Protein Components of Erythrocyte Membranes from Different Animal Species*

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ABSTRACT: Completely disaggregated polypeptide chains from erythrocyte membranes of different mammals were compared by polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate (Lenard, 1970). This procedure separates polypeptide chains according to molecular weight. The distribution of polypeptide chains was remarkably similar in red cell membranes obtained from man, pig, sheep, rat, and dog. Eight bands, ranging in molecular weight from 255,000 to 22,000 and accounting for well over two-thirds of the total protein of human membranes were present in all species examined. One of these bands, at *ca.* 108,000 molecular weight, which was previously shown to contain glycoprotein in human membranes (Lenard, 1970) was found by periodic acid–Schiff staining to contain glycoprotein in all other species studied. The intact membranes were labeled with *N*-ethylmaleimide-^{14}C before disaggregation in order to identify from the gels those polypeptide chains containing reactive sulfhydryl groups in the membrane. About 50% of the total label was associated with the *ca.* 240,000 and 255,000 bands in all species examined. The other major sulfhydryl-containing chains were also qualitatively and quantitatively similar in all species. It is concluded that the proteins of red cell membranes from different species show extensive similarities in over two-thirds of their total protein.

It has been shown in a previous paper that human red cell membranes can be completely disaggregated in SDS[1] solution after first dialyzing the membranes into hypotonic EDTA solution containing mercaptoethanol (Lenard, 1970). Electrophoresis of this preparation of disaggregated protein chains in polyacrylamide gels containing 1% SDS afforded separation of the chains according to molecular weight, and showed that human red cell membranes contain a complex population of protein chains of molecular weights ranging from 22,000 to 255,000. Of the total membrane protein, about one-third was contained in two major bands of *ca.* 240,000 and 255,000 molecular weight, another third was contained in two bands of *ca.* 86,000 and 108,000 molecular weight, and the remaining third was of lower molecular weight (Lenard, 1970).

In this paper, these observations have been extended to red cell membranes of other animal species, and it is shown that most of the prominent molecular weight classes of red cell membrane proteins are found in all species examined.

An additional characterization of red cell membrane proteins is also described in this paper. By labeling the intact membrane with NEM-^{14}C and then disaggregating the membranes and slicing the resulting gels it is possible to identify those protein chains which possess reactive sulfhydryl groups in the intact membrane. These sulfhydryl-labeling patterns are shown to be strikingly similar in all species examined.

Materials and Methods

All blood was used within 2 days after it was obtained from the animal. Citrate was used as anti-coagulant for the sheep and pig blood. Heparin was used as anti-coagulant for the human, rat, and dog blood. SDS was used from several commercial sources without purification.

Membranes from each species were prepared by the procedure of Dodge *et al.* (1963) using hypotonic phosphate buffer, and also by the procedure of Marchesi and Palade (1967) using hypotonic Tris–EDTA buffer adjusted to pH 7.4.

Membranes were disaggregated by dialysis into 5 mM EDTA–5 mM mercaptoethanol (pH 7.0), addition of SDS to a final concentration of 2–3%, and heating 3 min at 100° (Lenard, 1970). Gels were 10 cm long and contained 5% acrylamide and 1% SDS (Lenard, 1970). Ethylene diacrylate was used as the cross-linker (Choules and Zimm, 1966). Gels were run at 5–8 mA per gel until cytochrome *c* in a parallel gel had migrated to within 1 cm of the bottom of the gel. Gels were fixed overnight in 50% trichloroacetic acid stained in 0.25% coomassie blue in 20% trichloroacetic acid and destained over several days in repeated changes of 7% acetic acid (Lenard, 1970). PAS staining was carried out by the method of Zacharias *et al.* (1969) and destained in repeated changes of 7% acetic acid over a period of 1–2 weeks. Molecular weights shown in this paper are those previously calculated from the migration of several proteins of known molecular weight subjected to electrophoresis in parallel with the red cell membrane preparation (Lenard, 1970).

Radioactive labeling with NEM was carried out on the intact membranes in 5 mM Tris–1 mM EDTA (pH 7.4) at about 1×10^{-4} M NEM for 30–45 min at 37°. Similar results are obtained with NEM concentrations up to 1×10^{-3} M. NEM-2,3-^{14}C (Amersham-Searle) of specific activity 2.6 Ci/mole was used for all experiments. The gels were sliced into approxi-

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; PAS, periodic acid–Schiff.

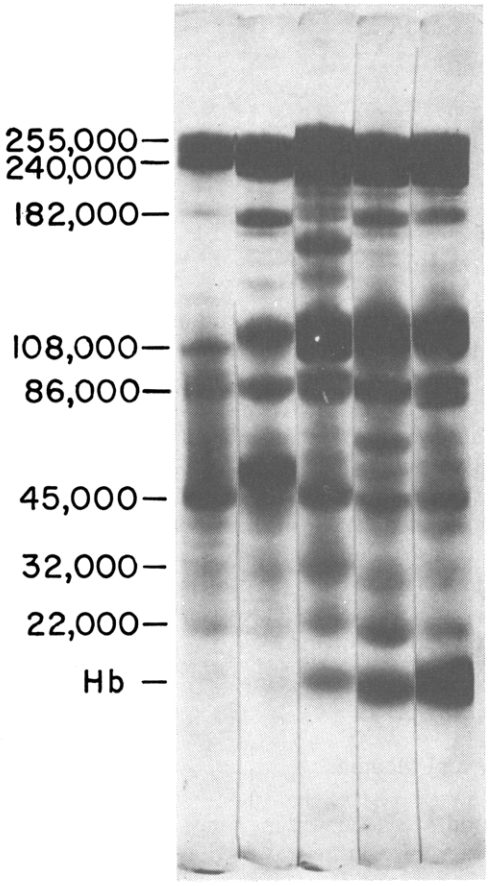

FIGURE 1: Gels of red blood cell membrane proteins from different species stained with coomassie blue. Membranes prepared by the procedure of Dodge *et al.* (1963). From left to right: man, pig, sheep, rat, and dog. The estimated molecular weights of the common bands are shown on the left. Hb, hemoglobin.

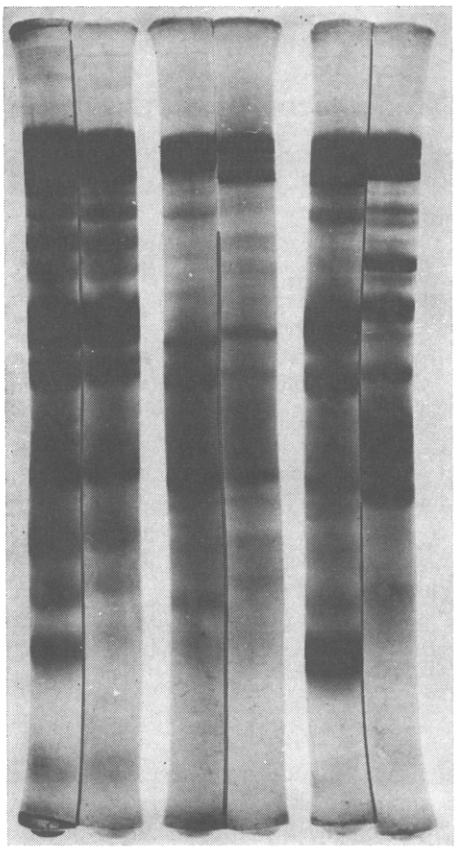

FIGURE 2: Comparison of gel patterns obtained from membranes prepared by the procedure by Dodge *et al.* (1963) (left gel of each pair) and by the procedure of Marchesi and Palade (1967) (right gel of each pair). Pairs, from left to right: sheep, human, and dog.

mately 50 slices and each slice was dissolved in 1 M piperidine before counting (Choules and Zimm, 1966). Approximately 70–85% of the total counts applied to the top of the gel were recovered in the fractions. The loss is thought to be due to inefficiency of layering of the sample on the top of the gel, and to decreased efficiency of counting of the piperidine-dissolved slices as compared to the unfractionated protein solution. No significant number of counts were found on the top of any of the gels.

NEM labeling was shown to be restricted to sulfhydryl groups by the following experiment. Labeled membranes were hydrolyzed for 72 hr in 5.7 N HCl at 110° (Smyth *et al.*, 1961).

The hydrolysate was separated by electrophoresis on Whatman No. 1 paper at pH 5.2 (2% pyridine–1% acetic acid) for 1 hr at 2500 V. The electrophoretogram was scanned for radioactivity using a Packard Model 7201 radiochromatogram scanner.

A single peak of radioactivity was obtained which ran to the same position as authentic *S*-succinylcysteine, but differently from Im-succinylhistidine or NH_2-succinyllysine, showing that NEM reacts quite specifically with the sulfhydryl groups on the membrane under the conditions used. Reference compounds were prepared as described by Brewer and Riehm (1967).

Results

The gel patterns from the membranes of five mammalian species are shown in Figure 1. The most striking similarities are the bands for which molecular weight estimations are shown (Lenard, 1970). A single band was previously seen at *ca.* 86,000 molecular weight (Lenard, 1970), but this is resolved into two closely spaced bands in the gels of human, sheep, and dog membranes shown in Figure 1. The differences between the species is most pronounced in the molecular weight regions *ca.* 108,000–240,000 and *ca.* 45,000–86,000. Among the prominent bands which appear to be species specific are the sheep band at *ca.* 160,000, the pig band at *ca.* 53,000 and the rat band at *ca.* 62,000 (Figure 1). Other less pronounced differences can also be seen in Figure 1.

The distribution of glycoproteins in the gels was scanned by PAS staining. A major glycoprotein band at *ca.* 108,000 previously reported in human red cell membranes (Lenard, 1970) was seen in all species examined. In addition a second major glycoprotein component of lower molecular weight was visible in gels from sheep and dog membranes. The molecular weight of the second PAS-reactive component from the sheep membrane was estimated to be *ca.* 50,000 while from the dog it was *ca.* 60,000.

The patterns obtained from membranes prepared by two different procedures of hypotonic lysis are compared in Fig

ure 2. Of the five species examined, only dog membranes (right pair, Figure 2) showed significant differences when prepared by the different procedures. It is noteworthy that dog red cell membranes, prepared by either procedure, showed a substantial amount of vesicularization and fragmentation upon examination by phase microscopy. All of the other membranes appeared to be completely intact when viewed in the phase microscope. The striking similarities in protein patterns from membranes obtained by these two preparative procedures is not inconsistent with the studies of Mitchell *et al.* (1965) or Burger *et al.* (1968), who showed that retention of protein, lipid, and specific enzyme activities by red cell membranes depended critically on pH and ionic strength. The two procedures used in this paper both use identical pH and closely similar ionic strength.

The NEM-labeling pattern of red cell membranes from four species (excluding the dog, where a population of intact red cell membranes was not obtained) is shown in Figure 3. In each case five discrete populations are seen, representing those protein chains with reactive sulfydryl groups in the intact membrane. Most prominent in every case is the region of the gel labeled I (Figure 3) corresponding to the two major bands at 240,000 and 255,000. The molecular weight and quantitative distribution of sulfhydryl groups from pig, sheep, and rat membranes are compared in Table I below. Although the

DISTRIBUTION OF N-ETHYL MALEIMIDE-C^{14} IN PROTEINS FROM LABELLED MEMBRANES OF DIFFERENT SPECIES

Fraction	M.W.	% of total label in gel		
		Pig	Sheep	Rat
I	> 200,000	48	50	47
II	100,000 - 115,000	8	5	9
III	79,000 - 86,000	14	14	11
IV	47,000 - 60,000	18[a]	12	15[a]
V	30,000 - 32,000	4	5	6
		82	86	88

[a] Two NEM-containing bands in this area

patterns from human membranes is very similar (Figure 3) it is not included in Table I since the pattern was obtained in a separate experiment.

In another experiment, NEM-labeled proteins from human red cell membranes were permitted to migrate a shorter distance down the gel, so that the lipid components and any low molecular weight peptides present in the membrane were retained on the gel (*cf.* Lenard, 1970, Figure 1). No label was found below 22,000 molecular weight in any of these gels. This finding is especially noteworthy in view of recent claims of a "mini-protein" fraction of *ca.* 6000 molecular weight in bovine red blood cell membranes (Laico *et al.*, 1970), and suggests that such proteins, if these exist in human red cell membranes, do not contain reactive sulfhydryl groups.

Discussion

In this paper it has been demonstrated that at least eight

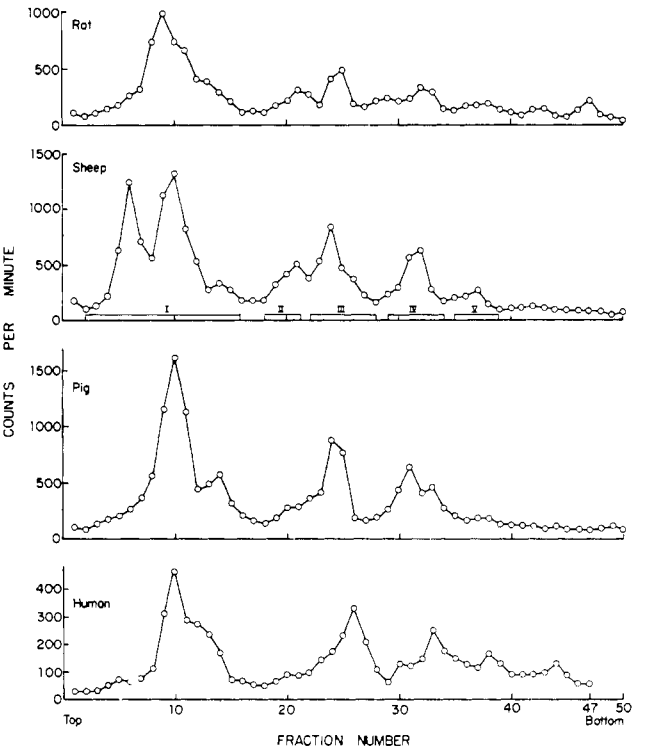

FIGURE 3: Radioactivity distribution in gels prepared from red blood cell membranes labeled with NEM-^{14}C.

molecular weight classes of proteins are similar in five species of mammals. The distribution of glycoprotein is also similar, as is the distribution of reactive sulfhydryl groups in all species examined.

These extensive similarities contrast with the findings of Zwaal and Van Deenen (1968) who studied membrane proteins from different species prepared by 1-butanol extraction and precipitation at pH 4.5. Their preparations showed differences between proteins of different species, both in sedimentation properties and upon polyacrylamide gel electrophoresis at low pH. It seems likely that under the conditions used by Zwaal and Van Deenen (1968) the protein chains were not completely disaggregated, although charge differences between membrane proteins of different species could result in the gel patterns they obtained, and would not be inconsistent with results reported here. It has previously been shown that the procedures used in the present communication effect complete disaggregation of human red cell membranes (Lenard, 1970).

Demus and Mehl (1970) have recently reported an analysis of pig erythrocyte membrane proteins solubilized in a phenol–formic acid–water solvent. They reported that 30% of the total protein had a molecular weight of 48,000 and 13% was of mol wt 27,000. Figure 1 shows a very prominent, but apparently species specific band in pig membranes, which, in the present paper, was assigned a molecular weight of *ca.* 53,000. Demus and Mehl (1970) were unable to demonstrate the very prominent higher molecular weight components shown in Figure 1.

It is noteworthy in this connection that the very high molecular weight components demonstrated in all the species

(Figure 1 and Lenard, 1970) has also been found in human membranes by Gwynne and Tanford (1970). These workers used guanidine hydrochloride to disaggregate the membranes. Thus, the observation that very high molecular weight protein chains exist in human red cell membranes has been made using two entirely different procedures for disaggregating the membrane.

It can be seen in Figure 1 that the intensity of staining appears to be comparable at most molecular weights in each of the gels. This suggests that the quantitative as well as the qualitative distribution of proteins is similar in the species studied. Since the major bands from 86,000 to 255,000 molecular weight account for about two-thirds of the total human membrane protein (Lenard, 1970) it appears that the molecular weight of well over two-thirds of the total membrane protein has been conserved in all five species. This very large degree of homology between distantly related mammalian species suggests that each of the conserved proteins plays a necessary role in the structure, function and viability of the intact red cell.

Acknowledgment

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