Biochimica et Biophysica Acta, 394 (1975) 540–549

BBA 77014

LOCALIZATION OF THE BINDING SITES FOR THE *RICINUS COMMUNIS*, *AGARICUS BISPORUS* AND WHEAT GERM LECTINS ON HUMAN ERYTHROCYTE MEMBRANES

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(Received January 13th, 1975)

SUMMARY

Freeze-etch electron microscopy has been utilized to localize the binding sites for the *Ricinus communis*, *Agaricus bisporus* and wheat germ lectins on human erythrocyte membranes and to determine the relation of these different glycoprotein receptors to the intramembranous particles. *A. bisporus* lectin, which could be visualized directly on the surface of erythrocyte membranes, and ferritin conjugates of wheat germ agglutinin showed a distribution that correlates exactly with the intramembranous particles at all lectin concentrations tested. The binding sites for both of these lectins are located on the major sialoglycoprotein of the membrane. The *R. communis* agglutinin-ferritin conjugate which binds to receptors on membrane glycoproteins that are distinct from the major sialoglycoprotein showed a close correlation with the intramembranous particles at low lectin concentrations and a poor correlation at high lectin concentrations. High concentrations resulted in virtually complete coating of the surface of trypsinized ghosts which displayed marked aggregation of the intramembranous particles. We conclude that the intramembranous particles of erythrocyte membranes contain at least two glycoproteins and that some membrane lectin receptors are not associated with the intramembranous particles.

INTRODUCTION

Freeze-etch electron microscopy has given considerable information about the structure of cell membranes and the arrangement of proteins in the membrane. Membranes are known to split during freeze-cleavage, exposing particulate interruptions in the fracture face, called intramembranous particles [1, 2]. Previous studies have indicated that the binding sites for *Phaseolus vulgaris* E-phytohemagglutinin and influenza virus on human erythrocyte membranes are associated with the intramembranous particles [3]. Since these binding sites are located on the major salioglycoprotein of the membrane [4, 5], it seems likely that this molecule comprises at least a portion of the intramembranous particles.

Recently, considerable information has become available concerning the nature of the binding sites on human erythrocytes for the lectins of *Ricinus communis*, *Agaricus bisporus* and wheat germ [6, 7]. Erythrocytes contain approx. $6.8 \cdot 10^6$ binding sites for the *A. bisporus* lectin which are located on the main sialoglycoprotein [7]. This cell type also contains approx. $8 \cdot 10^6$ binding sites for wheat germ agglutinin, with at least a portion of these receptors also being located on the major sialoglycoprotein [6]. In contrast, the binding sites for the *R. communis* lectins *R. communis* agglutinin I and ricin are present on at least two glycoproteins which are distinct from the sialoglycoprotein [6]. In this investigation we have examined the localization of native or ferritin-conjugated *R. communis*, wheat germ and *A. bisporus* lectins on human erythrocyte membranes in order to determine whether the carbohydrate binding sites for these lectins are located on molecules which are associated with the intramembranous particles of the membrane.

MATERIALS AND METHODS

Materials. Wheat germ and ovomucoid were obtained from Sigma Chemical Co. Six times crystallized ferritin was obtained from Miles Laboratories. Trypsin, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone-treated, (TPCK) and lima bean trypsin inhibitor were obtained from Worthington Biochemical Co. Na ^{125}I (reagent grade) was from Mallinckrodt Chemical Co. 8 % gluteraldehyde, ultra-pure, was obtained from Biodynamics Research Corp.

Lectins. The *R. communis* lectins *R. communis* agglutinin I and ricin were prepared by affinity chromatography on ovomucoid-Sepharose as previously described [6]. *A. bisporus* lectin was prepared as previously described [7]. Wheat germ agglutinin was purified from crude wheat germ by affinity chromatography on ovomucoid-Sepharose according to the method of Marchesi [8]. Ovomucoid was coupled to Sepharose 2B by the CNBr procedure of Cuatrecasas [9].

Iodination of lectins. The lectins were iodinated with ^{125}I by the method of Hunter [10] using a 10-s exposure to chloramine-T. Specific activities ranged from $1 \cdot 10^5$ to $2 \cdot 10^5$ cpm/μg protein.

Conjugation of lectins to ferritin and purification of lectin-ferritin conjugates. 60 mg of purified *R. communis* agglutinin, ricin or wheat germ agglutinin, in the presence of an excess of the appropriate monosaccharide hapten (0.01 M lactose for the *R. communis* lectins and 0.1 M *N*-acetylglucosamine for wheat germ agglutinin), 300 mg of six times crystallized ferritin, 2 mg of ^{125}I-labeled lectin and 0.025 % glutaraldehyde in a total volume of approx. 30 ml of 0.005 M phosphate buffer, pH 7.5, were reacted for 45 min at room temperature with constant stirring. The conjugation was stopped by adding 10 ml of 0.1 M NH_4Cl in 0.005 M phosphate buffer and allowing the reaction mixture to stir for 10 min at room temperature. The reaction mixture was then centrifuged at $12\,000 \times g$ for 10 min and the pellet, containing about 2 % of the lectin, was discarded. The supernatant was dialyzed overnight against 0.005 M phosphate buffer to remove the hapten. Unconjugated lectin was separated from the ferritin-lectin and unconjugated ferritin by two or three centrifugations at $100\,000 \times g$ for 90 min followed by resuspension of the pellets in 0.005 M phosphate buffer. Approx. 15–20 % of the iodinated lectin was recovered in the final suspension of the conjugate. This material was applied to a column

(2.5 × 3 cm) of ovomucoid-Sepharose (10 mg ovomucoid per g Sepharose) and unconjugated ferritin was washed through with 50 ml of phosphate-buffered saline. Elution of the lectin-ferritin conjugate was carried out with the appropriate monosaccharide hapten in the same buffer. Fractions (5 ml) were collected and assayed for radioactivity to determine lectin concentration and for absorbance at 340 nm to determine ferritin concentration, assuming that 5.85 absorbance units correspond to 1 mg/ml apoferritin (mol. wt 460000) [11]. Fractions containing approximately equimolar amounts of ferritin and lectin were combined, dialyzed overnight against 0.005 M phosphate buffer and stored at 4 °C in tightly stoppered tubes containing suspended vials of toluene. The final yield of lectin-ferritin conjugate was 2–10 % in various preparations. The binding affinity of the final lectin-ferritin conjugates to human erythrocyte membranes was approximately one fifth that of the native lectin, as determined from Scatchard plots [12].

Preparation of erythrocyte ghosts. Fresh human whole blood was collected in 0.1 M sodium citrate and washed three times in phosphate-buffered 0.85 % saline. Ghosts were prepared by lysis in dilute phosphate according to the procedure of Dodge et al. [13]. Trypsinized ghosts were prepared by treating 0.5 ml of freshly prepared packed ghosts with 1 mg of trypsin in 0.5 ml of 0.01 M Tris · HCl buffer, pH 7.5, for 20 min at 37 °C. Lima bean trypsin inhibitor (2 mg) was then added and the ghosts were washed two times in 0.005 M phosphate buffer, pH 7.5.

Treatment of normal and trypsinized ghosts with lectins and lectin-ferritin conjugates. Pyrex centrifuge tubes were soaked with 1 % bovine serum albumin in 0.005 M phosphate buffer, pH 7.5, for 1 h and emptied before use. 10-μl amounts of packed normal or trypsinized ghosts were added to 0.1- to 1.0-ml amounts of appropriately diluted lectin or lectin-ferritin and incubated for 1 h at room temperature with occasional mixing. The ghosts were centrifuged at 17000 × *g* for 10 min, resuspended and washed two times with 0.005 M phosphate buffer. The final pellet was resuspended in 10 μl distilled water and prepared for freeze-etching.

Freeze-etching procedure. Small droplets of treated red cell ghosts were placed on gold alloy specimen carriers and frozen in liquid Freon 22 (E.I. DuPont de Nemours and Co.) cooled by liquid nitrogen. Specimens were freeze-cleaved at −100 °C and were deep-etched for 1 min at −100 °C in a Balzers freeze-etching apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.). The specimens were shadowed with platinum-carbon and carbon and the replicas were floated off onto distilled water, cleaned with Clorox, picked up on electron microscope grids and examined in a Philips 300 electron microscope.

RESULTS

Localization of the binding sites for the A. bisporus lectin and wheat germ agglutinin on human erythrocyte membranes

Native *A. bisporus* lectin, with an estimated molecular weight of 58 000 [7], could be visualized directly on the erythrocyte membrane surface by freeze-etching. The pattern of the bound lectin correlated with the pattern of the slightly aggregated intramembranous particles in well-washed erythrocyte ghost preparations, and could be distinguished from the smooth outer suface of untreated ghosts (Fig. 1). When native *A. bisporus* lectin was bound to trypsinized ghosts, it could not be

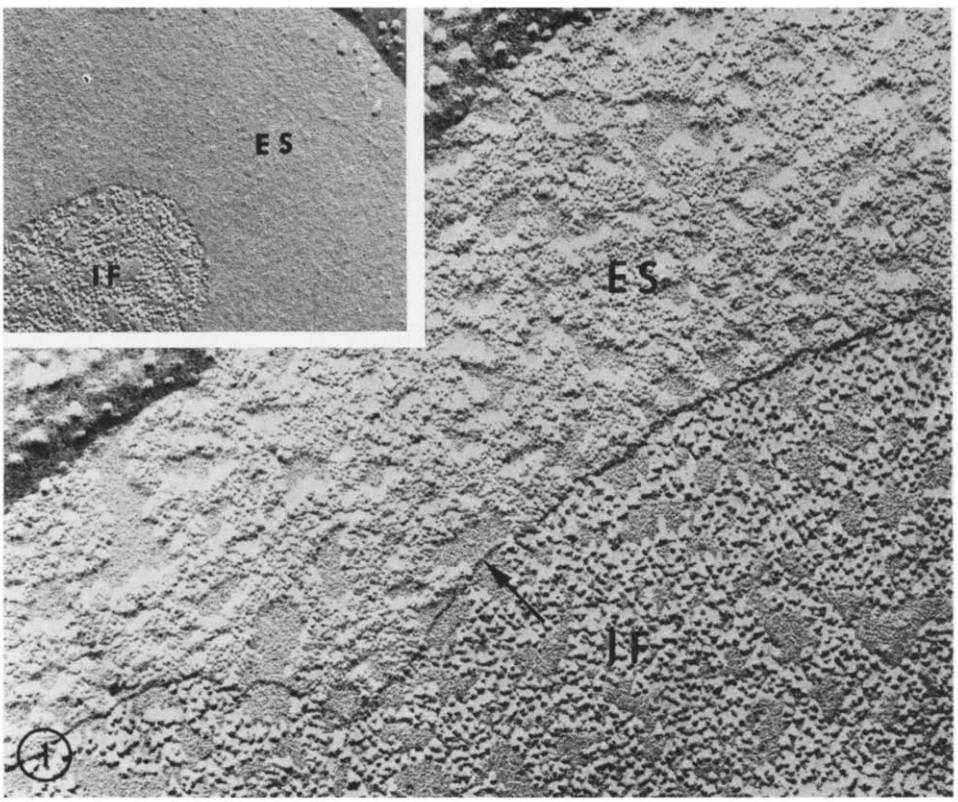


Fig. 1. Replicas of normal human erythrocyte ghost (insert) and ghost treated with native *A. bisporus* lectin. The external surface (ES) of the normal ghost (insert) is relatively smooth and the inner fracture face (IF) shows 70 Å intramembranous particles in a slightly aggregated pattern. The external surface of the ghost treated with native *A. bisporus* lectin shows granular material that is slightly aggregated and corresponds to the pattern of the intramembranous particles. Aggregates of lectin and intramembranous particles or bare areas appear continuous across the fracture line (marked with arrow). 100 000 ×; insert, 50 000 ×.

visualized on the surface of the membranes, even though the intramembranous particles of the trypsinized ghosts were markedly aggregated. In addition, binding curves with ^{125}I-labeled lectin showed only a 50 % decrease in binding to the treated ghosts. The failure to visualize the *A. bisporus* lectin under these conditions is not understood.

Ferritin conjugates of wheat germ agglutinin that had been purified on an ovomucoid-Sepharose column were used for ultrastructural localization. Wheat germ agglutinin-ferritin conjugates bound to the outer surface of the native erythrocyte membrane in a pattern similar to that of the intramembranous particles at all concentrations tested. When the intramembranous particles were aggregated by prior brief exposure to trypsin, the ferritin-wheat germ agglutinin conjugates were similarly aggregated on the outer-membrane surface (Fig. 2).

Fig. 2. Replica of a trypsinized human erythrocyte ghost treated with wheat germ agglutinin-ferritin conjugate. The intramembranous particles on the inner fracture face (IF) are markedly aggregated, and the wheat germ agglutinin-ferritin shows a similar marked aggregation on the external surface (ES) of the ghost. Aggregates of intramembranous particles and wheat germ agglutinin-ferritin appear continuous across the fracture line (marked with arrows). 61 000 ×.

Localization of the binding sites for R. communis lectin-ferritin conjugates on human erythrocyte membranes

Native *R. communis* agglutinin I was not well visualized on erythrocyte membranes; therefore ferritin conjugates were used in the localization studies. At low concentrations, *R. communis* agglutinin I-ferritin (8–50 μg lectin/ml) showed a scattered distribution on normal erythrocyte ghost surfaces and a reticulated distribution on trypsinized erythrocyte membranes similar to that of the aggregated intramembranous particles (Fig. 3). At high concentrations of the *R. communis* I-ferritin conjugate (100–200 μg lectin/ml) the conjugate densely covered the entire surface of the normal erythrocyte membranes and intramembranous particles showed a random distribution on the fracture faces of the membrane (Fig. 4A). Trypsinized erythrocyte membranes showed the usual aggregation of the intramembranous particles; however, the *R. communis* I-ferritin covered the entire outer surface of the trypsinized ghosts, such that no correspondence could be detected between the pattern of the aggregated intramembranous particles and the lectin conjugate (Figs 4B and 4C). In other experiments, it was demonstrated that the haptene lactose at a concentration of 10 mM inhibited virtually all binding of the lectin conjugate to the surface of normal and trypsinized ghosts.

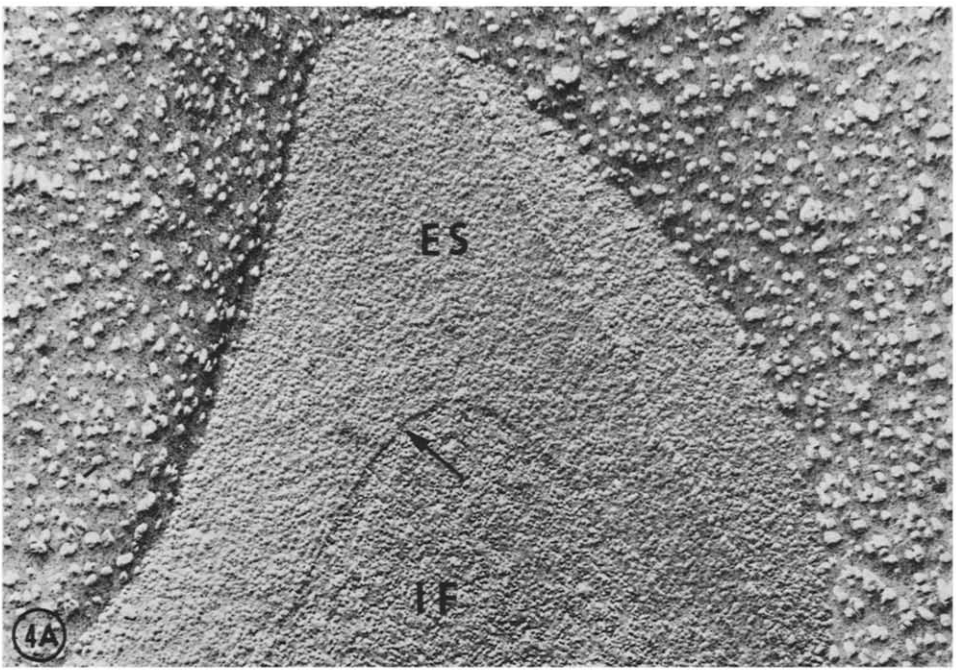
Ricin-ferritin showed a pattern of distribution similar to that of the intramembranous particles on trypsinized erythrocyte ghost membranes.

Fig. 3. Replica of trypsinized human erythrocyte ghost treated with *R. communis* agglutinin I-ferritin conjugate (50 μg lectin/ml). The trypsinized ghost shows marked aggregation of both the intramembranous particles on the inner-fracture face (IF) and the *R. communis* agglutinin I-ferritin on the external surfaces (ES) of the membrane, with close correspondence of the pattern of the conjugate and the intramembranous particles across the fracture line (arrows) at this concentration of lectin. 65 000 ×.

DISCUSSION

Previous investigations of the nature of the intramembranous particles of human erythrocyte membranes have shown an association of these particles with the binding sites for the kidney bean lectin E-phytohemagglutinin and with the sialic acid residues that act as receptors for influenza virus [3] and as binding sites for positively charged ferritin molecules [14]. These activities probably all reside on the major sialoglycoprotein of the human erythrocyte membrane [4, 5]. Our experiments extend these studies by demonstrating that the membrane binding sites for the *A. bisporus* lectin and for wheat germ agglutinin are also associated with the intramembranous particles. The receptor for the *A. bisporus* lectin is the *O*-glycosidically linked oligosaccharide chain of the major sialoglycoprotein. This oligosaccharide has the structure sialic acid α-2, 3 galactose β-1, 3 (sialic acid α 2, 6) *N*-acetylgalactosamine $\rightarrow$ Ser (Thr) [7]. The sialoglycoprotein also serves as the receptor for wheat germ agglutinin [6].

In addition, the present freeze-etch investigations show that *R. communis* agglutinin I-ferritin at low to moderate concentrations binds to receptors that are spatially related to the intramembranous particles of the membrane. Similar obser-


ES
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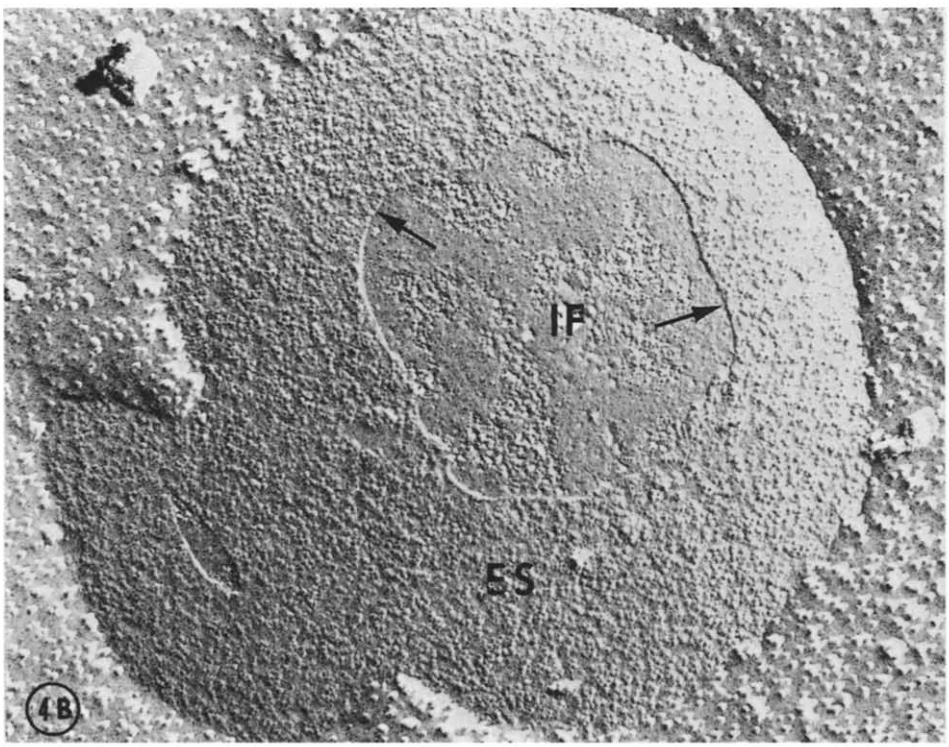
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4B

Fig. 4. Replicas of normal (A) and trypsinized (B and C) human erythrocyte ghosts treated with *R. communis* agglutinin I-ferritin conjugate (100 μg lectin/ml). The external surface (ES) of the normal ghost (A) is densely covered with *R. communis* agglutinin I-ferritin in no apparent relationship to the intramembranous particles on the inner fracture face (IF). Trypsinized ghosts (B and C) show aggregation of the intramembranous particles on the inner fracture face; however, the external surfaces are almost completely covered with *R. communis* agglutination I-ferritin and no correspondence can be observed between the dense *R. communis* agglutinin I-ferritin conjugate on the external surface and the aggregated intramembranous particles across the fracture lines (marked with arrows). A, 48 000 ×; B, 43 000 ×; C, 53 000 ×.

vations were made with ricin-ferritin. Since the binding sites for these two lectins are located on glycoproteins which are distinct from the major sialoglycoprotein, these data indicate that the intramembranous particles must contain at least two different species of glycoprotein molecules. Pinto da Silva and Nicolson [15] proposed that another membrane glycoprotein (band 3 seen on sodium dodecylsulfate-polyacrylamide gel electrophoresis of erythrocyte ghosts) is also associated with the intramembranous particles. These findings support the prediction of Bretscher [16] that the erythrocyte intramembranous particles must contain several types of proteins. Recent evidence also indicates that sialic acid-containing glycoproteins and the intramembranous particles may be structurally linked to spectrin molecules on the inner surface of the erythrocyte membrane [17, 18].

When higher concentrations of *R. communis* agglutinin I-ferritin were used, the lectin bound to regions of the external erythrocyte membrane that are free of underlying intramembranous particles. This is the first demonstration of a specific binding site on the erythrocyte membrane that is not related to the intramembranous particles. It is possible that the low affinity receptors for *R. communis* agglutinin I-ferritin that are responsible for the non-intramembranous particle-related binding may be glycolipid molecules randomly arranged on the external surface of the trypsinized erythrocyte membrane or be glycoprotein molecules not anchored into the intramembranous particles. The binding of *R. communis* agglutinin I-ferritin

is specific, however, because binding can be abolished by the hapten inhibitor, lactose.

Results from freeze-etch studies of ferritin-lectin labeling of other cell membranes do not demonstrate a complete correspondence of the intramembranous particles with ferritin-lectin binding sites on the membranes. Lymphocyte membranes, for instance, contain very few intramembranous particles in the 70 Å size range [19], although smaller, more poorly resolved particulate interruptions seem to be present. Freeze-etch experiments of *R. communis* agglutinin I and concanavalin A-ferritin binding to lymphocyte membranes do not show a spatial correspondence between intramembranous particles and ferritin-lectin molecules ([20] and Triche et al., unpublished). Lentil phytohemagglutinin-ferritin binding to human platelet membranes similarly did not show correspondence to the intramembranous particles by freeze-etch analysis or in number of binding sites [11].

We have previously demonstrated that the freeze-etch technique provides sufficient resolution to distinguish the native E-phytohemagglutinin lectin on the etched surface of erythrocyte ghosts from the slight protrusions in the outer membrane produced by the underlying intramembranous particles [3]. The resolution of the platinum-carbon shadow in the freeze-etch technique is approximately 20 Å, the size of the platinum grains. *A. bisporus* lectin molecules could be distinguished on the surface of erythrocyte membranes, although it is difficult to determine whether one is observing collections of molecules or individual molecules. Since there are $6.8 \cdot 10^6$ binding sites per erythrocyte for the *A. bisporus* lectin and only $5 \cdot 10^5$ sialoglycoprotein molecules per cell, 10 or more molecules of the native lectin may be binding to one sialoglycoprotein molecule. Native *R. communis* agglutinin I lectin could not be clearly distinguished on the external membrane surface.

ACKNOWLEDGEMENTS

Research supported by: Brown and Williamson Tobacco Corp.; Larus and Brother Co., Inc.; Liggett and Myers, Inc.; Lorillard, a Division of Loews Theatres, Inc.; Philip Morris, Inc.; R. J. Reynolds Tobacco Co.; United States Tobacco Co.; and Tobacco Associates, Inc. to Thomas W. Tillack; a U.S.P.H.S. training grant No. GM00897 to Timothy J. Triche and grant ROI CA08759 from the U.S.P.H.S. to Stuart Kornfeld. Dr Kornfeld is also the recipient of U.S.P.H.S. Research Career Development Award AM50298. We wish to acknowledge the skillful technical assistance of Shirley Carroll.

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