Isolation and Partial Characterization of Structural Protein Derived from Human Red Cell Membranes*

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ABSTRACT: A protein moiety has been isolated from human red cell membranes which corresponds in its physical and molecular properties to structural proteins obtained from other membrane systems. Parallel studies undertaken to compare this protein with structural protein derived from *Neurospora* mitochondria revealed considerable homology as well as certain specific differences. Structural protein prepared from human red cell membranes exhibited the characteristic insolubility in aqueous buffer systems except at pH extremes or in the presence of detergents such as Triton X-100. It sedimented as a single peak with an approximate *s* value of 2.3 S. In the ultraviolet range 280–290 mμ, structural proteins from human red cell membranes and *Neurospora* mitochondria membranes both showed a double peak whose characteristics changed similarly under different solvent conditions. Phospholipid binding curves were identical. On polyacrylamide gel electrophoresis, at pH 11, both preparations migrated equally as a single band. At pH 8.9 and pH 3, the two species of structural protein separated into a number of bands, some of which showed correspondence in migration, some of which did not. This phenomenon was interpreted as possibly representing the tendency of structural proteins to form different states of aggregation. Total amino acid analysis and two-dimensional peptide mapping indicated that while specific differences existed between the two structural proteins, the major portions were remarkably similar. That such homology exists between structural proteins from such diverse sources is in keeping with the concept of a "universality" of membrane systems.

Certain functions are common to all membrane systems while other functions are specific only to some membrane systems. Comparative studies of membranes of the sort described in this paper may offer an approach to the future elucidation of such structure–function relationships.

The concept of a "structural protein" in membrane systems arose from the observations of Green and coworkers (Green *et al.*, 1961; Criddle *et al.*, 1962) that a strict inventory of catalytically active mitochondrial membrane components accounted for only 25% of the total protein. Intimately associated with the electron transport enzymes they found a colorless, inactive protein which they called structural protein. Following these observations structural proteins were prepared from a variety of sources, including beef heart mitochondria (Green *et al.*, 1961; Criddle *et al.*, 1966), beef liver mitochondria, beef liver microsomes, bovine erythrocyte stroma (Richardson *et al.*, 1963), spinach leaf chloroplasts (Richardson *et al.*, 1963; Criddle and Park, 1964), and *Neurospora* mitochondria (Woodward and Munkres, 1966).

Structural proteins have been noted to share several physical and chemical properties: they polymerize readily, bind phospholipid, are devoid of cytochrome and enzymatic activity, and are almost completely insoluble in aqueous buffers at physiologic pH, requiring pH extremes or detergents to effect solubilization. These properties were in keeping with a predominantly structural role as opposed to catalytic role, and led to their consideration as the "matrix" of the membrane system (Richardson *et al.*, 1963). More recent data, however, indicate that integrated electron-transfer activity and membrane-forming capability persist in beef heart mitochondria in the absence of structural protein (Kopaczyk *et al.*, 1966; Bachmann *et al.*, 1966; Green *et al.*, 1966; Allmann *et al.*, 1966). Thus, although protein is clearly a major component in membrane structure, the exact role of the entity called structural protein is not well defined.

Current interpretation of electron microscopic and chemical studies of membranes has been to regard these structures as sheets of repeating globular macromolecular units containing both protein and lipid (Green and Perdue, 1966; Green and Tzagoloff, 1966; Korn, 1966; Green *et al.*, 1967). It has been further argued on genetic grounds that the primary event in membrane synthesis is probably the manufacture of the protein components, to which lipid is secondarily bound in a manner dictated by the specific amino acid sequences of the proteins (Korn, 1966).

Human red cells provide an abundant and available source of material for the study of membrane structure. An additional incentive to their study is the existence of at least two hereditary hemolytic anemias, hereditary spherocytosis and hereditary elliptocytosis, whose defects though unknown seem to be lodged within the cell membrane itself (see review by Jandl, 1965). Thus,

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the system provides the opportunity for studying structure–function relationships in both normal and altered states.

Earlier work in this laboratory led to the solubilization and electrophoresis of whole human red cell stroma (Schneiderman, 1965). Characterization of some of the protein components separated in such a system will be reported at a later date. In this paper we describe studies of a protein component of human red cell membranes obtained *via* a different approach, one which has led to the preparation of structural proteins from a variety of membrane sources. In order that we might gain further insight into our observations by comparison with another membrane system, we chose to run parallel investigations of structural protein derived from *Neurospora* mitochondria.[1]

Methods and Materials

Structural Protein Isolation. Human red cell membrane ghosts were prepared from 500-cc units of blood obtained from individual donors by methods previously described (Schrier and Doak, 1963; Schneiderman, 1965). The general approach to the isolation of structural protein from human red cell membranes followed the methods of (Green *et al.* 1961; Criddle *et al.*, 1962; D. E. Green, personal communication; D. O. Woodward, personal communication). Because of the poor solubility of human red cell membranes in many of the previously published detergent systems, certain modifications, particularly the use of Triton X-100, were found necessary to prepare an adequate yield of structural protein. Our procedure therefore will be described in detail.

Human red cell stroma suspended in 1.65×10^{-4} M Tris buffer (10 mg/ml) was homogenized, then sonicated for 3 min. To complete solubility an equal volume of 5% Triton X-100 was added and the suspension was again sonicated until clear, then centrifuged at 45,000*g* at 4° for 20 min. The supernatant was brought slowly to pH 3 with 75% acetic acid, then brought to 12% saturation with ammonium sulfate. The precipitate was stirred at room temperature for 30 min, centrifuged at 45,000*g* at 4° for 30 min, washed twice with distilled water, then suspended in 0.1 N NaOH–8 M urea at a concentration representing 10 mg/ml of original stroma. The suspension was sonicated 3 min until almost clear, allowed to stand at room temperature for 1 hr, then centrifuged at 45,000*g* at 4° for 30 min. The pH of the supernatant was again adjusted to 3 with 75% acetic acid and brought to 40% saturation with ammonium sulfate. The resultant precipitate was stirred at room temperature for 30 min, centrifuged at 45,000*g* at 4° for 30 min, washed three times with distilled water, then suspended and homogenized in 4:1 acetone–butanol (v/v) before being collected on a Büchner funnel equipped with Whatman No. 50 filter paper. This filtrate was thoroughly washed with the acetone–butanol, then given another thorough wash with 75% methanol heated to 50°. Suction was continued another 30 min to the filtrate which was then allowed to dry overnight at room temperature.

Ultracentrifugation. Human red cell structural protein was taken up in 0.1 N NaOH. Runs were made at 59,780 rpm at 5° in a Spinco Model E analytical ultracentrifuge and calculated sedimentation coefficients were corrected for temperature and density.

Carboxy-Terminal Amino Acid Studies. Various efforts were made to render structural protein of human red cell and *Neurospora* mitochondria origin susceptible to carboxypeptidase A digestion. Structural protein and cyanogen bromide treated structural protein (Steers *et al.*, 1965) were suspended in various basic buffers including ammonium carbonate, ammonium bicarbonate, and sodium hydroxide, sonicated, and incubated for various lengths of time, as well as boiled. The reaction with carboxypeptidase A took place at room temperature in 2 M NH_4HCO_3 and was observed over a period from 5 min to 3 hr. Supernatants obtained after trichloroacetic acid (100%) precipitation were heated to dryness, taken up in 0.2 M citrate buffer (pH 3.28), and examined for free amino acids on a Beckman amino acid analyzer, Model 120.

Spectrophotometric Studies. Structural protein derived from human red cell membranes and from *Neurospora* mitochondria were dissolved in 0.1 N NaOH and 75% formic acid at concentrations of 0.1 mg/ml and their spectral profiles in the base and acid solvents compared on a Beckman Model DB recording spectrophotometer in the ultraviolet range.

Polyacrylamide Gel Electrophoresis. Structural protein from human red cell membrane and from *Neurospora* mitochondria were run simultaneously on a variety of systems. Equal amounts (165 μg) of protein were applied at the origin. In our hands three buffer systems provided the most interesting results. (1) A modification of the method of Ornstein and Davis (1962) was used with upper buffers being 0.0509 M glycine and 0.053 M Tris (pH 8.9) and lower buffer being 0.05 M HCl and 0.1 M Tris (pH 8.1). Structural protein was solubilized in the same manner as was previously used for whole red cell stroma with the exception that 0.01 M dithioerythritol was substituted for 0.1 M β-mercaptoethanol (see Schneiderman, 1965). (2) The method of Ornstein and Davis (1962) was further modified in that the gel solution containing glycine and TEMED was brought to pH 11.3 by the addition of 10 N NaOH before mixing. In this system the upper buffer contained 2.45 ml of *N*-ethylmorpholine and 1.613 g of proline per l. of H_2O, and the lower buffer 2.53 ml of *N*-ethylmorpholine and 10 ml of 1 N HCl per l. of H_2O.[2] Both buffers were prepared immediately before use. (3) Structural protein samples from both sources were also solubilized and run by the phenol–acetic acid method of Takayama *et al.* (1964).

Amino Acid Analysis. Acid hydrolysates (36 hr) of human red cell membrane structural protein derived from individual donors were examined by the method

[1] Generously provided by D. O. Woodward.

[2] Suggested by T. Jovin.

of Spackman *et al.* (1958) and Moore *et al.* (1958) on a Beckman amino acid analyzer, Model 120.[3]

Two-Dimensional Peptide Mapping. Several different proteolytic enzymes (Worthington) and combinations of enzymes were systematically investigated, including trypsin, chymotrypsin, trypsin plus chymotrypsin, pepsin, leucine aminopeptidase, papain, trypsin plus papain, trypsin plus carboxypeptidase B, and trypsin plus carboxypeptidase B plus papain. Lyophilized structural protein served as substrate.

In addition, however, a number of modifications of structural protein were carried out in an effort to render it more susceptible to enzymatic attack. Structural protein was treated with cyanogen bromide (Steers *et al.*, 1965) prior to digestion with trypsin, trypsin plus chymotrypsin, and trypsin plus carboxypeptidase B plus papain. Cyanogen bromide treated structural protein was also suspended in 2 M urea prior to incubation with trypsin. Performic acid oxidation (Hirs, 1956; Criddle *et al.*, 1966) and succinylation (MacLennan *et al.*, 1965) were also employed prior to trypsin digestion. Papain digestion of lyophilized structural protein was carried out at pH 5.4 for 18 hr following the method of Konigsberg and Hill (1962). Chromatography was followed by high-voltage electrophoresis (Katz *et al.*, 1959) and papers were developed in a 0.1% ninhydrin solution containing collidine. Papers were also developed for tyrosine and histidine by the Pauly method and arginine by the Sakaguchi method.

Phospholipid Binding Experiments. The phospholipid binding characteristics of structural protein obtained from human red cells were compared with structural protein of *Neurospora* mitochondria following methods described by Richardson *et al.* (1964). The protein samples were sonicated and stored (1 mg/ml) in 0.1 N NaOH for 16 hr at 4°. Increments of mixed soybean phosphatides (Associated Concentrates) were suspended in distilled water. After storage, 5-mg samples of protein were incubated with 1-ml increments of the phosphatide suspension (10–90 μg/ml) for 30 min at room temperature and pH 12. Richardson *et al.* (1964) have previously shown that the incubation of structural protein prior to phospholipid addition and the maintenance of high alkaline pH provided optimal binding and reproducibility of results. The pH was then reduced to 7 by dropwise addition of 1 M Tris-HCl followed by 0.1 M HCl. Total phosphorus determinations on the washed, sedimented protein–lipid complex were made by the following modification of the methods of Bartlett (1959) and Robinson *et al.* (1961). The protein–lipid samples contained in 15-ml centrifuge tubes were placed in a 100° oven along with a water blank, two standard samples containing 2 and 4 μg of phosphorus, and samples of mixed soybean phosphatide suspensions in water at concentrations of 10, 30, 50, 70, and 90 μg/ml. Heating was continued for 3 days until dry. Distilled H_2O (1 ml) and 10 N H_2SO_4 (0.5 ml) were added, and after vigorous mixing, each tube was topped with a marble and placed in a 155° oven for 3.5 hr. The samples were removed, 2 drops of 30% H_2O_2 were added, and after vigorous mixing the samples were digested at 155° an additional 1.5 hr. They were then cooled and the volume was brought to 5 ml with distilled water with mixing. After addition of 0.5 ml of 10 N HCl and thorough mixing, the samples were boiled in a 100° water bath for 7 min. Again 0.5 ml of 10 N HCl was added, followed by 0.7 ml of 5% ammonium molybdate and 0.6 ml of Fiske–Subbarow reagent. The volume was brought to 10 ml with distilled water and after thorough mixing, the samples were incubated in a 70° oven for 15 min, cooled to room temperature, and read at 770 mμ.

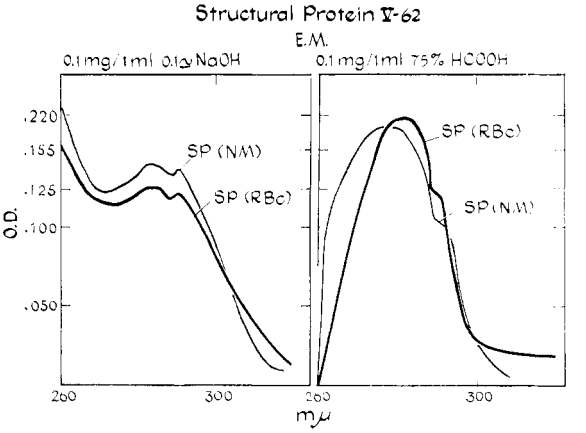

FIGURE 1: Comparative spectrophotometric profiles of structural protein of human red cell membrane (RBC) and *Neurospora* mitochondria (NM) in 0.1 N NaOH and 75% formic acid, illustrating the double-peak characteristics at 280–290 mμ in the two solvents.

Results

Structural Protein Isolation. By means of the isolation procedure described structural protein was obtained from human red cell stroma in about 20% of original dry weight. It was a white powder which proved to be resistant to solubilization in aqueous buffer systems except at pH extremes or in the presence of detergents such as Triton X-100.

Ultracentrifugation. In 0.1 N NaOH structural protein from human red cell membrane sedimented as a single peak with approximate *s* value of 2.3 S. This value closely corresponds with sedimentation coefficients observed for structural proteins of mitochondria (Criddle *et al.*, 1962; Woodward and Munkres, 1966).

Carboxy-Terminal Amino Acid Studies. These investigations did not provide a clear demonstration of the carboxy-terminal amino acid for either structural protein. In both preparations, exposure to carboxypeptidase A elicited a small peak coinciding with glycine at 5 min followed by a small peak coinciding with glutamic acid at 10 min. Within 30 min several other amino acid peaks appeared. Apparent in all the experiments, however, was the lack of increase in any amino acid peak after its initial presentation. The total calculated amino acids, as indicated by the heights of the peaks,

[3] We are grateful to Miss Geraldine Holland for performing these analyses.

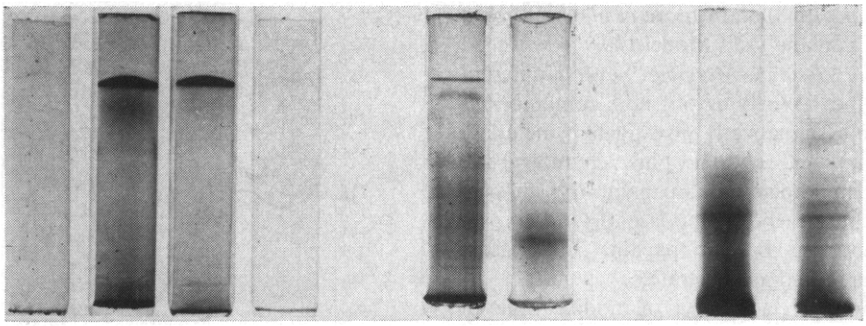

FIGURE 2: Polyacrylamide gel electrophoresis of structural protein derived from human red cell membranes and *Neurospora* mitochondria. Direction of migration is upwards. The group of four gels on the left shows the identical appearance of the two preparations at pH 11. The left pair of this group shows lipid and protein stain, respectively, of human red cell membrane structural protein and the right pair of the same group shows the protein and lipid stain, respectively, of *Neurospora* mitochondria structural protein. In both preparations a single dense protein band is evident, and at the same location a faint lipid-positive band can be seen. The second group of two gels shows the results of electrophoresis and protein staining at pH 8.9. The left member of this group is red cell structural protein, which has formed over a dozen visible bands. The right member of this group is *Neurospora* mitochondria structural protein, which persists as a single major band under these conditions. The third group of two gels shows the results of electrophoresis and protein staining at pH 11. The left member of this group is red cell structural protein, the right member is *Neurospora* mitochondria structural protein. Both samples appear as numerous bands under these conditions, with some correspondence of migration evident.

accounted for less than 1% of the applied protein sample. Thus, the results suggest that either a minute portion of the structural protein preparation was accessible to enzymatic attack or that the amino acids were being released from a trace contaminating protein. In any event, there was a striking similarity in the behavior of the structural proteins obtained from the two sources.

Spectrophotometric Studies. Figure 1 depicts the spectrophotometric profiles of human red cell membrane structural protein and *Neurospora* mitochondria structural protein in 0.1 N NaOH and in 75% formic acid. The double peak seen at 280–290 mμ and its changing character in the two solvents were nearly identical.

TABLE I: Total Amino Acid Composition of Human Red Cell Structural Protein as Determined from Four Separate 36-hr Acid Hydrolysates.

Amino Acid	Micromoles of Amino Acid/100 mg of Protein			
	1	2	3	4
Lys	33	36	35	35
His	12	13	14	13
Arg	35	37	36	35
Asp	62	63	65	67
Thr	37	38	39	40
Ser	43	45	46	47
Glu	95	98	100	105
Pro	37	38	34	36
Gly	51	52	53	57
Ala	60	62	63	66
Val	50	50	52	56
Met	13	16		
Ile	38	39	39	40
Leu	101	103	102	106
Tyr	20	21		
Phe	36	37	40	39

Polyacrylamide Gel Electrophoresis. Figure 2 illustrates the results of simultaneous electrophoretic runs of structural protein derived from the two sources in three different buffer systems. Over the pH range 3–11 the spectrum of similarity to diversity between the two preparations is displayed. At pH 11 and in the presence of *N*-ethylmorpholine the solubilized structural protein samples migrated equally as a single densely staining band. The presence of a small amount of bound lipid could be detected by oil red O stain in both preparations at the same protein site. At pH 8.9, solubilized structural protein of *Neurospora* mitochondria tended to remain concentrated in a single major band whereas structural protein of human red cell membranes resolved itself into at least a dozen fine bands. This latter pattern was reminiscent of, but not identical with, whole red cell stroma under the same conditions (Schneiderman, 1965). At pH 3, both structural protein preparations exhibited a number of bands, some of which showed correspondence in migration, some of which did not.

Amino Acid Analysis. Table I provides the results of four separate runs of structural protein derived from human red cell membranes. Total amino acid analysis of *Neurospora* mitochondria structural protein has previously been reported by Woodward and Munkres (1966). Comparison of these two preparations reveals an over-all similarity in the proportionate contribution of each amino acid to the total protein. Three neutral amino acids, glycine, alanine, and leucine, showed significant differences between the two structural proteins, as did the basic amino acid, lysine. Smaller but probably significant differences were also evident in the amino acids histidine, arginine, and glutamine. The remainder of the amino acids appeared to be identical within the limits of the procedure.

Two-Dimensional Peptide Mapping. Comparative fingerprints of structural protein derived from human

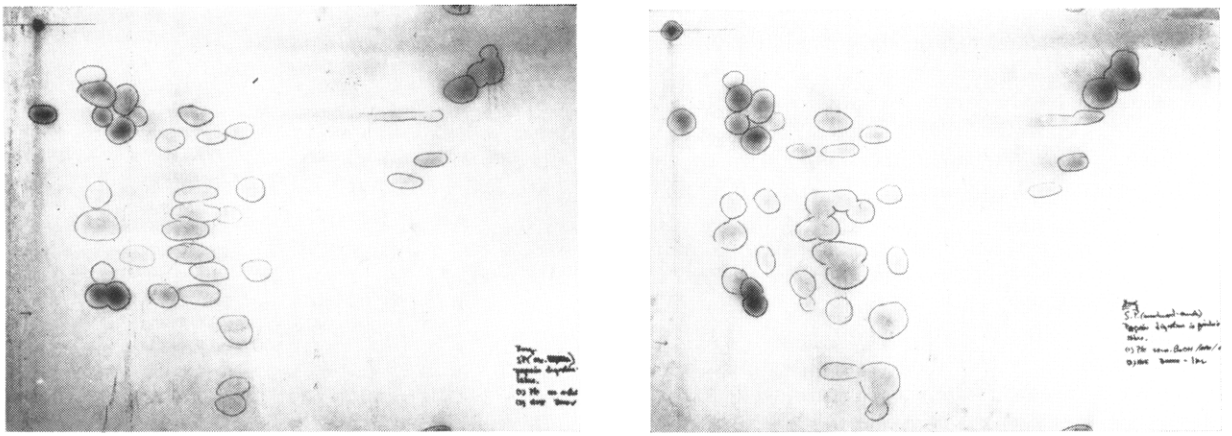

FIGURE 3: Two-dimensional peptide maps of papain-digested structural protein. Chromatography in butanol–acetic acid–water (down) was followed by high-voltage electrophoresis in pyridine–acetic acid–water (pH 3.7) (to the right). Structural protein derived from human red cell membranes is on the left, structural protein derived from *Neurospora* mitochondria is on the right.

red cell membranes and *Neurospora* mitochondria revealed generally similar patterns in the various systems employed. Both proteins, however, showed striking resistance to enzymatic digestion or gave smeared fingerprints of less than useful quality. In our hands the most successful demonstration of the major degree of similarity, as well as the small but unequivocal differences, between the two structural proteins occurred following papain digestion of the lyophilized material. With this enzyme, approximately 72% of the protein could be hydrolyzed and studied by two-dimensional analysis. As can be seen in Figure 3, the accessible portion of both proteins gave rise to many apparently homologous peptides. Nevertheless a few differences were evident in both densely stained and faintly stained spots. Specific amino acid stains revealed identical patterns and thus did not add further information to the differences observed.

Phospholipid Binding Experiments. Figure 4 illustrates the comparative phospholipid binding characteristics of the two structural proteins. Both samples showed the same ascending slope of optical activity representing protein-bound phospholipid following incubation with increments of mixed soybean phospholipid.

Discussion

Isolation of a structural protein from human red cell membranes has been achieved and chemical characterization has demonstrated its similarity to structural proteins reported from other membrane systems. Parallel experiments with *Neurospora* mitochondria structural protein were carried out which revealed a considerable homology between the two preparations.

Unfortunately the term, structural protein, suggests a more precise understanding of its role in biological membrane systems than is warranted. Nevertheless, a protein moiety can be obtained from a variety of membrane sources which shares a group of properties. Several of these properties, namely an insolubility in aqueous media under physiologic conditions, a capacity to bind phospholipid, and a tendency to aggregate, would be those expected within the current concepts of membrane structure.

Exactly what structural protein is, whether it is a single species of protein, a "family" of proteins, or even an artifact of preparation, has to be considered. The evidence gathered here is most consistent with structural protein existing as a single species of protein which tends to aggregate under certain conditions (see also Woodward and Munkres, in preparation). In a basic buffer, human red cell membrane structural protein sedimented and migrated electrophoretically as one band. In a molecular sieve system, such as polyacrylamide gel, under lower pH conditions, structural protein appeared as several, variously migrating bands.

Although we were unable to establish homogeneity of structural protein by demonstration of a single

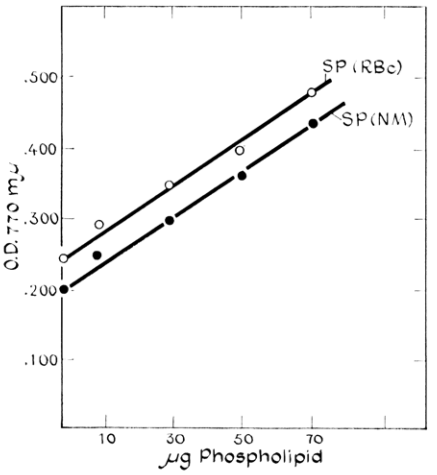

FIGURE 4: Comparative phospholipid binding curves of structural protein prepared from human red cell membranes (RBC) and *Neurospora* mitochondria (NM). Axis represents concentration of mixed soybean phospholipid incubated with aliquots of structural protein at pH 12. Ordinate indicates optical activity of phospholipid phosphorus in sedimented protein–lipid complex. (see Methods). Amount of phospholipid bound by both preparations increased similarly as functions of the amount of phospholipid added.

C-terminal amino acid, the results of carboxypeptidase A digestion militate against such bands representing different, discrete protein entities. All such proteins would have had to be equally resistant to the proteolytic enzyme (*e.g.*, either have had C-terminal arginine, lysine, or proline, or shared some other inhibitor of enzyme attack) to account for the negligible release of free amino acids.

No attempt was made to establish the N-terminal amino acid in view of the difficulties encountered by Criddle *et al.* (1962, 1966); instead experiments are in progress (Woodward and Schneiderman, 1968) to render structural protein more susceptible to carboxypeptidase A digestion following treatment with trypsin and carboxypeptidase B digestion following treatment with chymotrypsin, Hopefully, the action of the first enzymes will yield their characteristic C-terminal products which are resistant to the respective carboxypeptidase enzymes, while at the same time making possible the release of the original C-terminal amino acid.

As a whole, the data reported here tend to support the concept that structural protein represents a discrete, albeit poorly understood, component of membranes rather than a preparative artifact. Were structural protein merely a conglomerate of inactivated enzymes, for example, one would have expected considerable dissimilarity between the peptide patterns, reflecting the differences in enzyme content of the membrane sources. Such specificity of structural protein is further supported by the demonstration by Woodward and Munkres (1966) of a single amino acid difference in structural protein derived from mitochondria of a respiratory-deficient strain of *Neurospora*. Whether an altered structure–function analogy exists in the hereditary hemolytic anemias may become evident as the structure of the human red cell membrane is explored in greater detail.

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