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CYTOCHALASIN B-BINDING PROTEINS IN RABBIT ERYTHROCYTE MEMBRANES AND THEIR POST-NATAL CHANGE IN RELATION TO THE GLUCOSE CARRIER ACTIVITY

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Summary

Two distinct, carrier-mediated glucose uptake processes, a fast, cytochalasin B-sensitive and a slow, cytochalasin B-insensitive flux are identified in parallel in newborn rabbit erythrocytes. The fast, cytochalasin B-sensitive carrier function disappears as rabbits age, and only the slow cytochalasin B-insensitive carrier function is observed with adult rabbit erythrocytes.

Three different cytochalasin B binding sites are distinguished in newborn rabbit erythrocytes; a glucose-sensitive site (site I), a cytochalasin E-sensitive site (site II), and a site insensitive to both glucose and cytochalasin E. With adult rabbit erythrocytes, only a cytochalasin E-sensitive site is detected. The glucose-sensitive site disappears as rabbits age, with a time course which is comparable to that of the disappearance of the cytochalasin B-sensitive glucose carrier function. The cytochalasin E-sensitive cytochalasin B binding site does not increase during this change, thus the disappearance of the glucose-sensitive site is not due to its conversion to a cytochalasin E-sensitive site. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of rabbit erythrocyte ghosts revealed a partial decrease in each of the membrane polypeptides of approximate molecular weights of 240 000, 160 000 and 50 000 as rabbits aged. It is concluded that the cytochalasin B-sensitive glucose carrier of fetal rabbit erythrocytes, like that of the human erythrocyte, is tightly associated with the site I cytochalasin B-binding protein, while the cytochalasin B-insensitive glucose carrier, operative in adult rabbit erythrocytes, is not.

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Introduction

In human adults and fetal erythrocytes glucose transport across the membrane is carrier-mediated and is very rapid [1—3]. With most mammalia erythrocytes, however, the rapid glucose movement is seen only in fetal cells [1] and its transient presence after birth appears to be limited to the fetal cell lifetime [4]. Adult bovine [5] and rabbit [6,7] erythrocytes thus lack the rapid transport system found in human red blood cells.

Cytochalasin B inhibits this carrier-mediated, rapid glucose transport in human erythrocytes [8—11]. The inhibitor binds to human erythrocyte membranes at three different sites [11] and the binding at one of these sites (site I) is displaced specifically by D-glucose [11,12]. Based on this and other evidence, this site was tentatively identified as the glucose carrier [11]. Cytochalasin B also binds to adult bovine and adult rabbit erythrocytes, but this binding is not affected by D-glucose [12]. These suggested that study on cytochalasin B binding to fetal and post-natal erythrocytes and on its effect on their glucose transport function might provide important information regarding the molecular entity of the glucose carriers operating in these cells.

In this paper we have studied the cytochalasin B binding to baby and adult rabbit erythrocyte membranes and its effect on glucose transport as a function of the age of rabbits and the relationship of this change to the disappearance of the rapid glucose permeability. Our findings indicate that a glucose carrier similar to that of human erythrocytes is present in fetal rabbit erythrocytes; only the rapid flux seen in fetal rabbit erythrocytes, but not the slow flux seen in adult rabbit cells, is cytochalasin B-sensitive. The three types of cytochalasin B binding sites similar to that of human erythrocytes are present in fetal rabbit erythrocyte membranes. The fast component of the glucose uptake and the glucose-sensitive cytochalasin B binding both progressively disappear as rabbits age, with similar decay constants. Significant differences in some membrane polypeptides between neonatal and adult rabbit erythrocytes were revealed on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

Materials and Methods

Ten to twelve 4-day old rabbit littermates were obtained from an animal farm and kept with their mother in the VA Medical Center Animal Facility. Fresh blood of baby rabbits was obtained by cardiac puncture. With adult rabbits, the blood was obtained by tapping an ear vein. In both cases heparin was used as an anticoaggulant. Erythrocytes were washed three times in 10 vols. of physiological saline, stored in a coldroom and used within 36 h. White ghosts [13] were prepared from washed erythrocytes by the method similar to that of Dodge et al. [14]. Cytochalasins B and E were obtained from Aldrich Chemical Company. [^{3}H]cytochalasin B, [^{14}C]glycerol, D-[^{14}C]glucose, D-[^{14}C]galactose, 3-*O*-methyl-D-[^{14}C]glucose and [^{14}C]mannitol were from New England Nuclear.

Transport of sugars and related compounds across rabbit erythrocytes membranes was studied by following either a 60 min or a 15 s timecourse of the uptake of radioactive tracer of permeants at 23°C. Intact erythrocytes were used for all of the transport measurements in this study. Both the 60 min

uptake experiment [14] and the 15 s equilibrium experiment [15] were performed as detailed previously.

Equilibrium binding of cytochalasin B to the erythrocyte membrane was determined at 23°C by a tracer technique using [^{3}H]cytochalasin B, as described elsewhere [11]. White ghosts were used for all of the binding studies. Two types of the ligand binding experiements [11], the standard binding experiment and the displacement experiment, produced identical results.

SDS acrylamide gels were prepared with 5.6% acrylamide using 6 mm (inner diameter) tubes. Electrophoreses were run at pH 7.4 using pyronin Y as a tracking dye. Gels were stained with Coomassie blue according to Fairbank et al. [16]. Coomassie-stained gels were scanned at 550 nm with the Gilford 240 spectrophotometer equipped with a linear transport accessory. Protein was assayed using the method of Lowry et al. [17].

Experimental results

Age-related change in glucose carrier activity of rabbit erythrocytes

Fig. 1 shows 60-min time course of the uptake of several sugars and their analogs by the erythrocytes of an adult rabbit. Glycerol was completely equilibrated within less than two min, whereas D-mannitol was not taken up to any noticeable degree. D-glucose was taken up at an appreciable speed although this was much slower than that of glycerol. The rate of D-fructose uptake was approx. 10-fold lower than that of D-glucose, but significantly higher than that of D-mannitol. These results would indicate that a specific mechanism exists in adult rabbit erythrocyte membranes which selectively facilitates hexose uptake.

The rate of D-glucose uptake varied with individual rabbits ranging between 0.5—1.0 μmol/ml cells per h (at 23°C, with 0.5 mM D-glucose). It increase nonlinearly as the sugar concentration was raised, following a saturation kinetics with a K_m of 1.4 mM and a V of 0.04 μmol/ml cell per min at 23°C *. Since the energy of activation of the system was 18—23 kcal/μmol * these values are reasonably comparable with the reported values [7] for K_m of 6 mM and V of 0.23 μmol/ml cells per min at 37°C for this system. Compared with the human erythrocyte system [18], V is approx. 6000-fold lower and K_m is 2—5-fold lower. Confirming previous reports [7], this apparently carrier-mediated flux of D-glucose was insensitive to $HgCl_2$ at the concentration as high as 200 mM (data not shown), also indicating that the mechanism is different from that of the glucose carrier of human erythrocytes. Cytochalasin B, at the concentration range up to 10^{-5} M did not inhibit the D-glucose uptake by adult rabbit erythrocytes (data not shown). This is also in variance with the glucose uptake by human erythrocytes, which is completely inhibited by 10^{-5} M cytochalasin B [11].

Rates of the uptake of D-glucose by the erythrocytes of eight-day old rabbits appeared much greater than that of adult rabbits (Fig. 2). The time course of glucose uptake by the baby rabbit erythrocytes was biphasic, revealing a very fast component and a slow component (Figs. 2 and 3). The exact time course

* Data to be published.

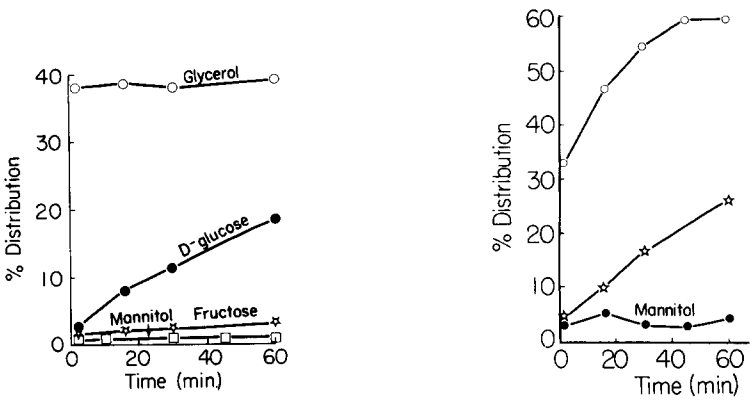

Fig. 1. Time course of the net uptake of glycerol, D-glucose, D-fructose, and D-mannitol by adult rabbit erythrocytes. To 2-ml suspensions of washed erythrocytes (50% hematocrit) in saline, pH 7.4, buffered with 20 mM Tris, 0.1 ml of the buffered saline containing appropriate amounts of a given permeant and its radioactive tracer (0.2 μCi) were added and immediately mixed (at $t = 0$). A series of 0.2-ml samples were taken into hematocrit capillary tubings at specified time points and packed cell columns and supernatants were separated by centrifugation (Hematocrit centrifuge, Clay Adams) in a coldroom for three min. The tubings were cut at the interface of the cell column and the supernatant and radioactivities of both portions were measured, from which the percent distribution of the permeants in cells was calculated. The time on abscessa indicates the time 1 min after each sampling. The concentrations of permeants in the suspension were 110 mM for glycerol and 0.5 mM for the sugars and mannitol. Temperature was 23°C.

Fig. 2. Time courses of the net uptake of mannitol and of D-glucose in the absence (○) and in the presence (★) of 10^{-5} M cytochalasin B by 8-day-old baby rabbit erythrocytes. Cytochalasin B was added to cell suspension 10 min before the addition of permeants. Experimental procedures were otherwise identical to those of Fig. 1.

of the fast component was studied by following 3-*O*-methyl-D-glucose equilibrium exchange (Fig. 4). A $t_{1/2}$ of 5.1 s (an average of five measurements, at 23°C, with 1 mM sugar) was estimated for this fast flux. The speed of the slow component (Fig. 3) was only slightly greater than that of the sugar uptake by adult rabbit erythrocytes. D-mannitol was not taken upt at all by the baby rabbit erythrocytes (Fig. 2). This would indicated that both the fast and slow components are mediated by mechanisms that are specific to sugars.

In contrast to the case with adult rabbit erythrocytes, both $HgCl_2$ and cytochalasin B affected the glucose uptake by the erythrocytes of an 8-day old rabbit (Fig. 4). However, this inhibition was selective, affecting only the fast component of the uptake (Figs. 2 and 4), the slow component remained practically unaltered (Fig. 2). Both the sensitivity of the fast component to those inhibitors and its fast flux rate are reminiscent of the glucose across human erythrocyte membrane. This suggest that the fast component of the glucose uptake by baby rabbit cells may be mediated by a carrier which is identical to that of the human erythrocyte.

Time courses of the glucose uptake by the erythrocytes of baby rabbits of varying ages are compared in Fig. 3. It is readily noted that the relative size of the fast uptake component decreases gradually as rabbits age. Replot of the change in the size of this component (Fig. 5) revealed an exponential decay.

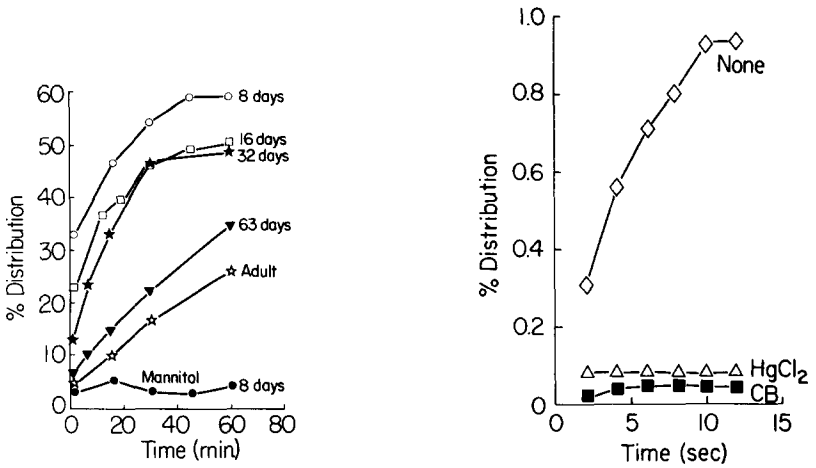

Fig. 3. Time courses of the net glucose uptake by the erythrocytes of rabbits of varying ages. Data of the glucose uptake experiments similar to that of Fig. 2 using erythrocytes of rabbits of specified age, were compared based on the two-min uptake of mannitol and 60-min uptake of D-glucose as shown in Fig. 2.

Fig. 4. Time course of 3-*O*-methyl-D-glucose equilibrium exchange by erythrocytes of 8-day-old baby rabbits in the absence (none) and in the presence of 10^{-3} M $HgCl_2$ or 10^{-5} M cytochalasin B. Washed erythrocytes were suspended in 10 ml of physiological saline, pH 7.4, buffered with 20 mM Tris at 4% hematocrit, and incubated at 37°C for 60 min with 0.5 mM 3-*O*-methyl-D-glucose. $HgCl_2$ or cytochalasin B, where used, was then added to the suspension in a small volume (0.1 ml). After 15 min at room temperature, the 15-s time course of the tracer uptake was followed by adding a tracer amount of [^{14}C]3-*O*-methyl-D-glucose to the suspension at $t = 0$. Six aliquots were taken at an equal time interval; each was immediately mixed with eight vols. of chilled containing 2 mM $HgCl_2$, and the cells were separated as a pellet from the supernatant by centrifugation (10 000 × *g*, for 10 min at 4°C with SS34 rotor in Sovall, RC-2B). Data presentation was the same as that of Fig. 1.

The half-time of this decay is fairly comparable to a reported half-life (21.3 days) of rabbit erythrocytes [19]. It appears that the fast component represents the glucose uptake by a distinct cell population, most likely prenatal erythrocytes, and that the disappearance of the fast component is a result of the disappearance of this population from circulation. A less drastic but significant reduction with age was also apparent in the rate of the slow uptake component (Fig. 3). The nature of this reduction was not studied further.

Age-related changes in cytochalasin B binding activity of rabbit erythrocyte membranes

Adult rabbit erythrocyte membranes bind cytochalasin B (Figs. 6 and 7). In the concentration range of cytochalasin B tested, the binding consisted of a linear component and a relatively small saturable component (Fig. 6). Scatchard analysis (data not shown) of the saturable binding revealed non-linearity with a range of values for the K_d (apparent dissociation constant) and B_o (the maximum binding capacity) of $0.1—1.0 \cdot 10^{-7}$ M and 35—40 pmol/mg dry mebrane (1.0 mg dry membrane represents 0.55 mg membrane protein), respectively. This binding was not affcted by 500 mM D-glucose (Figs. 6 and 7). More than 95% of this cytochalasin B binding, however, was displaced by cytochalasin E with an apparent displacement constant K_i of approx. 10^{-7} M (Fig.

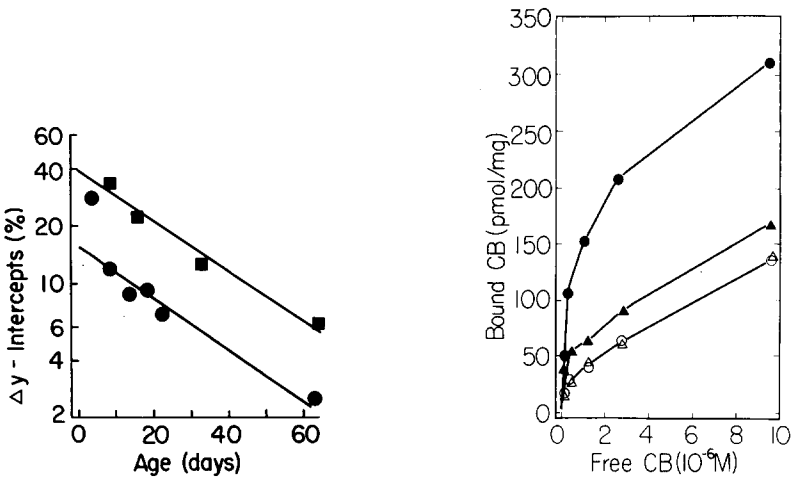

Fig. 5. Change in the relative sizes of the fast component of the D-glucose uptake (■) and of the glucose-sensitive portion of the cytochalasin B binding (●) by rabbit erythrocytes as a function of the age of rabbits. Relative size of the fast uptake component was estimated by taking the difference (% distribution) between the two-min uptakes of D-glucose and D-mannitol for each age from the data of Fig. 3. Relative size of the glucose sensitive component of cytochalasin B binding was estimated as fractional displacement of cytochalasin B binding by 500 mM D-glucose from the data of Fig. 7. Solid lines are drawn with a slope of 0.0325 day^{-1} and intercepts chosen arbitrarily to fit data points by eyes.

Fig. 6. Binding of cytochalasin B to adult (open symbols) and 5-day old (solid symbols) rabbit erythrocyte ghosts as a function of ligand concentrations. Incubation mixture contained 1.0 mg freeze-dried ghosts, 500 μmol, 0.01 μCi [^{3}H]cytochalasin B, and a given concentration of cytochalasin B in isotonic saline in a final volume of 1 ml. After a 30-min incubation at 23°C, the mixture was centrifuged (15 000 × *g*, 20 min at 4°C) and supernatants were separated from pellets. Trapped media in the centrifuge tubes containing pellets were removed by a careful wiping with cotton swabs. Radioactivities of both supernatants and pellets were assayed, from which the amounts of the free and bound (pmol/mg freeze-dried ghosts) cytochalasin B were calculated. Each data point represents an average of duplicate measurements, in the absence (circles) and in the presence (triangles) of D-glucose.

7). The insensitivity to D-glucose, the sensitivity to cytochalasin E and the value of K_i for cytochalasin E of this saturable cytochalasin B binding of adult rabbit erythrocyte membranes are all reminiscent of the site II cytochalasin B binding of the human erythrocyte membrane [11]. The fact that adult rabbit erythrocytes lack the glucose sensitive, site I cytochalasin B-binding component, which we have shown to be responsible for the inhibition of the glucose carrier in human erythrocytes by cytochalasin B [11], is consonant with the fact that cytochalasin B does not affect the glucose uptake in adult rabbit erythrocytes as observed above.

Erythrocyte membranes of five-day old rabbits also bind cytochalasin B (Figs. 6 and 7). The binding consisted of a linear component and relatively a large saturable component (Fig. 6). Scatchard analysis (not shown) of the saturable component of this data revealed a nonlinearity with a range of the K_d values of $0.5—5.0 \cdot 10^{-7}$ M and the B_o of approx. 200 pmol/mg dry membrane. It is of note that the B_o is approximately 5-fold greater than that of adult rabbit erythrocytes and is slightly less compared with that of human erythrocytes, [11]. In contrast to adult rabbit erythrocytes, approximately two-thirds

of this binding was displaced by D-glucose (Fig. 6) and only a part of this binding was displaced by cytochalasin E (see below).

At maximum, approx. 30% of the saturable binding was displaced by cytochalasin E when free cytochalasin B concentration was $3—4 \cdot 10^{-8}$ M (Fig. 7). The half-maximum of this displacement occurred at a cytochalasin E concentration of less than $2 \cdot 10^{-7}$ M. D-Glucose displaced approximately another 30% of the cytochalasin B bound, when free cytochalasin B concentration was $3—4 \cdot 10^{-8}$ M (Fig. 7). The half-maximum of this displacement was achieved at a glucose concentration of approx. 40 mM. Even if cytochalasin E and glucose affected entirely different subpopulations, about 40% of the binding was shown to be unaffected by either cytochalasin E or glucose (Fig. 7). It is apparent, then, that, like human erythrocyte membranes, 5-day old rabbit erythrocyte membranes bindg cytochalasin B at least three different binding sites, a glucose-sensitive site (site I), a cytochalasin E-sensitive site (site II), and a site which is by itself insensitive to either glucose or cytochalasin E (site III).

Relative contribution of each of these three sites to the overall saturable binding as assessed at 10^{-7} M cytochalasin B (overall concentration) changed as

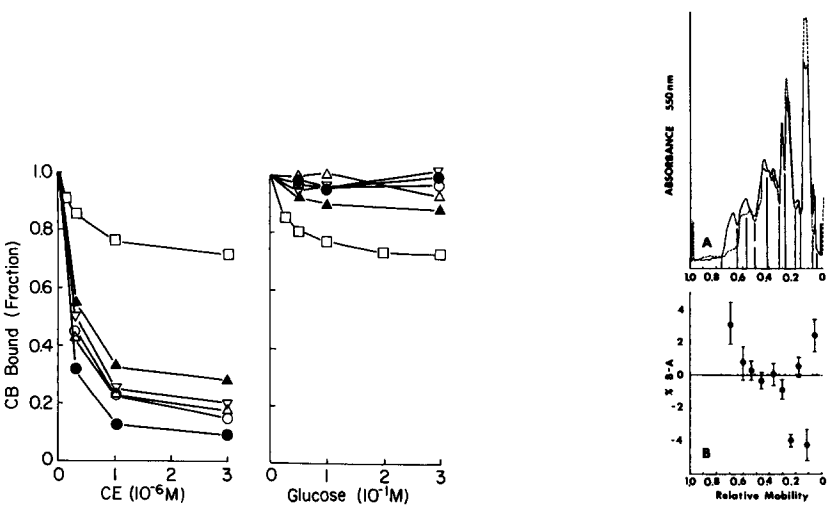

Fig. 7. Equilibrium binding of cytochalasin B to erythrocyte ghosts of rabbit at varying ages in the presence of an increasing concentration of cytochalasin E or D-glucose. Rabbits of 3 months (●), 26 days (○), 20 days (△), 14 days (▽), 8 days (▲), and 5 days (□) of age were used. Cytochalasin B concentration in the reaction mixture was fixed at 10^{-7} M. The binding was expressed as a fraction of the binding observed in the absence of cytochalasin E or glucose for each ghost preparation.

Fig. 8. Comparison of SDS acrylamide gel electrophoretic patterns of the erythrocyte membrane proteins of 5-day old rabbits and 3-month old rabbits. A 50 μg protein sample was applied to each gel. Absorbance scans of Coomassie-staining peptide patterns of the baby rabbit ghosts (solid line) and the adult rabbit ghosts (interrupted line) are superimposed in A after the mobilities for the two gels were normalized against each other. The traces were then divided into ten major band regions (as shown by vertical lines in A) and each region minus gel background (the absorbance at relative mobility 0.8—1.0) was cut out, weighed, and expressed as % total weight. For each band region, the % band weight of adult gel was subtracted from that of baby gel and the difference (B — A) was calculated. Similar analyses were carried out with the scans of four independent sets of the electrophoreses and the mean of the percentage differences ±95% confidence intervals are shown in B. The sume of the means of the percentaged (B — A) was —0.94, indicating that any point with percentage (B — A) greater than —0.94 represents an increase in peptide mass in baby cells over that in adult cells.

rabbits aged (Fig. 7). Cytochalasin E-sensitive binding drastically increased during the first week of neonatal life, and only gradually increased subsequently during maturation. Glucose-sensitive binding, on the other hand, decreased drastically during this period. Because of limited availability of baby rabbit cell samples, exact quantitation of these changes, which requires quantitation of the binding parameters at each age, was not attempted in this study. Nevertheless, the time course of the reduction in the glucose sensitive cytochalasin B binding and that in the size of the fast component of the glucose uptake appear to be similar (Fig. 5). This would strongly indicate that the glucose-sensitive, cytochalasin B bining and the cytochalasin B-sensitive fast component of the sugar uptake both occur in the same population that disappears as rabbits age.

Age-related changes in membrane polypeptides of rabbit erythrocytes

In an attempt to determine possible loss, gain or alteration in polypeptides of rabbit erythrocyte membranes causing the concomitant loss of the rapid glucose flux and the glucose-sensitive cytochalasin B binding, SDS polyacrylamide gel electrophoreses of erythrocyte membranes of baby rabbits and that of addult rabbits were compared (Fig. 8). The peptide bands [20] of apparent molecular weights 250 000—300 000, 160 000—180 000, 50 000—60 000 and 30 000—40 000 were found significantly more in baby rabbit cells than in adult rabbit cells. Polypeptides of apparent molecular weights of less than 20 000 were also noted in the baby cells. Whether any of these apparently age-related differences in polypeptide mass has relation to the age-related disappearance of the fast component of the glucose carrier activity and of the glucose-sensitive cytochalasin B binding is yet to be established.

Discussion

The uptake of sugar by erythrocytes of new-born rabbits is shown to be via two different carrier-mediated processes: a fast, cytochalasin B-sensitive process and a relatively slow, cytochalasin B-insensitive process. As rabbits age, the former gradually disappears; thus only the slow cytochalasin B-insensitive process is observable in adult rabbit erythrocytes. The fast process is very much similar to the carrier mediation of glucose by human erythrocytes, not only in its speed, but also in its sensitivity to cytochalasin B and $HgCl_2$.

Binding of cytochalasin B to the erythrocyte membranes of five-day old rabbits measured to 10^{-7} M cytochalasin B (overall concentration) (Fig. 7) indicates that the saturable binding of the ligand occurs at at least three different sites: a glucose-sensitive site (site I), a cytochalasin E-sensitive site (site II), and a site (site III) insensitive to both glucose and cytochalasin E when tested separately. This shows a striking resemblance to the cytochalasin B binding of the human erythrocyte membrane [11]. With human erythrocytes we have identified the site I cytochalasin B binding as being responsible for the inhibition of glucose carrier function by cytochalasin B. The total saturable binding sites of 5-day-old rabbit erythrocytes (200 pmol/mg dry weight) is about two-thirds of that of human erythrocytes. Estimation of the number of sites (B_o) and apparent dissociation constants (K_d) for each of these different sites was not possible in the present study because of a limited availability of the baby rabbit cells. It

was shown in the present study, however, that as baby rabbits age, the cytochalasin B binding to each of these sites changes in a characteristic manner: relative contribution of site I binding was gradually reduced while that of site II was increased (Figs. 6 and 7). Thus, the cytochalasin B binding to the erythrocyte membranes of 3-month-old rabbits showed striking differeneces from that of 5-day-old baby rabbits (Fig. 7). The total saturable binding of adult rabbit erythrocytes (40 pmol/mg dry weight) was reduced to only one-fifth of that of the baby rabbits. The glucose-sensitive, site I binding was totally missing and practically all of the saturable binding was now due to cytochalasin E-sensitive site II. Although exact quantitation was not made, the change is not at all due to a stoichimetric conversion of site I to site II. No indication of site III was appreciable with adult rabbit cells.

Age-related loss of the cytochalasin B-sensitive fast glucose flux and that of the glucose-sensitive, site I cytochalasin B binding reveal similar decay constants. These are in turn comparable with mean life span of rabbit erythrocytes. This would sugest that the fast glucose flux meditaed by the cytochalasin B-sensitive carrier and the site I cytochalasin B binding coexist in a specific population of erythrocytes in neonatal circulation. Fetal erythrocytes have been suggested to be responsible for the fast glucose permeation seen at neonatal life of many mammals [1,4]. The loss of fetal erythrocytes as a fraction of the total circulating erythrocytes during neonatal growth is a function of not only the lifespan of the fetal erythrocytes but also the rate of increase in the total number of erythrocytes in circulation as well [4]. Because of lack of relevant information on these parameters the exact analysis of the expected temporal change was not attempted in the present study.

We have previously proposed with human erythrocyte system that the site I cytochalasin B-binding protein is glucose carrier [11]. We now propose that this same site I-binding protein occurs transiently in fetal rabbit erythrocyte membranes and that this protein is responsible for the fast glucose uptake seen in newborn rabbit erythrocytes. With human erythrocytes, several molecular species of membrane polypeptides have been proposed to be the glucose carrier. These include polypeptides of molecular weights 240 000—300 000 [21,22], 180 000 [11], 100 000 [23] and 50 000—65 000 [22,24—26]. If the age-related disappearance of the fast glucose carrier activity and of the site I cytochalasin B binding activity is due to corresponding disappearance of a protein rather than its inactivation, a parallel decrease or disappearance of this protein may be detected on SDS gel electrophoresis. Results of the present study indicate that such an age-related reduction in mass occurs with a number of distinct membrane polypeptides, and that these include three of the four proposed molecular species of glucose carrier referred to above, only an apparent exception being a 100 000 molecular weight peptide. Although it is difficult to quantitate these reduction by the stain-intensity of the gel alone, the reduction was most significant with the 240 000—300 000 molecular weight region and least significant with 50 000—65 000 molecular weight region. Significance of the reduction in 20 000—40 000 molecular weight region (Fig. 8) is not immediately clear. One resonable interpretation would be that all of these three peptides are involved in the cytochalasin B-sensitive glucose carrier as a functional unit. It is possible, however, that a subunit or subunits of this

functional unit may be responsible to the site I cytochalasin B binding.

The cytochalasin B-insensitive glucose carrier of adult rabbit erythrocytes is chemically a less defined entity at present, although its kinetic behavior has been studied in some detail [7]. Whether this molecule is distinct from the cytochalasin B-sensitive carrier or is the same molecule but under control of a certain functional modification [27] is yet to be examined.

Acknowledgements

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