuged at 100 000 $\times g$ for 1 h at 4 °C; S, serum obtained from 12-day chick embryo, note absence (see arrow) of material of p*I* 5.5. The pH gradient formed in the gel is shown on the left of the figure. A, position of anode wick; C, position of cathode wick.

ated liver cells, though in the case of these cells, with a relatively larger quantity of starting material being available, it was possible to use heavier loadings and thus detect minor components (Fig. 4). When five separate liver plasma membrane preparations were examined, between 32 and 40 different components were discernible. Additional bands to those detectable in the blastoderm preparations were present with p*I* values between 3.5 and 4.3, as well as components focusing in the pH range 5.8–6.5. Centrifugation of Triton X-100-solubilized membranes did not alter the band pattern obtained using isoelectric focusing (Fig. 4, L–C).

Plasma membranes derived from trypsin-dissociated liver cells gave very varied results from one preparation to another and many fewer bands were present in trypsin- than in EDTA-treated material. Those bands that were detectable focussed at pH 4.9–6.2, and in some cases in the range 6.6–6.8, and correspond to components present in plasma membranes derived from EDTA-dissociated liver. These bands may represent those components which are more resistant to trypsin degradation. However, band patterns obtained from membranes of trypsinized liver cells were extremely variable between experiments. In the one case examined of cell membranes derived from EDTA-dissociated 12-day brain, 19 Coomassie brilliant blue-staining components over pH range 4.5–6.5 were detected, 16 of which correspond to bands

present in a sample of plasma membranes prepared from 12-day EDTA-dissociated liver examined on the same gel block.

Samples of serum obtained from 12-day embryos, as well as yolk material immediately underlying the blastoderm, were also examined. An examination of Coomassie brilliant blue staining bands in both cases indicated components of identical p*I* to those present in the plasma membrane fractions. However, the overall band pattern, as well as the relative amounts of stained material within each sample, differed from those present in the membrane fractions.

Under the conditions used here, 86 % of the protein present in a 12-day liver plasma membrane fraction was found by experiment to be solubilized by the non-ionic detergent, Triton X-100. The total protein, being determined on a portion of the same membrane sample, solubilized in 1 M NaOH at 70 °C for 5 min, under which conditions no pellet was recoverable after centrifuging the digest at 100 000 × *g* for 1 h. A preparation of human erythrocyte stroma was mixed with a preparation of plasma membranes isolated from 12-day EDTA-dissociated liver cells and then

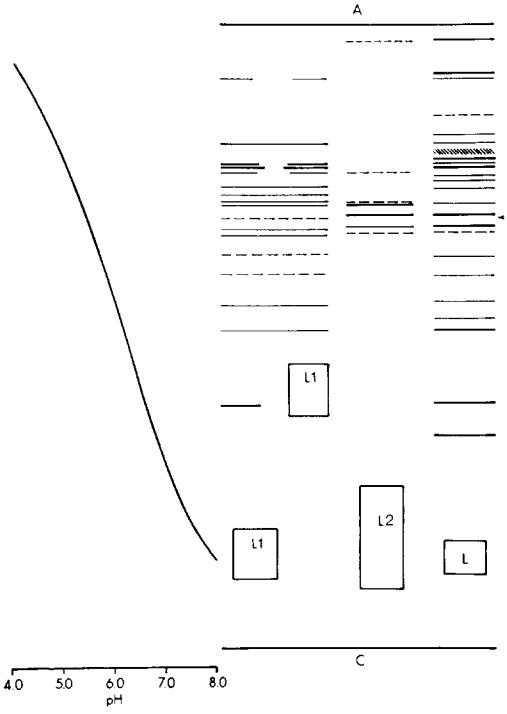

Fig. 5. Isoelectric focusing of liver plasma membrane material (64 embryos) eluted from wheat germ agglutinin-agarose columns with GlcNAc (L-2). Non-specific fractions (L-1) were initially eluted from the column with buffer containing no hapten inhibitor. This figure is a map of the Coomassie brilliant blue-staining bands present in the analytical gel slab after isoelectric focusing. The figure shows that aliquots of the non-specific fractions gave rise to identical band patterns irrespective of their position of application to the acrylamide gel. The specific and non-specific fractions are compared with a sample of unfractionated liver plasma membrane (L) preparation. The pH gradient is shown to the left of the band pattern and the arrow on the right hand side indicates the region where components of p*I* 5.5 would focus. A, position of anode wick; C, position of cathode wick.

solubilized in 1 % (v/v) Triton X-100. This mixture was then examined in an analytical isoelectric focusing gel. The Coomassie brilliant blue-stained band pattern corresponded to a combination of the banding pattern for each sample when solubilized and analyzed separately. A number of the human and chicken membrane components had identical p*I* values at pH 4.9, 5.0, 5.05, 5.4 and 6.2 and were, therefore, indistinguishable in the mixed sample. However, a "doublet" in the human erythrocyte stroma sample not present in the chick material, banding at pH 4.8, was clearly reproduced in the mixed solubilization sample. Also, a minor component of the stroma of p*I* 5.1 was still clearly present in the mixed sample. Under the above conditions of Triton X-100 extraction, in excess of 40 % of the total protein, containing glycoproteins of the erythrocyte stroma, and approaching 100 % of the sialic acid would be solubilized (Pratt, R. S., personal communication).

An examination of liver plasma membrane-derived material, eluted by GlcNAc from the wheat germ agarose column, was possible with two separate batches. In one experiment, material isolated from 24 livers was examined and an analysis of the GlcNAc-containing eluate showed the presence of a faint Coomassie brilliant blue-stained band at pH 5.5 and a trace of material at pH 5.6. When this experiment was repeated with membranes obtained from 64 chick embryo livers, material with p*I* values of 5.4–5.5 was clearly demonstrable as well as a faint band at pH 5.6. Traces of stain were also seen at p*I* 4.0, 5.1 and 5.3 (Fig. 5).

DISCUSSION

In the experiments described in this paper, the agglutination technique has been used to test for the presence of a number of lectin receptor sites at the surface of early chick blastoderm cells. In common with other authors [1, 2], we conclude that the clumping of the blastoderm cells we observe following treatment with lectins is a result of an agglutination reaction rather than a simple measure of flocculation. It is well known that freshly dissociated embryonic tissue cells will reaggregate under appropriate conditions; therefore, in all our experiments we established that in the absence of the lectin under examination no appreciable clustering of cells occurred. Additionally, we have demonstrated that the presence of the appropriate haptenic inhibitor prevents or greatly reduces the degree of lectin-induced clumping, and we conclude that the results of our agglutination studies provide information as to the presence in such a configuration, as to render the cells susceptible to agglutination, of various saccharide residues at the periphery of the embryonic chick blastoderm cell.

A suspension of EDTA-dissociated blastoderm cells is rapidly agglutinated by concanavalin A, wheat germ and *R. communis* agglutinins, reactions which are inhibited by the appropriate haptens, suggesting that α-D-glucosyl or α-D-mannosyl, *N*-acetyl-D-glucosaminyl and β-D-galactosyl-like residues are present at the cell surface. The fact that wheat germ agglutinin-induced agglutination is inhibited completely with GlcNAc only after neuraminidase treatment suggests that some sialic acid residues may also be involved in wheat germ agglutinin-mediated agglutination as reported by others [23], though the possibility that the removal of sialosyl residues causes steric reorientation of membrane-associated material cannot be overlooked. On visual observation it would appear that the majority of the chick blastoderm cells undergo lectin-induced agglutination. However, the possibility

exists that a small number of cells which do not agglutinate and react differentially with these lectins are present and not detectable under our experimental conditions. Earlier experiments using cell electrophoresis [9] had suggested that sialic acid residues were absent at the surfaces of cells from the unincubated chick blastoderm and contributed to a very small extent to the surface charge density of cells from 24 h incubated blastoderms. The finding that blastoderm cells at stages 1–4 are agglutinated by soybean agglutinin after neuraminidase treatment suggests that this enzyme uncovers lectin-binding sites at the cell surface which were previously masked by sialic acid residues. The fact that soybean agglutinin-induced agglutination is inhibited by GalNAc suggests that these binding sites are presumably *N*-acetylgalactosaminyl groups occurring subterminally to sialic acid. However, subsequent reorientation of soybean agglutinin-binding sites previously present in a condition precluding agglutination, due to rearrangement of cell surface components after neuraminidase treatment, cannot be ruled out by these experiments.

Our results on agglutination induced by concanavalin A and *R. communis* agglutinin are in agreement with those of Moscona [1] and Kleinschuster and Moscona [2] for late embryonic and fetal embryonic chick tissue and those of Krach et al. [24] for the developing sea urchin embryo. However, in addition to agglutinating with the above-mentioned lectins, we have shown that during early development of the chick embryo, when cells are involved in extensive migratory movements of gastrulation, wheat germ agglutinin receptors available for agglutination are also present at the surfaces of mechanically dissociated cells. Moscona [1] and Kleinschuster and Moscona [2] have demonstrated that with progressive differentiation of the developing liver and retina, concanavalin A receptors become masked or sheltered by trypsin-sensitive materials, while wheat germ agglutinin receptors were present in a condition which precluded agglutination. It appears from our work and that of the above mentioned investigators [1–3] that as cell differentiation is taking place, wheat germ agglutinin receptors are the first to change over to a non-agglutinable condition due to the appearance of trypsin-sensitive material at the cell surface. Whether wheat germ agglutinin-induced agglutination in blastoderm and differentiating embryonic tissues involves the same receptors remains to be established.

That differentiating cells obtained by mechanical dissociation of liver and brain are not agglutinated by wheat germ agglutinin should not necessarily be interpreted as being due to the absence of an appropriate receptor at the surfaces of these cells. In agreement with the results of other investigators [1–3], we find that such cells following treatment with trypsin are then agglutinable by wheat germ agglutinin, a finding which these authors attribute to the wheat germ agglutinin receptor sites being sheltered or masked by a trypsin-sensitive material. Alternatively a proteolysis-induced change in the distribution of surface agglutinin-binding sites favouring agglutination [25] would be in accord with our finding that appreciable quantities of haptenic specific wheat germ agglutinin are bound to embryonic liver cells, even though they remain inagglutinable.

To investigate the nature of the wheat germ receptor on the cells under investigation, we isolated quantities of plasma membrane from both early blastoderm and differentiating embryonic cells in order to examine this material by a combination of analytical isoelectric focusing and affinity chromatography procedures. That the gel support used in the isoelectric focusing does not exert any molecular sieving effect

on the separation of the detergent-solubilized membrane components was confirmed by showing that the resulting band pattern obtained with embryonic liver was the same irrespective of the point of application of the sample to the block. The latter experiment would also indicate that no localized pH-induced alteration of the components of the mixture under examination is taking place.

Though the band patterns obtained on isoelectric focusing of blastoderm and liver membranes show striking similarities it is not possible in the present work to distinguish between different proteins of identical p*I*. It is unlikely, however, that the bands visualized by Coomassie brilliant blue are artefacts of the solubilization procedure, representing a micelle composed of detergent and a mixture of different proteins. If the latter were the case it would be expected that the mixture of human erythrocyte stroma and embryonic liver membranes, when examined in the analytical isoelectric focusing system, would have yielded an entirely new band pattern bearing no resemblance to that produced by either membrane: such is not the case. In the case of embryonic liver it was possible to obtain sufficient isolated membranes to attempt an isoelectric focusing examination of Triton X-100 solubilized material fractionated on a column of wheat germ agarose. Examination of material (L2) eluted from the column with GlcNAc showed a number of Coomassie brilliant blue-staining bands of p*I* 5.4–5.6 not present in serum from 12-day chick embryo or the actual wheat germ agglutinin preparation. As regards wheat germ receptors, this experiment demonstrates that glycoproteins are present in the non-trypsin-treated differentiated liver cells with the appropriate receptor specificity. It is interesting that membranes isolated from trypsin-dissociated liver cells though possessing many fewer components as judged by analytical electrofocusing than EDTA-treated material, nonetheless still possess a protein component of identical p*I* (5.5) to material eluted by GlcNAc from liver membrane material adsorbed onto wheat germ agarose. The significance of this finding will no doubt await further experimentation, though it is tempting to speculate that this may be the component present in the native liver plasma membrane with which the lectin binds and that, following trypsin treatment, it is still available and in such a configuration within the cell periphery that wheat germ agglutinin-induced agglutination may take place.

Whether the wheat germ agglutinin receptors in the blastoderm and differentiating cells are identical is as yet unknown, though the methodology described here may well help answer some of these questions. Nevertheless, the results reported in this paper show that differentiation-related changes in lectin-binding sites at the cell surfaces, resulting in agglutination, are not just confined to concanavalin A receptors [1–3, 26, 27] but also apply to wheat germ agglutinin receptors, albeit at much earlier stages in embryogenesis.

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