Journal of Supramolecular Structure
and Cellular Biochemistry 17:163–181 (1981)
Differentiation and Function of Hematopoietic Cell Surfaces 39–57

Member-Associated Changes During Erythropoiesis. On the Mechanism of Maturation of Reticulocytes to Erythrocytes

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The mature mammalian erythrocyte has a unique membranoskeleton, the spectrin-actin complex, which is responsible for many of the unusual membrane properties of the erythrocyte. Previous studies have shown that in successive stages of differentiation of the erythropoietic series leading to the mature erythrocyte there is a progressive increase in the density of spectrin associated with the membranes of these cells. An important stage of this progression occurs during the enucleation of the late erythroblast to produce the incipient reticulocyte, when all of the spectrin of the former cell is sequestered to the membrane of the reticulocyte. The reticulocyte itself, however, does not exhibit a fully formed membranoskeleton. In particular, the in vitro binding of multivalent ligands to specific membrane receptors on the reticulocyte was shown to cause a clustering of some fractions of these ligand-receptor complexes into special mobile domains on the cell surface. These domains of clustered ligand-receptor complexes became invaginated and endocytosed as small vesicles. By immunoelectron microscopic experiments, these invaginations and endocytosed vesicles were found to be specifically free of spectrin on their cytoplasmic surfaces.

These earlier findings then raised the possibility that the maturation of reticulocytes to mature erythrocytes in vivo might involve a progressive loss of reticulocyte membrane free of spectrin, thereby producing a still more concentrated spectrin-actin membranoskeleton in the erythrocyte than in the reticulocyte. This proposal is tested experimentally in this paper. In vivo reticulocytes were observed in ultrathin frozen sections of spleens from rabbits rendered anemic by phenylhydrazine treatment. These sections were indirectly immunolabeled with ferritin-antibody reagents directed to rabbit spectrin. Most reticulocytes in a section had one or more surface invaginations and one or more intracellular vesicles that were devoid of spectrin labeling. The erythrocytes in the same sections did not exhibit these features, and their membranes were everywhere uniformly labeled for spectrin. Spectrin-free surface invaginations and intracellular vesicle were also observed with reticulocytes within normal rabbit spleens. Based on these results, a scheme for membrane remodeling during reticulocyte maturation in vivo is proposed.

Key words: spectrin-actin complex, membranoskeleton, immunoelectron microscopy, membrane remodeling, endocytosis

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Received March 18, 1981; revised and accepted June 9, 1981.

0275-3723/81/1702-0163$05.50

The mammalian erythrocyte is in many respects a unique cell. Many of the properties associated with its membrane are not duplicated in any other cells of the body. These properties include two that we address in this paper: its biconcave disc shape, and the highly restricted mobility of its integral proteins in the plane of the membrane. These two special properties are generally attributed to a unique membranoskeleton* attached to the cytoplasmic surface of the erythrocyte membrane, the spectrin-actin complex. The detailed molecular and ultrastructural organization of this skeleton and its membrane attachments are subjects of great current interest, and our present understanding of these problems is discussed in papers from the laboratories of V.T. Marchesi and D. Branton in this volume.

The critical role of the membranoskeleton in determining the biconcave disc shape of the erythrocyte is indicated by several observations. For example, lysed and resealed erythrocyte ghosts under appropriate conditions can themselves adopt the biconcave disc shape [1, 2]. Furthermore, Triton treatment of erythrocytes, which removes the membrane constituents and cytoplasm, leaves behind a shell of the membranoskeleton that largely retains the outline of the intact cell [3, 4]. The most likely explanation of how the membranoskeleton determines cell shape is as follows. The ion pumps and leaks in the membrane generate a steady-state osmotic pressure gradient across the membrane that, in isotonic media, maintains the volume of the intact cell well below that of the equivalent sphere. The cell surface is thus constrained to adopt some steady-state nonspherical shape. The membranoskeleton, however, offers mechanical resistance to bending, and energy must be expended against that resistance to give the cell membrane a particular shape. If the resistance to bending is everywhere uniform over the surface of the membrane, the biconcave disc is uniquely that shape which requires the least expenditure of bending energy [5].

This mechanical picture must be expanded to take into account the fact that the membranoskeleton of the erythrocyte is a *mechanochemical* structure, and not simply an inert mechanical sheath. It is chemically and structurally responsive to cell metabolism [2, 6] and exists in a characteristic steady state under normal physiological conditions. In this view, therefore, the biconcave disc shape of the erythrocyte should be treated as corresponding to a mechanochemical energy minimum.

The involvement of the spectrin membranoskeleton in severely restricting the lateral mobility of integral proteins in the mammalian† erythrocyte membrane has

*The term cytoskeleton is often used in connection with the spectrin-actin complex of erythrocytes This seems to us inappropriate because in other eukaryotic cells, the cytoskeleton refers to a cytoplasmic matrix of filaments (microfilaments, intermediate filaments, and microtubules) that is only sporadically attached to the membrane, whereas in the erythrocyte, the skeleton is attached to the membrane and is not present in the cytoplasmic matrix For the erythrocyte, therefore, the term membranoskeleton is more appropriate than cytoskeleton

†Although the integral components of the membranes of normal mammalian erythrocytes show little evidence of lateral mobility in the plane of the membrane, components in the membranes of nucleated avian erythrocytes exhibit substantial lateral mobility (see Fig. 6 in [7]).

been amply documented. In all cells other than mammalian erythrocytes, lacking such a membranoskeleton, integral proteins are freely mobile over long distances in the membrane [8]. Furthermore, although membrane components of normal reticulocytes and erythrocytes show very restricted degrees of lateral movement in the plane of the membrane, in circulating erythrocytic cells from mice with hereditary spherocytosis, which are deficient in spectrin [9, 10], substantially greater degrees of lateral movement of surface components can be observed. It is extremely interesting, however, that while the lateral mobility of particular membrane components is restricted in normal mammalian erythrocyte membranes, the same components show essentially completely free rotational mobility about an axis perpendicular to the membrane [11, 12]. The picture that emerges, therefore, is that the spectrin-actin complex forms a kind of continuous chicken-wire meshwork attached to and immediately under the mammalian erythrocyte membrane. This meshwork does not impede rotational motions of individual integral proteins that are not connected to it, but it probably restricts the lateral motions of these proteins to within the individual units of the meshwork.

SPECTRIN IN CELLS OF THE ERYTHROPOIETIC SERIES

The cellular developmental sequence leading to the mature mammalian erythrocyte has been well delineated. All of these cells have amorphous shapes that are in no way similar to the biconcave disc of the mature erythrocyte end cell. Spectrin is uniquely characteristic of erythroid cells [13, 14] and is synthesized early in the developmental sequence [15]. It is clearly of interest to determine what is the amount, disposition, and function of spectrin in the cells of this series, and to understand how the spectrin-actin membranoskeleton of the mature erythrocyte arises in development. To understand this problem fully requires knowledge of the appearance and fate of all the cellular components throughout this developmental pathway, clearly a formidable task. It seems appropriate, however, in view of its importance to the mature erythrocyte, to begin such studies with spectrin. To this end, Geiduschek and Singer [16] examined the problem with cells obtained from mouse bone marrow. In this highly heterogeneous cell population, cells of the erythropoietic series were detected by their specific intracellular staining with fluorescently labeled antibodies to mouse spectrin. Early and late erythroblasts were differentiated by their difference in size and by the intensity of membrane-associated spectrin labeling, the late erythroblasts being smaller and substantially more intensely labeled than the early ones. If the living cells were first incubated with concanavalin A (con A) labeled with one fluorophore for 20 min at room temperature, and were then fixed, permeabilized, and immunolabeled with a second fluorophore for spectrin, the extent of the con A-induced redistribution of con A-binding proteins in the cell membranes of the early and late erythroblasts could be determined. These experiments showed that the con A-binding proteins in the early erythroblast could be capped onto one pole of the cell, while those in the late erythroblast formed small patches over the entire cell surface but did not form caps. These membrane components therefore appeared to be more restricted in the

extent of their lateral mobility in the late as compared to the early erythroblast, which correlated with the apparently larger density of spectrin associated with the membranes of the late erythroblast.

In a subsequent stage of differentiation of mammalian erythropoietic cells, the late erythroblast undergoes enucleation, the nucleus taking with it a surrounding sheath of the erythroblast membrane and leaving behind the newly formed reticulocyte. The reticulocyte then leaves the marrow and enters the circulation. The remarkable finding was made that the spectrin of the mouse late erythroblast appeared to be regularly and completely segregated during enucleation to the membrane of the incipient reticulocyte. The newly formed reticulocyte, no longer capable of synthesizing spectrin [15], thereby substantially increases the density of spectrin associated with its membrane over that of the intact late erythroblast. Correlated with this increase in spectrin density is an apparent increase in the restriction of mobility of its con A-binding proteins, since incubation of reticulocytes with fluorescent-labeled con A no longer induces even the patching that is observed on the membranes of the late erythroblast*.

This sequence of developmental stages observed in mouse bone marrow cells therefore shows that a regular increase in the density of membrane-bound spectrin occurs, accompanied by an increasing restriction on the extent of the lateral mobility of the con A-binding proteins in the membrane. (A similar increased restriction was observed for wheat germ agglutinin (WGA)-binding membrane components.)

SOME MEMBRANE PROPERTIES OF MAMMALIAN RETICULOCYTES

Although fluorescent microscopic observations with reticulocytes did not reveal patching or capping of con A- or WGA-binding components in the membrane, suggesting that significant restrictions are imposed on the extents of lateral movement of the majority of these components in the reticulocyte membrane, it had already been demonstrated by higher resolution electron microscopic experiments that at least some fraction of the reticulocyte membrane components did exhibit lateral mobility, albeit limited in extent [18]. The original purpose of these particular experiments was to investigate further the finding [19] that antibodies to blood group A substance were endocytosed by mature erythrocytes of human newborns, but not by the erythrocytes of adults. With ferritin-labeled con A (fer-con A), it was confirmed that neonatal erythrocytes in cord blood endocytosed fer-con A, whereas human adult cells did not [18]. This itself is a most interesting observation. However, for our present purposes, we will not discuss it further here, but will rather concentrate on the reticulocytes in the cord blood. Because the content of reticulocytes in that blood is quite high ($\approx 10\%$ of the erythrocytes), it was readily possible to show that a 20–50-fold greater amount of endocytosis of fer-con A was induced in neonatal reticulocytes than in neonatal erythrocytes. This endocytosis was a reflection of the lateral mobility of con A-binding proteins in the membranes of these cells, which were clustered together by the fer-con A into invaginations on the cell surface that were subsequently endocytosed. This was

*Redistributions of integral membrane proteins, including con A-binding proteins, also occur in the erythroblast membrane during enucleation [17]

demonstrated by the fact that succinylated fer-con A, which is incapable of inducing such cross-linking, did not induce invaginations of the cell surface and was not endocytosed. However, if anti-ferritin antibodies were added to cells that had bound succinylated fer-con A, then clustering, surface invaginations, and endocytosis were again observed.

It was clear in these experiments, however, that at a maximum only a small fraction of the con A-binding proteins in the reticulocyte membrane could be endocytosed. This was interpreted [18] to mean that "there exist, or are induced upon ligand binding, *discrete domains* in the intact [reticulocyte] cell membrane within which receptor proteins can exhibit lateral mobility. These mobile domains would be interspersed within a matrix of immobilized receptors, and might be so few and far between as to remain essentially independent of one another. Receptor clustering and endocytosis would be confined within those domains. . . . Such mobile domains are absent, or cannot be induced by ligand binding, in the membrane of the normal intact adult erythrocyte."

In order to pursue this idea further, we turned to rabbit reticulocytes, which can be generated in large amounts by phenylhydrazine-induced anemia. It was found [20] that fer-con A was endocytosed to a limited extent by rabbit erythrocytes. Furthermore, however, it was observed that the extent of endocytosis among the reticulocytes decreased with increasing maturation, the age of particular reticulocytes in the sample being measured by the ribosome configurations in the cell interior. This suggested that "during reticulocyte maturation, these [mobile] domains are gradually eliminated, eventually disappearing upon formation of the mature erythrocyte" [20].

In a parallel further investigation of the neonatal human cells, we investigated the structural relationship of the spectrin membranoskeleton to these mobile domains [21]. In order to do this, we employed the techniques developed in this laboratory [22, 23] for the immunoelectron microscopic labeling of ultrathin frozen sections of cells and tissues. Unlabeled con A was incubated with human cord blood cells, which were then fixed, ultrathin frozen-sectioned, and directly labeled for spectrin with ferritin-conjugated antibodies. The rabbit anti-human spectrin antibodies used in these experiments, although raised by immunization with human spectrin containing equimolar amounts of both bands 1 and 2, were directed predominantly to band 1 [24]. The striking finding was that the surface invaginations and endocytosed vesicles induced by con A in both neonatal erythrocytes and reticulocytes were specifically devoid of spectrin labeling. Although strictly speaking this reflected only upon the absence of spectrin band 1, we assumed that, since bands 1 and 2 exist as a heterodimer in the membranoskeleton, this meant that both bands 1 and 2 were absent from these con A-induced invaginations and endocytosed vesicles.

AN IMMUNOELECTRON MICROSCOPIC STUDY OF THE DISTRIBUTION OF SPECTRIN IN RABBIT SPLENIC RETICULOCYTES AND ERYTHROCYTES IN SITU

The results of the effects of in vitro treatment of intact reticulocytes with con A led us to ask whether related events might not occur in vivo. In particular, could the process of maturation of reticulocytes to mature erythrocytes in the circulation include the progressive loss of plasma membrane fragments *devoid of asso-*

ciated spectrin? Such a process might go on until it eliminated the mobile domains of the reticulocyte membrane. The concentration of spectrin attached to the erythrocyte membrane would thus increase to some maximum value, which promoted the formation of a continuous intact membranoskeleton in the mature erythrocyte membrane would thus increase to some maximum value, which promoted the formation of a continuous intact membranoskeleton in the mature erythrocyte. Such an intact skeleton might then produce the still more restricted lateral mobility of surface components that characterizes the mature erythrocyte and, in addition, contribute to the conversion to the biconcave disc shape.

The idea that membrane loss and remodeling is involved in reticulocyte maturation is an old one. Rat reticulocytes from stressed animals have been shown to lose approximately one-third of their membrane lipids during in vivo maturation [25]. Radiolabeling experiments by Come et al [26] showed quite elegantly that reticulocytes from phenylhydrazine-treated animals lost a disproportionate amount of membrane relative to cytoplasmic components during maturation in vivo. These results suggest that some membrane vesiculation process might occur during reticulocyte maturation, but the detailed nature of that process, and in particular whether spectrin was involved in it, could not be ascertained from such experiments.

The spleen is known to play an important role in the remodeling of the surface membranes of reticulocytes and erythrocytes [25–29]. The remodeling includes the removal of various intracellular debris and inclusion bodies, as well as selected membrane constituents such as the high molecular weight membrane complex described by Lux and John [30]. Erythrocytes from splenectomized animals have characteristically larger and more distorted membranes than normal, and they contain numerous intracellular vesicles and inclusions. It thus seemed likely that the site where reticulocyte membrane remodeling events might occur would be the spleen. We therefore decided to examine reticulocytes and erythrocytes in situ within the red pulp of rabbit spleen by ultrathin frozen sectioning of the fixed spleen and immunoelectron microscopic labeling for spectrin in these sections. These experiments have revealed splenic reticulocytes in both phenylhydrazine-treated rabbits and (with more difficulty) in normal animals; invaginations of the membrane surface and intracellular vesicles existed that were specifically devoid of spectrin labeling. Such spectrin-free regions were not observed on mature erythrocyte membranes or on other specialized portions of reticulocyte membranes, such as those associated with Heinz bodies or those under mechanical stress. We have proposed a scheme for the remodeling of the reticulocyte membrane during maturation in vivo based on these results.

MATERIALS AND METHODS

Antibodies

Rabbit erythrocyte spectrin was isolated and purified by the methods of Marchesi [31]. Goat anti-rabbit spectrin was obtained by subcutaneous injection of a goat with 14 mg rabbit spectrin in complete Freund's adjuvant, followed 4 weeks later by a subcutaneous booster injection of 9 mg rabbit spectrin in incomplete Freund's adjuvant. Highest titer antisera were obtained 2 weeks after this booster injection, and these were used for all subsequent experiments. Immunoelectrophoresis against purified spectrin bands 1 and 2, following the methods of Con-

verse and Papermaster [32], revealed that approximately 70% of the antibodies were directed against band 1 and 30% against band 2. Antibodies were affinity purified using spectrin (bands 1 and 2 together) coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia), following the method of Nicolson and Painter [33]. Rabbit anti-goat IgG was obtained by injection of rabbits with 1 mg goat IgG in complete Freund's adjuvant subcutaneously, followed by subcutaneous booster injections of 1 mg IgG every month. Antisera were collected 10–12 days after the first booster injection, weekly thereafter, and the antisera were then pooled for use. These antibodies were affinity purified using goat IgG bound to glutaraldehyde-activated Ultragel (Pharmacia), following the method of Ternyck and Avrameas [34].

Protein Conjugation Procedures

Ferritin-conjugated rabbit antibodies to goat IgG were prepared by the two-step glutaraldehyde coupling procedure of Kishida et al [35].

Immunoelectron Microscopy

Generally, the procedures used followed the methods of Tokuyasu and Singer [23]. Rabbits weighing ≈2.5 kg were made anemic by treatment with phenylhydrazine; on 4 consecutive days 1 ml of 30 mg/ml phenylydrazine in physiological saline was injected subcutaneously. The spleens were excised, and minced into cubes approximately 1–2 mm on a side in a solution of 0.1 M Na phosphate buffer, pH 7.4, containing 3% formaldehyde and 0.1 M ethyl acetimidate (EAI) at room temperature. The cubes were left in this first-stage fixative at 4°C for 30 min. They were then washed in the pH 7.4 phosphate buffer for 5 min, and were placed in the second stage fixative, which was a solution of 0.1 M Na phosphate buffer, pH 7.4, containing 3% formaldehyde and 0.2% glutaraldehyde, for 2 hr. The cubes were then washed in a pH 7.4 phosphate-buffered solution containing 3% formaldehyde, and stored overnight at 4°C in this solution. Following treatment ≈ ½ mm cubes of the red pulp portion of the spleen were removed under the dissecting microscope and equilibrated at room temperature in a solution of phosphate buffer containing 0.8 M sucrose for 30 min. Freezing, frozen-sectioning, and indirect immunoferritin labeling were performed as described in Tokuyasu and Singer [23]. Specimens were then adsorption stained in a 0.02% uranyl acetate-1% methylcellulose solution for 10 min [36]. The grids were then picked up in embedding loops, and the methylcellulose was removed from the loops with filter paper lightly touched to the side of the loop until the grids suspended in the loops looked flat. Upon proper drying, a gold layer (≈80 nm thick) of methylcellulose was deposited upon the grid. The grids were then examined and photographed in a Philips EM300 electron microscope.

RESULTS

Localization of Spectrin in Reticulocytes Within the Rabbit Spleen

To verify the specificity of the ferritin immunolabeling, ultrathin frozen sections of the rabbit spleen were immunolabeled as described in Materials and Methods, except that 200 μg/ml of normal goat IgG was substituted for the 200 μg/ml goat anti-spectrin antibodies normally used. The results are shown in Figure

1. There was essentially no labeling of erythroid cell membranes under these conditions. Nonspecific background was also very low.

A low magnification view of an ultrathin frozen section of red pulp from the spleens of rabbits in a state of phenylhydrazine-induced stress anemia is shown in Figure 2. At the time the spleen was removed, the reticulocyte concentration in the circulating blood was about 20%. The majority of the cells present in this particular section are erythrocytes, most of which contain Heinz bodies induced by the phenylhydrazine treatment. The reticulocytes and erythrocytes appear to be distorted, probably because of steric constraints imposed by the spleen. (The cells may not retain their original positions with respect to the splenic structures after the ultrathin frozen sections are prepared and transferred to the grids.) For illustrative purposes, three of the reticulocytes present in this section are indicated by the enclosed areas a, b, and c. Area d shows a typical erythrocyte containing a Heinz body. Any erythrocyte apparently in the process of having its Heinz body phagocytosed by a reticular cell is shown in area e. In the following figures, higher magnifications of selected portions of these cells are shown.

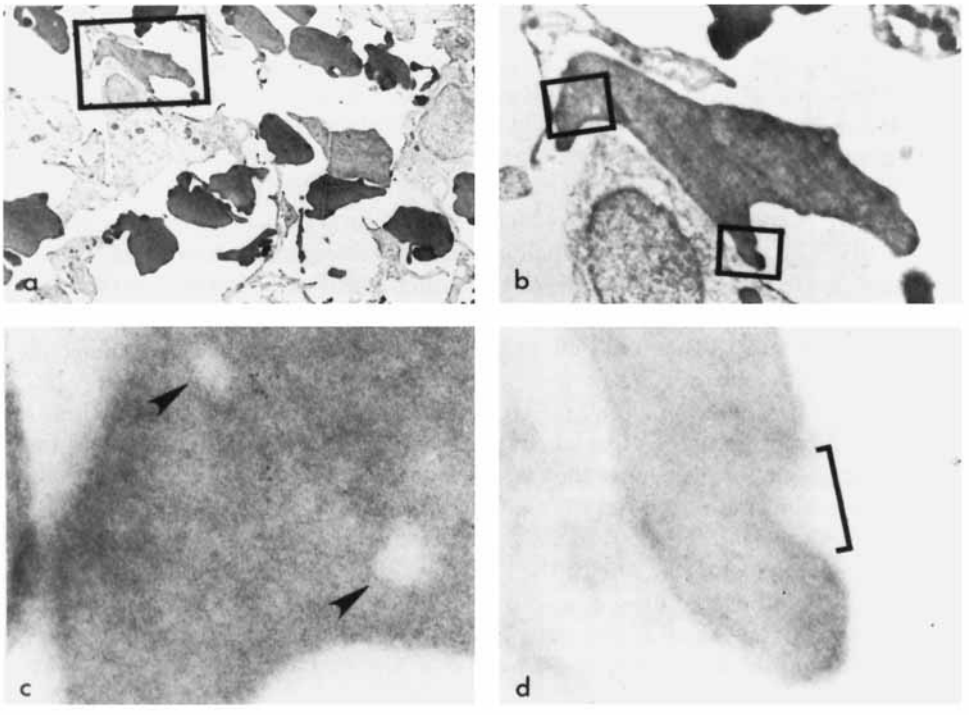


Fig 1 Normal goat IgG control Red pulp from a phenylhydrazine-induced anemic spleen was sectioned and immunoferritin-labeled as described in Materials and Methods, except that 200 μg/ml of normal goat IgG was substituted for the 200 μg/ml goat anti-rabbit-spectrin IgG normally used (a), (b) low and somewhat higher magnification views of a reticulocyte, (c), (d) high magnification view of selected areas of the reticulocyte shown in b, (c) two endocytotic vesicles (large arrowheads) and a small amount of nonspecific immunoferritin label, (d) a surface invagination (bar) No significant degree of nonspecific labelling is observed on these membrane sites Magnification (a) × 2,300, (b) × 8,800, (c), (d) × 70,000

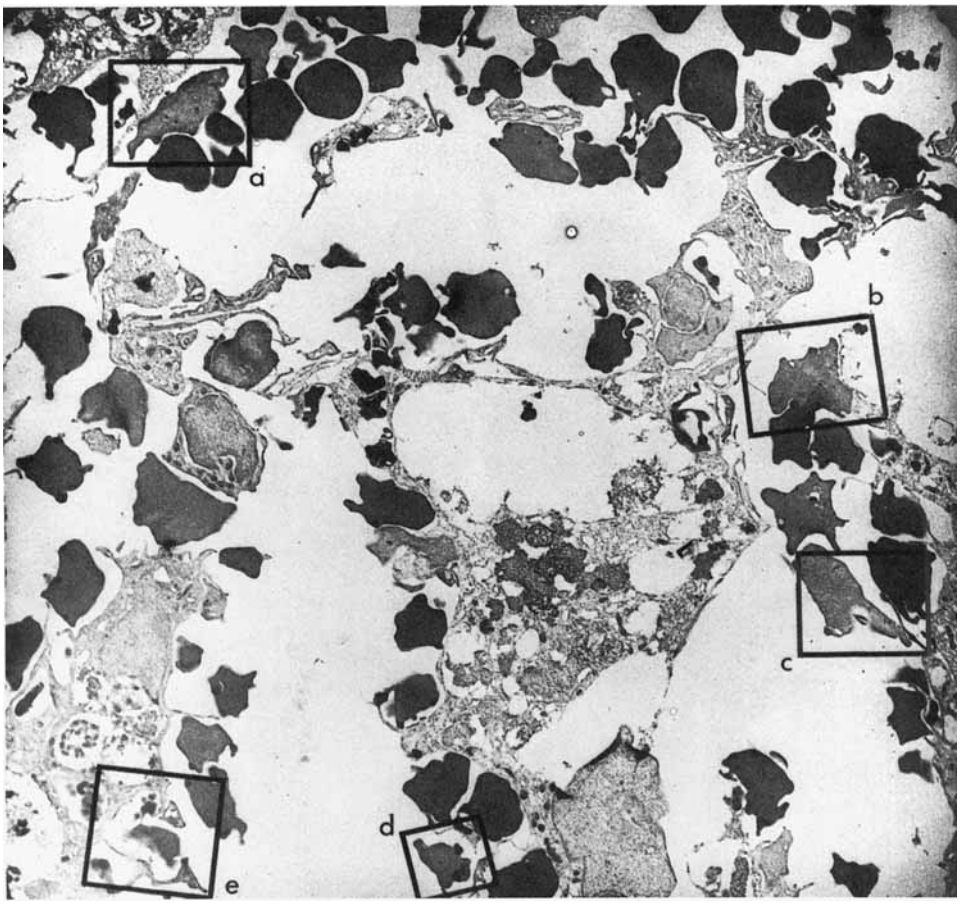
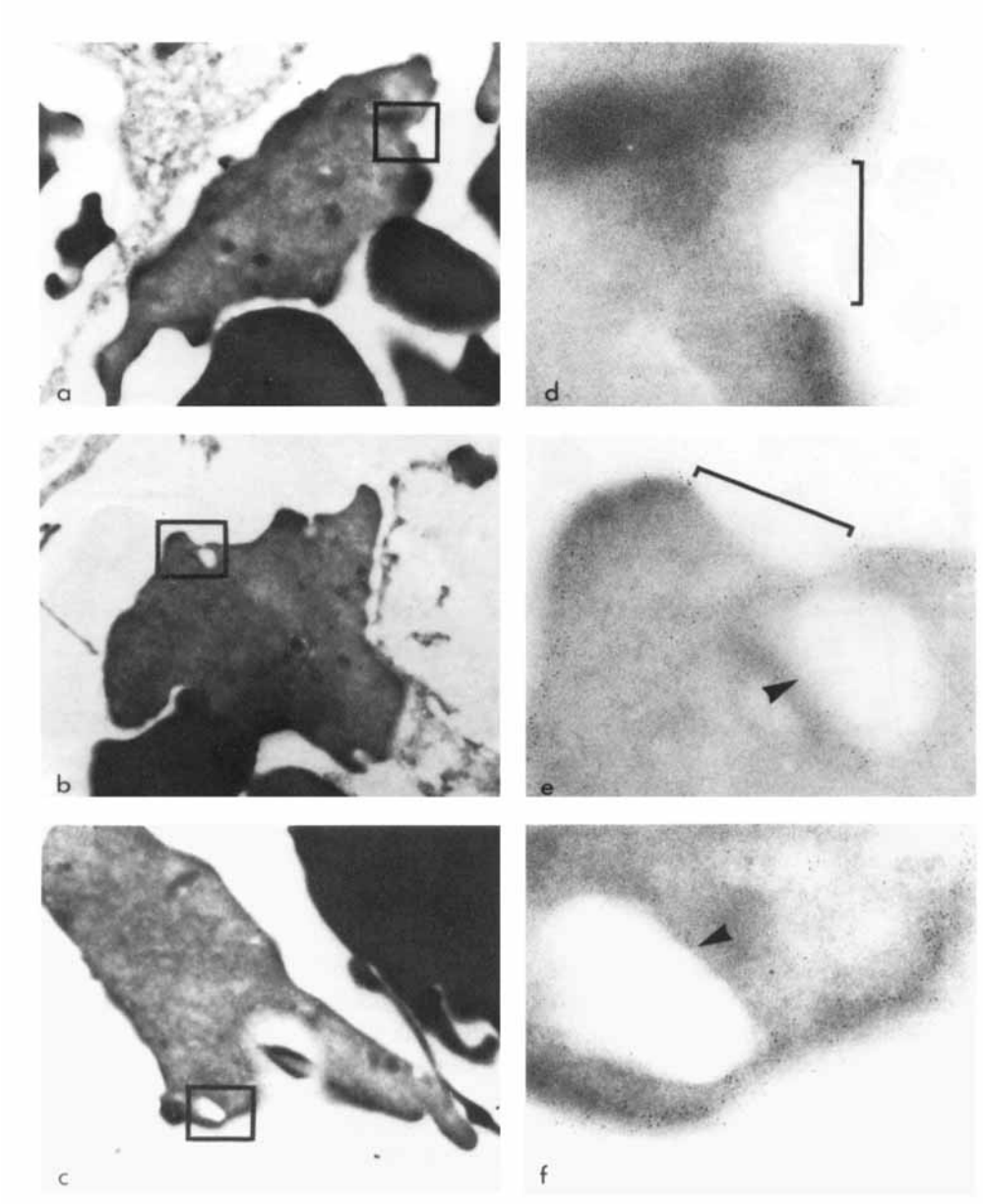


Fig. 2. Low magnification view of the red pulp of a phenylhydrazine-treated rabbit spleen, immunoferritin-labeled for spectrin. The enclosed areas indicate selected cells shown in greater detail in subsequent figures. Areas (a), (b), and (c) are stress reticulocytes (detailed in Fig. 3). Area (d) is of an erythrocyte containing Heinz bodies (detailed in Fig. 4). Area (e) contains an erythrocyte in the process of having its Heinz body phagocytosed by a reticular cell. (detailed in Fig. 6). Magnification, × 2,800.

In Figure 3 are shown higher magnification views of the reticulocytes in enclosed areas a, b, and c of Figure 2. That these are indeed reticulocytes is indicated by the hemoglobin density in their cytoplasms, which is clearly less than that of nearby erythrocytes; and by the presence of mitochondrial and other debris inside the cells (Fig. 3 a, b, c). In all three cells there is evidence of endocytotic activity, including cell surface invaginations (Fig. 3 d, e) and intracellular vesicles* near the cell surface (Fig. 3 e, f). These and approximately 50 other such invaginations seen

*In a parallel study, spleens from phenylhydrazine-treated animals were embedded in plastic and serially sectioned. The results (not shown) demonstrated that >95% of the membrane-bound vesicular structures inside the reticulocytes were indeed intracellular vesicles and not plasma membrane invaginations that had been sectioned. This was true of membrane-bound structures located at different distances from the plasma membrane in the section.

on reticulocyte membranes were all remarkably free of spectrin labeling (Fig. 3 d, e, f), although at the immediate edges of the invaginations, the spectrin labeling always sharply increased to a density that of unperturbed regions of the same cell membranes. Similarly, the intracellular vesicles of the reticulocytes were devoid of spectrin labeling (Table I, top row).



enclosed areas (a), (b), (c) of Figure 2 Selected areas of these reticulocytes are in turn indicated by enclosures, which are magnified in (d), (e), and (f) These show that the spectrin label is highly depleted on both surface invaginations (bars) and intracellular vesicles (arrows heads) Magnification (a), (b), (c), × 11,000, (d), (e), (f), × 70,000

Erythrocytes in this preparation showed much less evidence of endocytotic activity than reticulocytes. The incidence of apparent intracellular vesicles in erythrocytes was more than 20-fold lower than that of reticulocytes, as previously demonstrated [18], and some of these might have been sectioned-through surface invaginations. In any event, they were invariably labeled for spectrin (Table I, second row), in contrast to the vesicles within the reticulocytes. The erythrocyte indicated in Figure 2d is shown in higher magnification views in Figure 4. Areas of surface indentations shown in Figure 4b and c were, by contrast with the reticulocytes, uniformly labeled for spectrin. Where a Heinz body was closely apposed to

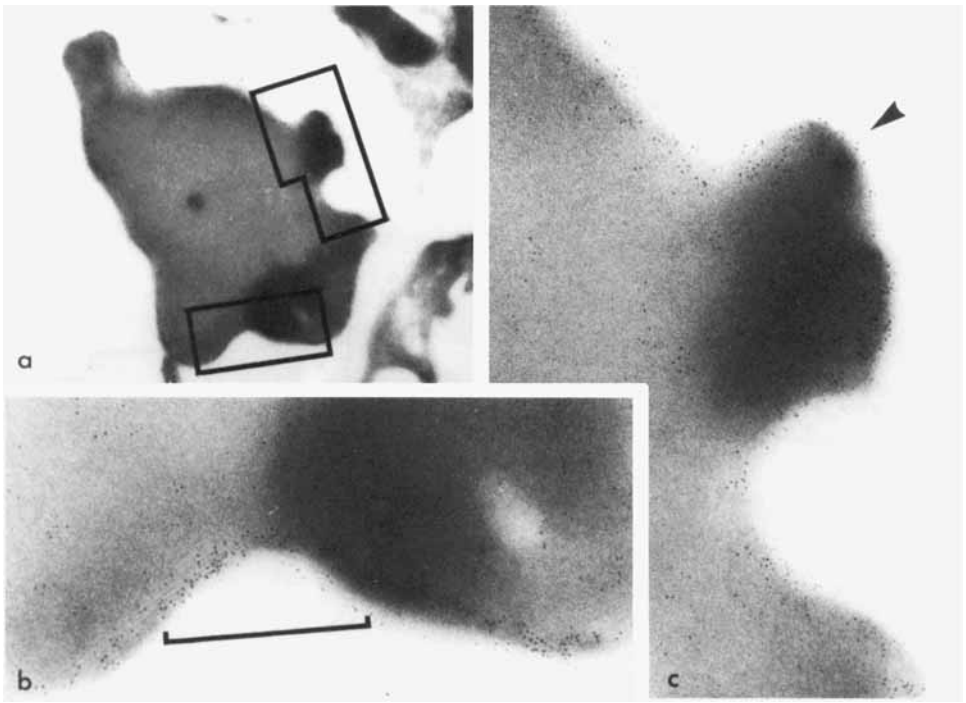


Fig. 4. Higher magnification view of the erythrocyte shown in Figure 2, enclosure (d). (a) View of the entire erythrocyte. A Heinz body and a concave portion of the membrane are indicated by enclosed areas. (b) High magnification view of the lower enclosed area in (a), showing dense spectrin labeling in the concave portion indicated by the bracket. (c) High magnification view of the upper enclosed area in (a), showing a Heinz body (arrowhead) and surrounding membrane exhibiting dense spectrin labeling throughout the Heinz body region. Magnification: (a), × 20,000; (b), (c), × 70,000.

TABLE I. Spectrin Labeling of Vesicles in Splenic Erythroid Cells

Treatment	Cell type	Numbers of cells examined	Vesicles without spectrin labeling	Vesicles with spectrin labeling
Phenylhydrazine	Reticulocyte	33	27	2
	Erythrocyte	267	0	11
Normal	Reticulocyte	10	7	0
	Erythrocyte	100	0	1

the distorted cell membrane (Fig. 4c, arrowhead), there was likewise no diminution of spectrin labeling.

These experiments were carried out with phenylhydrazine-treated animals in order to observe a high frequency of reticulocytes. However, such so-called stress reticulocytes are known to have properties that are different from normal reticulocytes [26]. In order to determine whether the results described above also apply to normal cells, spleens from untreated normal rabbits were then examined. Reticulocytes are, of course, much less frequent in the normal than in the anemic spleen sections. Some typical results are presented in Figure 5. A reticulocyte is shown that has two surface invaginations, both of which at high magnification were devoid of spectrin labeling along their contours. In other normal reticulocytes (not shown, see Table I, third row) intracellular vesicles were observed that were not labeled for spectrin. No significant numbers of vesicles were seen in the normal erythrocytes (Table I, fourth row).

Upon detailed examination, significant quantitative differences between the properties of stressed and normal reticulocytes could be demonstrated. With in-

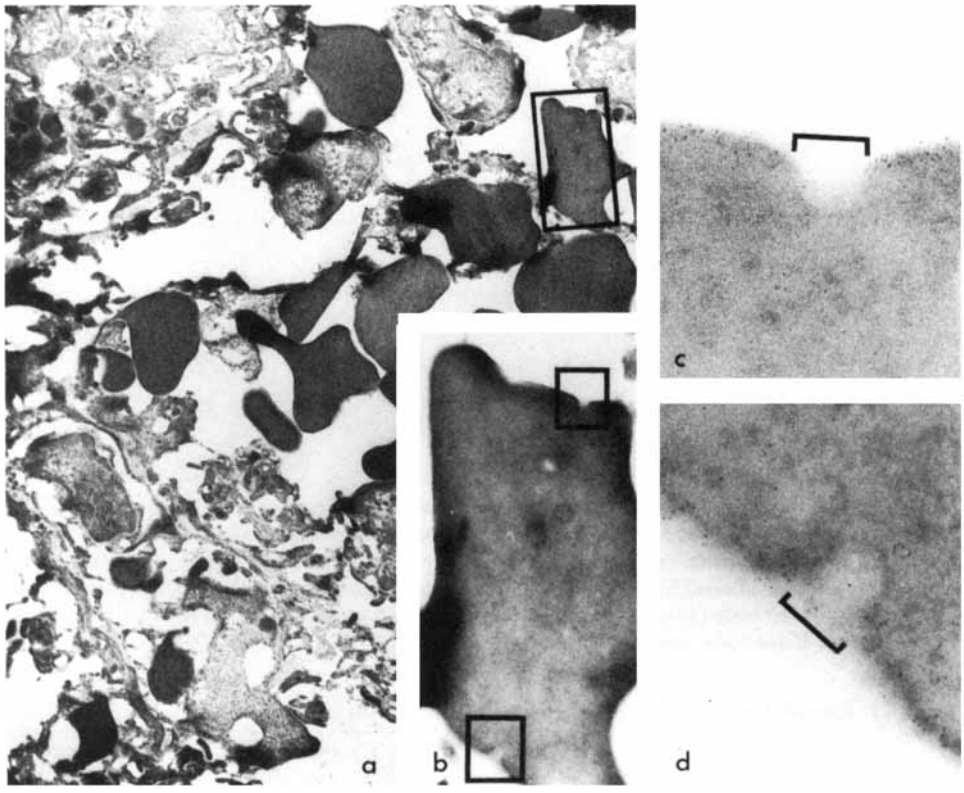


Fig. 5. Normal rabbit spleen (a) Low magnification view of the red pulp, showing a reticulocyte (indicated by the enclosed area). (b) Higher magnification view of the normal reticulocyte shown in (a). Two presumed endocytotic invaginations are present (indicated by enclosures). (c), (d) Higher magnification views of the areas indicated by the enclosures in (b). The spectrin label is highly depleted in the membrane lining of the endocytotic invaginations indicated by the brackets. Magnification: (a), × 4,700; (b), × 15,000; (c), (d), × 70,000.

dividual sectioned cells, the length of the cell membrane that was in surface invaginations devoid of spectrin labeling was divided by the total length of the membrane that was spectrin labeled, to give the fraction f_i. This was done for 10 randomly selected normal reticulocytes and 13 stress reticulocytes. The value of f_i was 0.012 ± 0.010 for the normal cells and 0.018 ± 0.015 for the stressed cells. This difference cannot be considered significant. However, we also measured the fraction f_v of the membrane perimeter that was in the form of intracellular vesicles free of spectrin labeling in the same cells. The value of f_v was 0.024 ± 0.030 for the normal reticulocytes and 0.066 ± 0.030 for the stressed cells. Therefore, the relative amount of spectrin-free membrane found in intracellular vesicles in stressed reticulocytes at any one time was significantly greater than in normal cells.

Further indication that the spectrin depletion of invaginations on reticulocyte surfaces is significant is given by observations that even those membrane regions of reticulocytes and erythrocytes that were highly deformed and were presumably under considerable stress still exhibited the normal density of spectrin labeling. Figure 6 contains higher magnification views of the erythrocyte in Figure 2e, apparently undergoing removal of its Heinz body by phagocytosis. The Heinz body is being pulled away from the main portion of the erythrocyte, leaving a thin connection between them (Fig. 6a). The membrane of this connection was labeled normally for spectrin (Fig. 6b). Figure 7a shows a different area of the same specimen shown in Figure 2. This area contains an erythrocyte (b) and a reticulocyte (c), por-

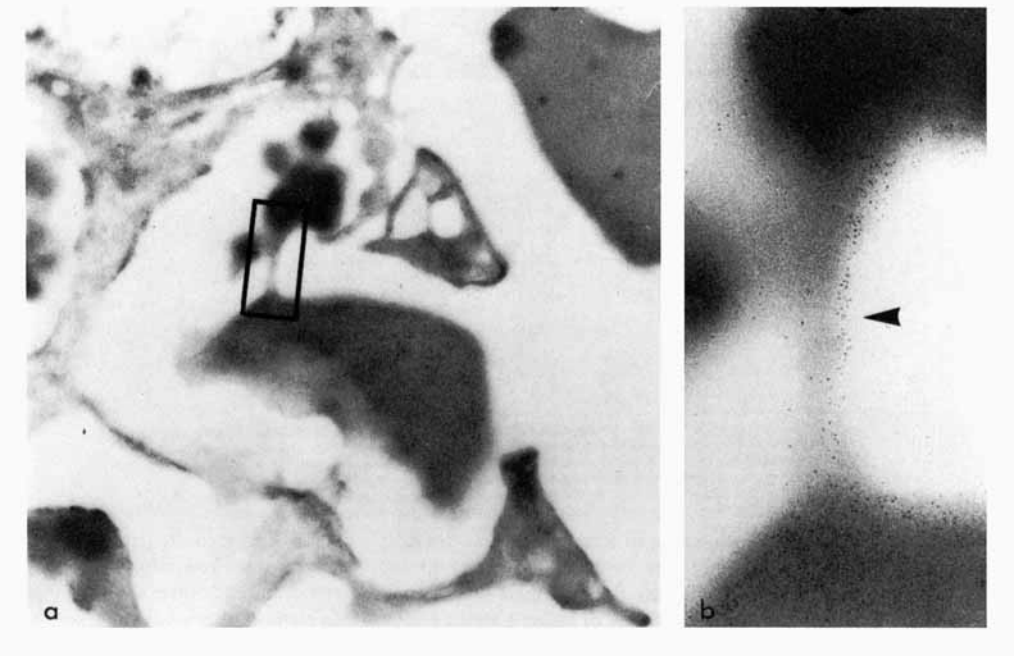


Fig. 6. (a) Higher magnification view of the erythrocyte shown in Figure 2, enclosure (e), with a Heinz body undergoing phagocytosis by a reticular cell. The membrane bridge connecting the Heinz body to the bulk of the erythrocyte is indicated by an enclosed area. (b) High magnification view of the enclosed area in (a), showing that a normal high concentration of spectrin is present in the connecting membrane bridge (arrowhead). Magnification, (a), × 17,000; (b), × 40,000.

tions of whose membranes were severely distorted. These distorted regions at higher magnification [(d) and (e), respectively] showed the normal density of spectrin labeling. Mechanical deformation alone, therefore, would not appear to account for the depletion of spectrin from the invaginations on the reticulocyte membranes.

DISCUSSION

The new experimental results described in this paper demonstrate that rabbit reticulocytes found within the spleen regularly exhibited surface invaginations and intracellular vesicles whose membrane elements were depleted of spectrin. Such

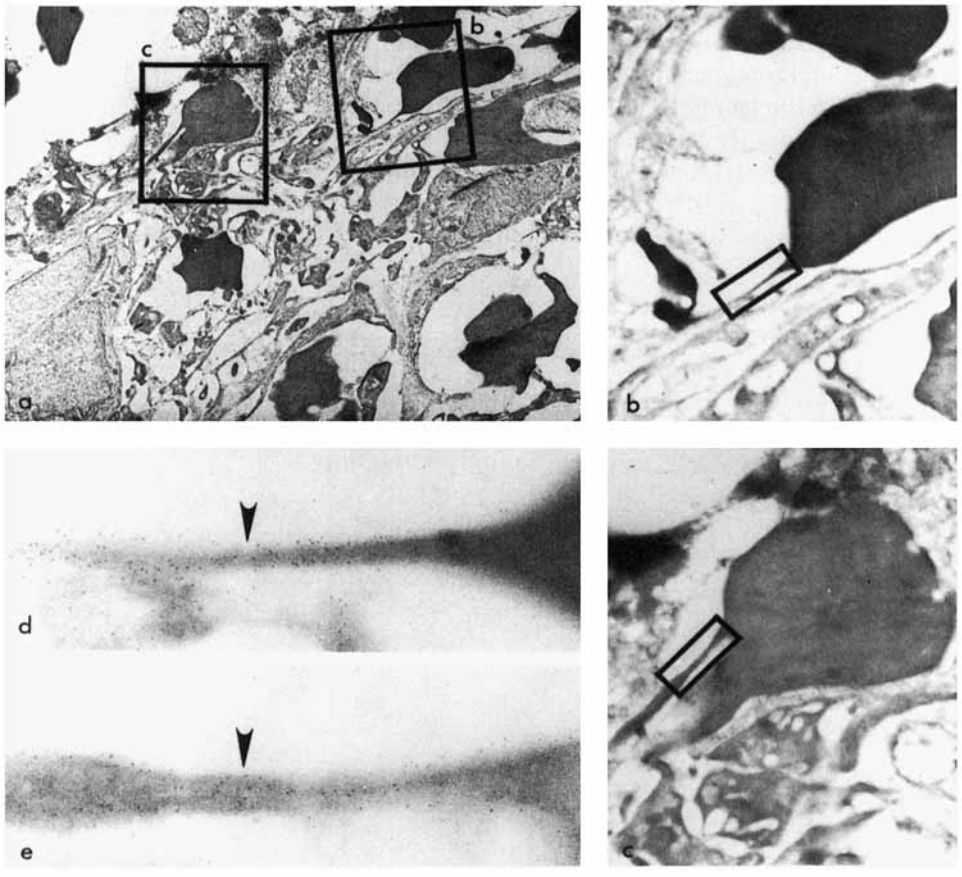


Fig 7 **(a) Low magnification view of a reticulocyte** (enclosed area c) **and an** erythrocyte (enclosed area b) undergoing severe membrane distortion in passage through the cords of the red pulp of a phenylhydrazine-treated rabbit spleen (b), (c) Higher magnification views of the enclosures shown in (a) (b) Erythrocyte apparently in the process of losing a Heinz body A thin membrane bridge is indicated by the enclosure and is shown magnified in (d) (c) Reticulocyte under conditions of membrane distortion A stretched portion of the membrane indicated by the enclosure is shown magnified in (e) (d), (e) High magnification views showing normal concentrations of spectrin in the stretched portions of both erythrocyte (d) and reticulocyte (e) membranes Magnification, (a), × 3100, (b), (c), × 11,000, (d), (e), × 71,000

spectrin-free membrane elements were not seen in erythrocytes present in the same splenic sections. Even when portions of reticulocytes or erythrocyte membranes were placed under stress, as during the envelopment and removal of Heinz bodies or during severe distortion in the spleen, they retained their normal densities of associated spectrin. The spectrin-free surface invaginations and intracellular vesicles of reticulocytes are therefore considered to be physiologically meaningful structures.

The mechanisms that generate these spectrin-free structures in vivo are not known. The parallelism of these in vivo structures, however, to the spectrin-free invaginations and intracellular vesicles that are induced in vitro upon incubation of reticulocytes with con A (as described above) suggests that similar mechanisms may be involved in both cases. In the case of con A induction of these phenomena, the evidence indicates that after attachment of the con A to con A-binding proteins in the membrane, the latter are cross-linked and induced to become clustered into the mobile domains of the membrane, which are – or which become – spectrin free. These regions of the membrane are then invaginated and endocytosed by some unknown mechanochemical mechanism.* We may speculate, therefore, that in vivo some ligand(s) or factor(s) bind to their specific receptor(s) on the reticulocyte membrane; such binding induces those receptor molecules to become clustered within the mobile domains of the membranes, which are – or which become – free of spectrin. This is followed by the invagination of these membrane regions and endocytosis of the invaginations. This process as described not only would remove spectrin-free membrane from the reticulocyte surface, but also would remove specific surface membrane receptor(s) as maturation proceeded to the erythrocyte stage.

It is known that certain membrane components of mammalian reticulocytes are markedly decreased in mature erythrocytes. There is, for example, a large reduction in the numbers of receptors for transferrin [38] and for insulin [39] in the membranes of erythrocytes compared to those of reticulocytes. It has also been reported [40] that a membrane protein of molecular weight 33,000 and of unknown function is detected in reticulocyte, but not erythrocyte, membranes. The process of specific ligand-induced endocytosis of surface components could be involved in the removal of one or more of these membrane proteins with reticulocyte maturation. This proposal could be tested in vitro by the application to reticulocytes of the appropriately labeled ligands for the receptors mentioned above, to determine by electron microscopic observations whether the ligand-receptor complexes are indeed endocytosed by reticulocytes, and whether this endocytosis occurs by way of spectrin-free invaginations [21].

The experiments described in this paper were carried out with reticulocytes found within the red pulp of rabbit spleens, but they were not performed on tissues from elsewhere in the circulation. We therefore have no evidence that the process

*One possibility is that a coated-pit mechanism [37] is involved in the invagination and endocytosis of the con A-clustered con A-binding proteins. We have no direct evidence, however, that coated pits are present in these reticulocytes, let alone whether they are involved in these events. Another possibility is that the spectrin-actin meshwork immediately surrounding a mobile domain in the reticulocyte membrane acts as a mechanochemical "noose" around the neck of the invagination, responding to some signal induced by the clustering event to cause a pinching off of the invagination as an intracellular vesicle.

of invagination and the production of intracellular vesicles is confined to the spleen. If the putative ligand(s) or factor(s) responsible for inducing these processes were to be soluble components present throughout the circulation, these events might occur outside as well as within the spleen. However, if such ligand(s) or factor(s) were released and concentrated in the spleen, these processes might be confined there.

If the formation of spectrin-free invaginations and their endocytosis were indeed involved in the physiologically relevant membrane remodeling events occurring during reticulocyte maturation, they should be able to account quantitatively for the magnitudes of the surface membrane changes that are produced during maturation. Unfortunately, there is too little information available from these experiments to make such calculations. The electron micrographs are "snapshots" of the reticulocytes. While they reveal that invaginations and intracellular vesicles are regular features of these cells, we do not know the residence times or fates of these structures. A particular invagination, furthermore, could be on its way into the cell interior, or it could be newly arrived at the cell surface by the fusion of an intracellular vesicle with the surface membrane. Nor do we have any idea how long a vesicle remains inside the reticulocyte before it fuses back to the surface membrane, is degraded intracellularly, or is exocytosed. Therefore, the *net* rate of removal of spectrin-free membrane from the reticulocyte occurring during the 2–3 days required for its maturation in the circulation cannot be reliably estimated from our present experiments. Qualitatively, however, if 1–2% of the reticulocyte surface is in the form of spectrin-free invaginations at any one time, it would seem that the formation of invaginations is a sufficiently frequent occurrence to allow it to be a predominant factor in reticulocyte membrane remodeling.

The higher frequency of intracellular vesicles in stress reticulocytes formed in phenylhydrazine-induced anemia as compared to normal reticulocytes is consistent with the observations of Come et al [26] that stress reticulocytes, having on the average a larger area of surface membrane than normal cells, undergo a larger extent of membrane loss during maturation.

It is of interest that while spectrin-free invaginations on reticulocyte surfaces were found with reasonably high frequency, we rarely observed any evaginations, and never observed any spectrin-free evaginations on the same cell surfaces. Exocytosis of Heinz bodies was frequently seen, but the regions of the cell membrane enveloping the bodies invariably had the normal amounts of spectrin associated with them. There is therefore no evidence from our results for the proposition that reticulocyte membrane remodeling involves an exocytosis of spectrin-free evaginations [41, 42].

On Membrane Remodeling Events in In Vivo Maturation of Reticulocytes to Erythrocytes

On the basis of these results and considerations, we propose that the mechanism of the in vivo maturation of reticulocytes to erythrocytes includes the following critical features (Fig. 8):

1) The progressive endocytosis of spectrin-free regions of the reticulocyte membrane (Fig. 8, panel 1). This endocytotic process may arise as a result of the binding of ligands or factors to their specific receptor molecules in the reticulocyte membrane, the clustering of these receptors within the mobile domains of the

membrane, and the subsequent invagination and pinching-off of these spectrin-free membrane regions to form intracellular vesicles. This process, possibly reversible, continues until the mobile domains of the reticulocyte are eventually eliminated. It is likely that these events are not confined to the spleen, but occur throughout the circulation.

2) The progressive elimination of the endocytosed vesicles. This could occur by intracellular degradation of the vesicles, by autophagy [27, 29], or by an exocytotic process such as is suggested schematically in the lower two panels of Figure 8. It should be emphasized that we have no direct evidence for the exocytotic process depicted in Figure 8. The endocytosed spectrin-free vesicles may become associated with nonspecific sites on the inner face of the reticulocyte membrane

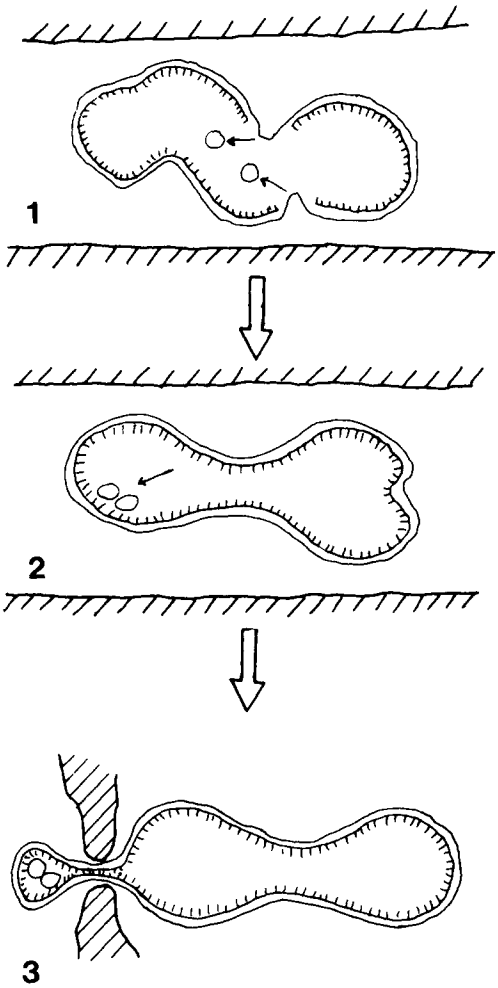


Fig. 8. A proposed scheme for the remodeling of reticulocyte membranes during the process of reticulocyte maturation to erythrocytes in vivo. Panel 1 depicts the invaginations of spectrin-free domains of the reticulocyte membrane occurring in the circulation, and their subsequent endocytosis. In panel 2, the endocytosed vesicles are pictured as associating with the membrane either to fuse with it or to be exocytosed. In panel 3, the exocytosis of the enclosed vesicles is pictured as occurring by mediation of the spleen. See text for further details.

(panel 2, Fig. 8). Following this stage, and probably mediated by the spleen, the vesicles could then be eliminated by exocytosis along with a surrounding portion of the reticulocyte membrane (panel 3, Fig. 8), pictured as retaining its normal density of spectrin (see Fig. 6b). The exocytotic process depicted, therefore, does not itself produce any changes in the density of spectrin or of integral proteins associated with the remaining cell membrane.

That the endocytosed spectrin-free vesicles may indeed be eliminated from the maturing reticulocyte by the process proposed in Figure 8 is suggested by the finding of many investigators [25–29] that the erythrocytes in the circulation of a splenectomized animal are larger than the corresponding cells of normal animals, and contain a large number of vesicular and other inclusion bodies.

The net result of these two membrane remodeling phenomena put forward in this hypothesis of reticulocyte maturation would be to contribute to 1) the reduction of the surface area of the reticulocyte; 2) the elimination of the mobile domains in the reticulocyte membrane and an increase in the density of the spectrin complex under the membrane. This increase in spectrin density might then promote the eventual acquisition of the biconcave disc shape of the mature erythrocyte; and 3) possibly the removal of certain specific membrane components from the reticulocyte membrane and their consequent depletion in the mature erythrocyte membrane.

None of this implies that many other phenomena may not also play prominent roles in reticulocyte maturation. In particular, metabolic changes must certainly occur inside the reticulocytes as they mature, and these changes may also be important in determining the properties of the resultant erythrocyte. Nevertheless, the hypothesis discussed above contains specific features that can be experimentally tested, and it may therefore eventually contribute to a deeper understanding of the molecular basis for the maturation of mammalian reticulocytes.

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