Identification of the Transferrin Receptor of the Rabbit Reticulocyte†

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ABSTRACT: Reticulocytes were separated on the basis of density by isopycnic centrifugation in dextran gradients. This parameter was shown to correlate with the degree of maturity of the cells. Lactoperoxidase iodination of cells of different densities followed by sodium dodecyl sulfate ($NaDodSO_4$) electrophoresis revealed a 190 000 molecular weight protein which was well labeled in early reticulocyte membranes. Efficiency of labeling decreased as the cells increased in density, and high specific activity iodination of mature erythrocytes did not result in the labeling of any species near this molecular weight. Inclusion of rabbit transferrin prior to the iodination procedure resulted in a specific loss of labeling of this 190 000 molecular weight species. When steps were taken to clear endogenous transferrin from the membranes, the labeling of this species was enhanced. These observations are consistent with the concept that transferrin can block the lactoperoxidase catalyzed iodination of this membrane protein by specifically associating with it. Coomassie blue and periodic acid–Schiff staining of $NaDodSO_4$ gels of these membranes revealed that a glycoprotein present at this molecular weight is lost during the course of reticulocyte maturation. It is concluded from these studies that a glycoprotein of molecular weight 190 000 constitutes the transferrin receptor in the reticulocyte membrane.

Iron, destined for hemoglobin synthesis, is known to be delivered to developing erythroid cells by the plasma protein, transferrin (Jandl & Katz, 1963). Although this relationship has been recognized for over 15 years, the actual process of iron transfer from the transport protein into the interior of the immature cell remains obscure. Indeed, a fundamental controversy exists over the point in the protein–cell interaction at which the iron is removed from transferrin. On the one hand, a substantial body of evidence indicates that the transferrin molecules can actually penetrate the cell membrane and enter the cytoplasm (Morgan & Baker, 1969), while other studies have demonstrated that the removal of iron takes place at the level of the plasma membrane (Fielding & Speyer, 1974). The resolution of this problem is essential to a more complete understanding of intracellular iron metabolism.

It is now generally accepted that there are receptors present in the plasma membrane of immature red cells that are specific for the binding of diferric transferrin. The role of this putative receptor subsequent to its interaction with transferrin is not certain. Supporters of the hypothesis that the transferrin molecule is internalized propose that the entire complex is taken into the cell (Hemmaplardh & Morgan, 1974), possibly by micropinocytosis (Morgan & Appleton, 1969; Sullivan et al., 1976), whereas other evidence indicates that iron may be removed from transferrin by the receptor (Fielding & Speyer, 1974) or while the transferrin is bound to the receptor (Leibman & Aisen, 1977). It is expected that characterization of this membrane will provide insight into the first steps of the process of iron transfer from protein to cell.

Recent studies have indicated that it is possible to extract, by the use of nonionic detergents, a transferrin binding polypeptide from the plasma membrane of reticulocytes, but the reported characteristics of this species have not, in general, been in agreement. For instance, the molecular weight estimates reported for the extracted receptor have ranged from 35 000 (Light, 1977) to 350 000 (Leibman & Aisen, 1977). This descrepancy may result from variations in methodology or may reflect artifacts produced by differences in the detergent:protein ratio. Although it seems clear that the transferrin–receptor complex is brought into solution, molecular weight determination has been shown to be difficult under these nondenaturing conditions (Tanford et al., 1974).

In the studies reported here, a different approach has been applied utilizing the combined methodologies of lactoperoxidase catalyzed iodination and $NaDodSO_4$[1] gel electrophoresis. This obviated the use of nonionic detergent extraction.

Experimental Procedures

Materials. $Na^{125}I$ and $^{59}FeCl_3$ were from New England Nuclear. Lactoperoxidase, glucose oxidase, and type 60C Dextran were from Sigma Chemical Co. (St. Louis, Mo.). Sodium dodecyl sulfate was electrophoresis quality from Pierce Chemical Co. (Rockford, Ill.). Acrylamide was from Bio-Rad (Richmond, Calif.). All other chemicals used were reagent grade or better. Glass-distilled water was used in the preparation of all solutions.

Purification of Transferrin. Transferrin was fractionated from rabbit plasma by a previously described method (Baker et al., 1968) and then further purified by isoelectric focusing twice, first as diferric transferrin, and then in the iron-free form. This second step was necessary to remove hemopexin which cofocused with diferric transferrin. Transferrin prepared by this method was greater than 99% pure as judged by analytical gel electrophoresis (Smith, 1968) and by $NaDodSO_4$ electrophoresis (Fairbanks et al., 1971). The purified protein was stored frozen in the iron-free form. Iron was added to it as $Fe(ClO_4)_2$ in the presence of 20 mM $NaHCO_3$ and 1 mM nitrilotriacetate. Radioiodination of the diferric transferrin was by the iodine monochloride method (McFarlane, 1963).

Preparation of Reticulocytes. Reticulocytosis was induced in young New Zealand rabbits of either sex by daily bleeding

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[1] Abbreviations used: CBB, Coomassie brilliant blue R-250; EDTA, ethylenediaminetetraacetate; PAS, periodic acid–Schiff; $NaDodSO_4$, sodium dodecyl sulfate.

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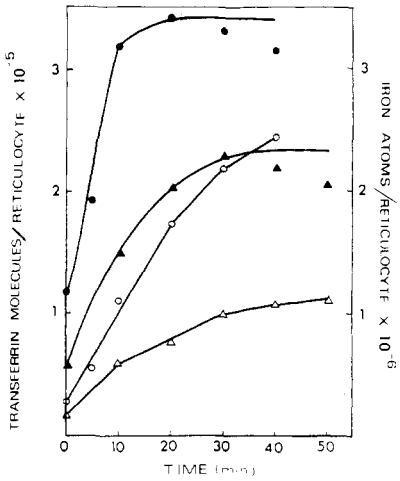

FIGURE 1: Transferrin binding and iron uptake by cells of different density. Reticulocyte rich blood cells were separated on the basis of density into seven fractions. Fraction I reticulocytes (●, ○) were compared with fraction III reticulocytes (▲, △) with regard to both [^{125}I]transferrin binding (●, ▲) and iron uptake (○, △). Transferrin was labeled with both ^{59}Fe and ^{125}I for this experiment; final concentration in the incubation mixture was 20 μM. Reticulocytes were determined independently by two workers after staining with New Methylene Blue and results were subsequently adjusted to a per reticulocyte basis.

of 10–15 mL/kg of body weight. This was accomplished either by venisection of the marginal ear vein or by cardiac puncture. Reticulocyte enriched blood cells were harvested when the level of circulating reticulocytes reached a maximum value, usually about 30%. The heparinized cells were washed thrice in an isotonic medium of 0.15 M NaCl, 0.01 M sodium phosphate, 1 mM EDTA, pH 7.4, and the buffy coat was carefully removed. They were then centrifuged in a discontinuous dextran density gradient to achieve separation on the basis of specific gravity (Schulman, 1968). The gradient was constructed in a 40-mL polycarbonate centrifuge tube and consisted of six 5-mL layers ranging from 21 to 27% dextran in 1% increments. The dextran was made up in the same medium used for washing the cells. The reticulocyte enriched cells were layered over this gradient and centrifuged at 13 000g for 30 min in a Sorvall HB-4 rotor. The cells could then be seen to form bands at the interfaces between the layers of the gradient. The six fractions were removed separately and washed thrice in the phosphate-buffered isotonic saline. Reticulocytes were determined by staining with New Methylene Blue (Brecher, 1949).

Binding and Uptake Studies. The interaction of doubly labeled [^{125}I, ^{59}Fe]transferrin with reticulocytes was studied in a medium consisting of Earle's salts, 6 mM glucose, and 2.5% bovine serum albumin under a flow of 95% air–5% CO_2 at 37 °C (Williams & Woodworth, 1973). Binding studies were done by sampling at timed intervals, dispersing the 100-μL aliquot into 1 mL of cold medium, centrifuging for 10 s in a Beckman Microfuge B, and washing the pellet two additional times with cold medium. The activity associated with the pellet was then determined by counting directly in a Beckman Biogamma scintillation counter.

Lactoperoxidase Iodination. Cells to be surface iodinated were suspended in 4 mL of phosphate-buffered saline, pH 7.0. To this suspension was then added 5 U of lactoperoxidase and 1–2 U of glucose oxidase followed by 25 μmol of glucose and 0.01 μmol of KI containing 20–70 μCi of Na^{125}I. Incubation was at 25 °C for 20 min. The iodinated cells were then washed five times in cold phosphate-buffered saline and lysed in cold 5 mM phosphate, pH 7.5, after the method of Dodge et al. (1963). This procedure was capable of producing hemoglobin-depleted ghosts from both erythrocytes and reticulocytes.

Removal of Endogenous Transferrin. In some experiments, endogenous transferrin was removed from the reticulocytes prior to surface iodination. This was accomplished by sequential incubation and washing. Incubation was for 15 min at 37 °C in a medium consisting of 0.15 M NaCl, 0.05 M sodium phosphate, pH 7.65, 0.02 M $NaHCO_3$ under a flow of 95% air–5% CO_2 at 37 °C.

To the suspension was then added 4 volumes of cold medium and the cells were collected by centrifugation. This process was repeated four times and was capable of removing most of the cell-bound transferrin (Hemmaplardh & Morgan, 1974).

$NaDodSO_4$ Electrophoresis. Sodium dodecyl sulfate electrophoresis was carried out in gradient gels ranging from 4 to 12% acrylamide using the buffer system described by Fairbanks et al. (1971). Samples of ghost membranes were prepared for electrophoresis by incubation at 37 °C for 1 h in the presence of 1% $NaDodSO_4$ and 40 mM dithiothreitol. The gels were cast and run in a Hoefer electrophoresis apparatus (Bio-Rad). Staining was by Coomassie brilliant blue for protein and by the periodic acid–Schiff technique (Fairbanks et al., 1971) for carbohydrate. Molecular weight calibration was based on parallel electrophoresis of galactosidase, glycogen phosphorylase, bovine serum albumin, alcohol dehydrogenase, glycerol-3-phosphate dehydrogenase, carboxypeptidase, carbonic anhydrase, myoglobin, and hemoglobin as well as on the reported molecular weights of five major membrane proteins of the erythrocyte (Steck, 1974). Activity profiles of the gels were determined by slicing into 1-mm fractions and counting in a Beckman Biogamma counter.

Results

When rabbits were bled daily, a regular pattern of increasing reticulocytes could be observed. An initial steady increase in reticulocytosis was observed up to about 100 h; this was followed by a more rapid increase to a level of about 30% of the circulating red cells. This plateau level persisted for several days.

Reticulocytes were harvested during this phase of rapid increase (i.e., 110–120 h following the onset of bleeding). The reticulocyte enriched population of cells was then separated into six fractions on the basis of specific gravity by isopycnic centrifugation. It was clear that the less dense regions of the gradient were selectively enriched with cells staining as reticulocytes, but it was important to demonstrate that these less dense reticulocytes represented a younger population of cells than those banding in the more dense layers of the gradient. To this end, reticulocytes from fraction I were compared with those from fraction III with regard to their ability both to bind transferrin and to take up iron. Figure 1 demonstrates that the low density reticulocytes from fraction I bind as average of 3.3×10^5 transferrin molecules/cell, while those from fraction III bind only about 2.0×10^5 molecules/cell. In addition, the less dense reticulocytes are over twice as active in acquiring iron from the cell-bound transferrin as their more dense counterparts. This clearly demonstrates the correlation of the specific gravity of the reticulocytes with their degree of maturity.

Considerable evidence has supported the concept that the transferrin receptor is exposed on the external surface of the reticulocyte plasma membrane. This being the case, it should be possible to label this species by lactoperoxidase iodination (Phillips & Morrison, 1971). To test this possibility, plasma membranes from cells of fractions I, II, IV, and V were surface labeled by this method and, after hemolysis, subjected to

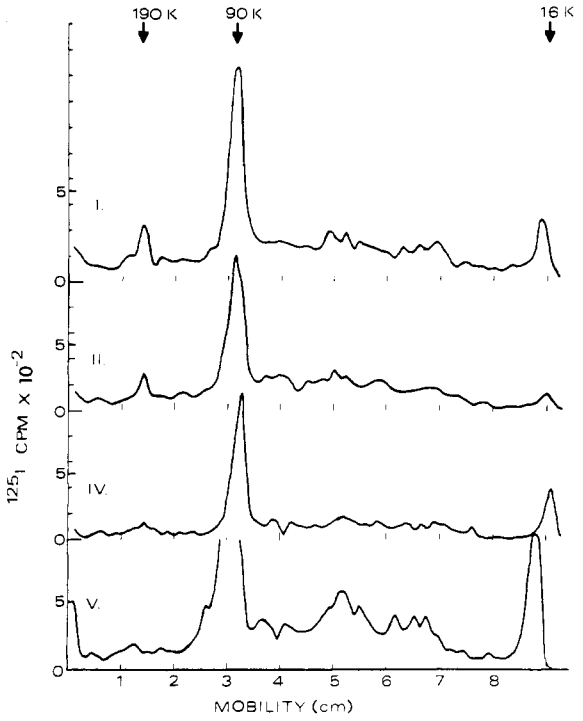

FIGURE 2: $NaDodSO_4$ electrophoresis of lactoperoxidase iodinated reticulocytes of different densities. The Roman numerals with which the activity profiles are numbered refer to the fractions from which the cells originated. The labeled species at 90 000 (3.1 cm) is band 3 and that at 16 000 (9.0 cm) is the hemoglobin subunit. Electrophoresis was for 2.5 h at 60 mA on a gradient gel ranging from 4 to 12% polyacrylamide.

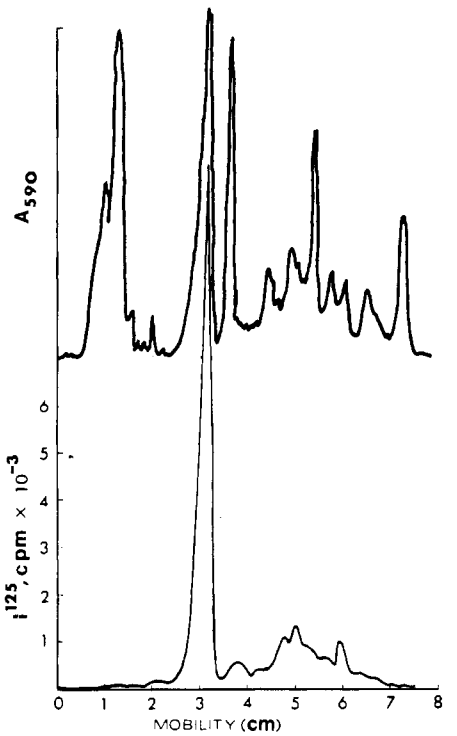

FIGURE 3: $NaDodSO_4$ electrophoresis of lactoperoxidase iodinated membrane proteins from mature rabbit erythrocytes. Erythrocytes (reticulocytes <2%) were obtained from a normal rabbit. These cells were lactoperoxidase iodinated and hemolyzed and the labeled membranes prepared for $NaDodSO_4$ electrophoresis. Electrophoresis was as before on a gradient gel of 4–12% polyacrylamide. A densitometric scan of the Coomassie blue stained gel is shown in the upper panel. Band 3 (mol wt 90 000) at 3.1 cm is the most heavily labeled species. Other labeled proteins appear at 4.9 cm (55 000), 5.0 cm (50 000), and 6.0 cm (37 000). Note that no label is incorporated into any proteins of mol wt >100 000.

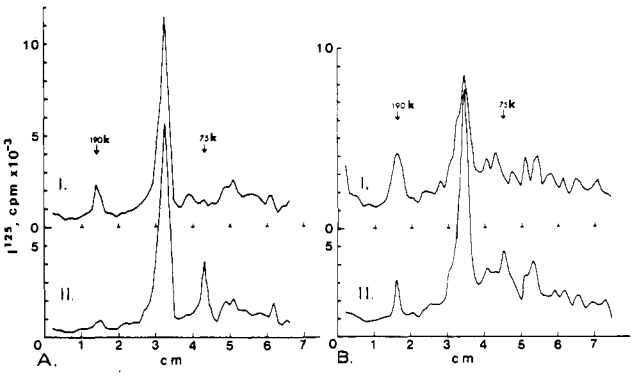

FIGURE 4: Lactoperoxidase iodination of reticulocytes which had been previously either loaded with or cleared of transferrin. Cells from fraction III (A) or fraction I (B) were either exhaustively cleared of endogenous transferrin (I) or incubated with 20 μM exogenous transferrin (II) prior to lactoperoxidase iodination. $NaDodSO_4$ electrophoresis was as before on 4–12% polyacrylamide gels. The labeled protein at 190 000 is present in all preparations but with different relative activity. Prior incubation of the cells with transferrin lowered the activity of this species. A considerably higher relative activity was obtained in the younger reticulocytes of fraction I. An additional labeled protein is also seen at mol wt 75 000 in those cells which had been previously incubated with transferrin. This most likely represents transferrin which was iodinated while bound to the receptor.

$NaDodSO_4$ gel electrophoresis. As the ^{125}I activity profiles in Figure 2 demonstrate, there is a loss of labeling detectable in a membrane protein with a molecular weight of 190 000. High specific activity iodination of mature rabbit erythrocytes drawn from the same animal before the onset of bleeding showed no labeling in the region of the gel representing mol wt >100 000 (Figure 3). The possibility that this species might represent the transferrin receptor was further investigated.

It was reasoned that the presence of transferrin on the receptor might sterically hinder the labeling of that membrane protein since the formation of a complex between the lactoperoxidase molecule and a tyrosine residue is a prerequisite for enzymic iodination (Morrison & Bayse, 1970). Low density reticulocytes were either cleared of endogenous transferrin or loaded with exogenous transferrin prior to lactoperoxidase iodination. Clearing endogenous transferrin was a necessary step since it had been demonstrated that reticulocytes harvested from the circulation of anemic rabbits retained a substantial amount of bound transferrin (Hemmaplardh & Morgan, 1974). This endogenous transferrin could not be removed by repeated washing at 4 °C and only sequential incubation and washing was able to release the major portion of the bound protein.

Fraction III reticulocytes which had been either cleared of transferrin or loaded with exogenous transferrin were lactoperoxidase iodinated and analyzed as before. The activity profiles of the gels are shown in Figure 4A. A selective enhancement of labeling may be seen in the mol wt 190 000 species from the plasma membranes of the cells which have been cleared of transferrin. In addition, a labeled band appears at mol wt 75 000. No other major differences are apparent.

The experiment was repeated with fraction I reticulocytes to ascertain whether the differences in the pattern of labeling would be further enhanced in younger cells. Figure 4B demonstrates that the difference in labeling of the mol wt 190 000 species is considerably more dramatic in the lower density reticulocytes. In this experiment the total amount of label incorporated into the mol wt 190 000 species in cells previously cleared of transferrin was over 50% of that found in band 3, the major external membrane protein of the rabbit erythrocyte (Lodish & Small, 1975).

Membrane preparations from low density reticulocytes which had been electrophoresed on $NaDodSO_4$ gels were stained with both Coomassie brilliant blue and PAS (unpublished data). Coomassie blue staining revealed a protein with mol wt 190 000 that tended to decrease with reticulocyte maturation. This species comigrated with the peak of ^{125}I activity seen in labeled membrane preparations from young reticulocytes. PAS staining of duplicate gels revealed that this protein or a species comigrating with it was positive for carbohydrate. There were no PAS positive bands present in this region in gels run on mature erythrocytes.

Discussion

The biphasic development of reticulocytosis in the rabbit that has been reported here suggests that about 100 h are required for the differentiation of an erythropoietic stem cell from the point of its initial stimulation to its release from the bone marrow. The slower portion of the curve probably represents the release of already partially differentiated precursors, while the sudden increase in rate observed at 100 h following the onset of bleeding would represent the release of reticulocytes whose differentiation was initiated by the original blood loss.

By harvesting cells at the peak of this rapidly increasing portion of the curve, before the development of steady-state reticulocyte levels, it was possible to enrich the reticulocytes with particularly young cells. These cells were subsequently separated into six fractions on the basis of specific gravity, a parameter that was shown to correlate with the degree of maturity of the reticulocyte. The earliest population of reticulocytes tested was found to bind an average of 3.3×10^5 transferrin molecules/cell. This figure is in close agreement with previously reported values (Baker & Morgan, 1969) and would be considerably higher if extrapolated to infinite transferrin concentration. As maturation progresses there is a decrease in cell size and an increase in hemoglobin content (Bessis, 1973). Both of these factors contribute to an increased cell density.

A direct comparison of the externally presented membrane proteins of cells that had been separated on the basis of specific gravity has revealed that there is a major species (mol wt 190 000) present in early reticulocytes that cannot be as well labeled in more mature cells. Mature erythrocytes demonstrated no labeling in this region of the gel. Whether this observation represented an actual loss of this portion or a modification (chemical, structural, or both) that rendered it unavailable to the enzyme was not certain. Staining with Coomassie blue revealed that there is a decrease in protein banding at this apparent molecular weight, while carbohydrate staining demonstrated a loss in PAS positive material. These observations tended to support the conclusion that this reticulocyte-specific species disappears upon maturation. Other membrane proteins that could be labeled by this method in rabbit erythrocytes (mol wt: 90 000, 55 000, 50 000, and 37 000) were also found in reticulocytes and no other major losses could be detected.

The possibility that this mol wt 190 000 species represented the transferrin receptor was further investigated. That the transferrin receptor should be capable of being detected by lactoperoxidase iodination seemed likely for several reasons. First, it would be expected that for the receptor to enter into a highly specific interaction with transferrin, a mol wt of 80 000, a substantial portion of the polypeptide chain must exist external to the cell. The external location of the transferrin–receptor complex has been shown by the agglutination of transferrin-loaded reticulocytes by antitransferrin antibody (Ecarot-Charrier et al., 1977). In addition, the presence of >300 000 molecules of receptor/reticulocyte membrane would make this one of the major membrane proteins and, as such, it should be easily detected by lactoperoxidase labeling, even if its iodination were a relatively inefficient process.

It also seemed reasonable that the lactoperoxidase catalyzed iodination of the receptor might be inhibited by its specific association with transferrin. Since the formation of a complex between the activated enzyme and a tyrosine residue in the protein is a prerequisite for iodination, it would be expected that the interaction of transferrin and its receptor would shield at least some of the tyrosine residues from attack by the lactoperoxidase molecule.

It was possible to demonstrate an enhancement of iodination in this mol wt 190 000 species by "difference iodination," in which the pattern of labeling of reticulocyte membranes which had been preloaded with transferrin was compared with membranes which had been previously cleared of bound transferrin. This effect was shown to be even more pronounced in younger cells. No other major differences were observed by this technique.

Leibman & Aisen (1977), by examining Triton X-100 extracts of reticulocyte membranes, have inferred that a glycoprotein of mol wt 176 000 is the specific transferrin receptor and that the carbohydrate portion of this molecule may be lost during development. In addition, another glycoprotein of mol wt 95 000 may be involved in the complex (Aisen et al., 1978). The heavy labeling of band 3 in our experiments would have obscured any differential labeling of such a 95 000 peptide. Our studies do not indicate the involvement of any species other than that at mol wt 190 000 but other possibilities cannot be ruled out.

It is concluded from these studies that the receptor for transferrin in the rabbit reticulocyte membrane is a glycoprotein of mol wt 190 000. This species appears to be lost from the membrane during erythroid maturation as judged by loss of reactivity with both protein (CBB) and carbohydrate specific (PAS) stains. This is supported by its loss of ability to be iodinated by lactoperoxidase, although it is conceivable that the latter reflects an alteration in the receptor molecule or its microenvironment rather than its actual disappearance.

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The Procuticle of *Drosophila:* Heterogeneity of Urea-Soluble Proteins†

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ABSTRACT: Proteins, soluble in 7 M urea, 4 M guanidine hydrochloride, or 2% sodium dodecyl sulfate, have been extracted from untanned larval cuticles of *Drosophila melanogaster.* A major protein fraction, apparent molecular weight 8000–10 000, is resolved into eight different components (five major, three minor) by gradient gel electrophoresis under nondenaturing conditions. Proteins extracted in 7 M urea have been resolved by diethylaminoethylcellulose chromatography into five fractions, three of which are greatly enriched for electrophoretically homogeneous proteins. The five fractions have different amino acid compositions. Electrophoretic variants involving four of the five major proteins have been obtained. Preliminary genetic analysis indicates that at least three of the five proteins are specified by separate structural genes.

The cuticle of insects is an extracellular structure which serves functionally as both skin and skeleton. The cuticle is composed of an outer epicuticle and an inner procuticle. The epicuticle, as seen by transmission electron microscopy, is a trilayered structure with two electron dense layers separated by an electron transparent layer. The procuticle is composed of helicoidally arranged fibers and has a laminated appearance in sectioned material. The fibers in the procuticle are composed of chitin, a β-(1,4)-linked unbranched polymer of *N*-acetylglucosamine, and protein. In sclerotized cuticle the proteins are cross-linked, while in unsclerotized cuticles there is little cross-linking. X-ray crystallographic analyses of unsclerotized cuticles suggest that in some cases the proteins exist in repeated structures associated with three or four chitobiose residues [see Rudall (1976) for review]. Extraction with solvents such as 7 M urea eliminates the repeating structure and releases proteins into the solvent (Rudall and Kenchington, 1973). Indeed, 7 M urea has been found to release proteins from all kinds of unsclerotized cuticles which have been investigated (Hackman, 1972, 1974a, 1976). The extracted proteins bind to chitin when exposed to the polysaccharide in the absence of urea at pH 7 (Hackman, 1976). For most insects studied, the urea-soluble protein fraction is heterogeneous, as judged by $NaDodSO_4$[1]–gel electrophoresis and isoelectric focusing (Hackman, 1972, 1974a, 1976). Comparisons among several genera demonstrate that these proteins are not conserved during evolution.

The cuticle is deposited by an underlying layer of epidermal cells in response to ecdysteroids.[2] Cuticle deposition by epidermal tissues cultured in vitro with ecdysteroids has been demonstrated by workers in several laboratories [for review, see Marks and Sowa (1976)], including imaginal disks of *Drosophila* (Mandaron, 1976; Fristrom, unpublished observation). Current evidence indicates that ecdysteroids act on target tissues by interacting with the chromatin and modulating transcription (Ashburner et al., 1973; Fragoulis and Sekeris, 1975; Yund and Fristrom, 1975a,b) in a manner similar to that proposed for vertebrate steroid hormones (reviewed by Gorski and Gannon, 1976; Yamamoto and Alberts, 1976).

The different urea-soluble cuticle proteins may be derivatives, variants, or multimers of a single polypeptide (Hackman, 1975, 1976) or involve polypeptides with different primary structures. If the last is the case, cuticle formation in insects, particularly in *Drosophila* where genetic analysis is possible, may serve as an excellent system for the study of coordinate gene regulation in eukaryotes. We report in this paper characteristics of procuticle proteins isolated from unsclerotized larval cuticles of *D. melanogaster.* We conclude that at least five different polypeptides specified by three or more genes are present.

Experimental Procedures

An *Ore. RC* strain of *D. melanogaster* maintained in mass culture in the Genetics Research Laboratories was used in

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[1] Abbreviations used are: 7 MUT, 7 M urea, 0.005 M Tris, pH 8.6; Tris, tris(hydroxymethyl)aminomethane; PUT, phenylthiourea; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; binding buffer, 0.005 M NaCl, 0.005 M Tris, pH 7.0; CP, cuticle protein.

[2] The term ecdysteroid refers to any member of a family of steroids with molting hormone activity.

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