Age-dependent Accumulation of Protein Residues Which Can be Hydrolyzed to D-Aspartic Acid in Human Erythrocytes*

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We have measured the rate of accumulation of amino acid residues in human erythrocyte membrane and cytosolic proteins which give D-aspartic acid upon acid hydrolysis. These residues would include D-aspartic acid, D-asparagine, as well as the β-transpeptidation product, D-isoaspartic acid. Measurements made using age (density) fractionated cells indicate that racemization at these residues occurs on membrane proteins with a 1% (the time required to convert 1% to the D configuration) of about 38.6 days. Fractionation of membrane components revealed a faster rate of racemization for intrinsically proteins than for extrinsic proteins. On the other hand, significant age-dependent racemization was not detected for cytosolic proteins, and the calculated 1% value for these proteins is at least 4 times larger. These results suggest that in the 120-day life span of an erythrocyte, significant racemization of membrane (but not cytosolic) proteins can occur. We have also determined that the rates of accumulation of these residues for erythrocyte membrane and cytosolic proteins incubated in vitro are similar to those observed in vivo. These observations are discussed in terms of the possible cellular metabolism of racemized proteins.

D-Aspartic acid has been detected in hydrolysates of the long lived proteins of teeth (1, 2) and eye lens (3). Although the exact origin of this uncommon amino acid has not been established, it appears to be derived from L-aspartyl and/or L-asparaginyl residues in proteins that have undergone spontaneous racemization reactions. The presence of D residues may contribute to the decline in cell function associated with the aging process (4).

To examine whether significant protein racemization reactions can occur more generally in mammalian tissues and whether a cell can limit the accumulation of racemized asparagine and its derivatives, we chose to study this process in human erythrocytes. The membrane and cytosolic proteins of these cells are synthesized in erythrocyte precursors; mature cells then exist in the bloodstream for 120 days with essentially no protein synthesis or degradation. We were particularly interested in the erythrocyte system because recent studies had indicated that enzymes present in these cells could catalyze the formation of protein D-aspartic acid β-methyl esters and L-isoaspartic acid α-methyl esters (5–10). These results suggested that these methyltransferases recognize chemically altered aspartyl residues and that the methylation reaction could be the first step in the repair or selective degradation of proteins containing such residues (5, 10–12). Thus, it is possible that the cell is able to metabolize racemized proteins and that the net accumulation of D-aspartic acid in protein hydrolysates would then reflect both the rates of the spontaneous formation of these residues and their metabolism.

Mechanistic considerations further suggest that racemization and isomerization-prone intermediates may be important in the formation of D-aspartyl and L-isoaspartyl residues in proteins (5–8, 10–12). One such intermediate is the L-succinimide product of the intramolecular attack of the peptide nitrogen on the side chain carbonyl carbon of aspartyl and asparaginyl residues. This intermediate can not only racemize to the D-succinimide, but each of these forms can be readily hydrolyzed to give a mixture of about 75% of the isoaspartyl residue and 25% of the normal aspartyl residue (7–8, 10–12). If this is the case, a significant fraction, or even the majority, of D-aspartic acid in hydrolysates of aging proteins may be derived from D-isoaspartyl residues.

In this study we have measured the rates of D-asparaginyl/D-isoasparaginyl/D-isoaspartyl accumulation in red blood cells under both in vitro conditions, where metabolism of D-aspartyl residues by enzymatic methylation reactions does not occur, and under in vivo conditions, where methylation does occur.

**MATERIALS AND METHODS**

**RESULTS**

In Vivo Accumulation of D-Asparaginyl/D-Aspartyl Residues in Erythrocyte Membrane and Cytosolic Proteins—Erythrocytes were separated by density into different age fractions and the relative amounts of D- and L-aspartate in acid hydrolysates of each fraction were determined by HPLC as described under "Materials and Methods." We find a steady increase in the amount of D-aspartic acid in hydrolysates of the membrane fraction as a function of cell age (Fig. 4A). Rate constants determined from these data for the inter-
conversion of L-aspartic and D-aspartic acid were found to be similar in the three separate experiments performed using blood from three individuals (Table I), although there were differences (up to 1.4-fold) in the absolute value of the D/L ratio at a given cell age. It is possible that these differences are due to variable amounts of background racemization that occur during the acid hydrolysis step itself; small changes in the hydrolysis temperature can give rise to significant differences in the amount of racemization at this step.

The accumulation of D-residues was also measured for the cytosolic fraction of these erythrocytes, which contains predominantly hemoglobin. Fig. 4A shows the results of DL-aspartate analysis performed on cytosol prepared from the same age-fractionated cells whose membranes were used above. These data show that if there is any accumulation of D-aspartyl/D-asparaginyl/D-isoadaspartyl residues in the cytosol it is below the limits of detection of our system.

Although the hydrolyses of membrane samples and cytosol samples of a given age fractionation were done simultaneously, the absolute values of the D/L-aspartate ratios for the membrane samples, even at the earliest ages, are greater than those obtained for the cytosolic samples. This difference is too large to be due to the presence of free L-aspartate and D-aspartic acid fractionations; the blood used was from different individuals. O, membranes, fractionation 1; Δ, membranes, fractionation 2; □, membranes, fractionation 3; ▲, cytosol, fractionation 2; ■, cytosol, fractionation 3. B, change in D/L-aspartate ratio as a function of cell age in intrinsic and extrinsic membrane proteins. Hydrolyses were samples done simultaneously. ▪, intrinsic membrane fraction, fractionation 4; □, intrinsic membrane fraction, fractionation 5; ●, extrinsic membrane fraction, fractionation 4; ○, extrinsic membranes fraction, fractionation 5. The error bars represent the range of duplicate or triplicate samples analyzed in duplicate runs on the HPLC. The slopes indicate the linear best fit of all data points.

![Figure 4](image_url)

**Fig. 4.** Age-dependent accumulation of residues which give D-aspartic acid upon hydrolysis in intact erythrocytes. Erythrocytes were fractionated into age classes by isopycnic density centrifugation. Samples of whole membranes, cytosol, and both intrinsic and extrinsic membrane proteins for each age fraction were prepared as described under "Materials and Methods." Aliquots of each sample were acid hydrolyzed, the released aspartate isolated, and the ratio of D- to L-aspartate determined by HPLC as described under "Materials and Methods." Hydrolyses were done simultaneously for all samples derived from a given age fractionation. A, change in D/L-aspartate ratio as a function of cell age in whole membranes and cytosol. The data shown are derived from analyses of three separate density (age) fractionations; the blood used was from different individuals. O, membranes, fractionation 1; Δ, membranes, fractionation 2; □, membranes, fractionation 3; ▲, cytosol, fractionation 2; ■, cytosol, fractionation 3. B, change in D/L-aspartate ratio as a function of cell age in intrinsic and extrinsic membrane proteins. Hydrolyses were samples done simultaneously. ▪, intrinsic membrane fraction, fractionation 4; □, intrinsic membrane fraction, fractionation 5; ●, extrinsic membrane fraction, fractionation 4; ○, extrinsic membranes fraction, fractionation 5. The error bars represent the range of duplicate or triplicate samples analyzed in duplicate runs on the HPLC. The slopes indicate the linear best fit of all data points.

Table I: Accumulation of D-aspartyl/D-asparaginyl residues in erythrocyte proteins in vivo and in vitro

<table>
<thead>
<tr>
<th>Sample</th>
<th>$k_{	ext{ave}}$ (slope)$^a$</th>
<th>Average $k_{	ext{ave}}$ $^b$</th>
<th>$t_1$%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. In vivo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 1</td>
<td>0.208 ± 0.518 ± 0.161 ± 0.423</td>
<td>&gt;171 ±</td>
<td></td>
</tr>
<tr>
<td>Trial 2</td>
<td>0.113 ± 0.830 ± 0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membranes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 1</td>
<td>2.44 ± 1.18 ± 2.59 ± 0.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 2</td>
<td>2.81 ± 0.66 ± 3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intrinsic membrane fraction</td>
<td>2.94 ± 0.43 ± 3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extrinsic membrane fraction</td>
<td>0.715 ± 0.713 ± 1.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>II. In vitro</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.098 ± 0.995 ± 0.423</td>
<td>&gt;90 ±</td>
<td></td>
</tr>
<tr>
<td>Membranes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 1</td>
<td>1.43 ± 1.05 ± 1.48 ± 0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 2</td>
<td>1.53 ± 0.27 ± 1.67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Error expressed represent 95% confidence limits calculated using Student's t test. The rate constant describing the interconversion of residues which give L-aspartate and D-aspartate upon hydrolysis is defined by the following equation:

$$ k_{	ext{ave}} = \frac{\ln \left[ \frac{(1 + D/L)}{(1 - D/L)} \right] - \ln \left[ \frac{(1 + D/L)_0}{(1 - D/L)_0} \right]}{2t} $$

where $t = $ time of racemization. For small values of the D/L ratio, the rate constant closely approximates the slope of the line when the D/L ratio is plotted as a function of time as described in the legends to Figs. 4 and 5.

$^b$ Error expressed represents the mean of the 95% confidence limits for replicate experiments.

$^c$ Not statistically significant at 95% confidence limit.

$^d$ Calculated using the upper 95% confidence limit.

The effects of such variation can be estimated using the Arrhenius equation, assuming an activation energy (Ea) of 21.5 kcal/mol for the fully protonated free aspartate species (22). We have calculated that the observed maximal difference in absolute D/L-aspartate ratios could result from only a 5°C variation in oven temperature during the acid hydrolysis step, and this variation is within the ±3°C range that we measure for this oven.

We have also considered the possibility that the rate of racemization of red cell proteins during the acid hydrolysis step itself may be dependent upon the age of the protein and would contribute to the accumulation of racemized residues. Although this possibility cannot be ruled out conclusively at this time, control experiments with "native" protein and peptides have demonstrated that the degree of racemization during hydrolysis is generally less than 3% under these conditions.
significantly larger than that of the extrinsic proteins (Table I).

**In Vitro Accumulation of D-Aspartyl/D-Asparaginyl/D-Isospartyl Residues in Membrane and Cytosolic Proteins**—To compare the rate of D-residue accumulation in intact cells with the rate in *vitro*, we fractionated red blood cells into both a membrane and a cytosolic fraction, and then artificially aged “aged” each fraction by incubating aliquots for appropriate times in isotonic phosphate at physiological pH in a 37 °C constant temperature water bath as described under “Materials and Methods.” The rate of D-aspartate accumulation was then determined as described above; the results are shown in Fig. 5. With both the membrane and cytosolic fractions the rate of D-aspartate accumulation is similar to that of equivalent fractions prepared from cells aged in the bloodstream (Table I).

**DISCUSSION**

**Detectable Accumulation of D-Aspartyl/D-Asparaginyl/D-Isospartyl Residues in Aging Erythrocyte Membrane but Not Cytosolic Proteins**—We show here the time-dependent accumulation of residues which give D-aspartic acid upon hydrolysis in erythrocyte membrane proteins *in vivo* and *in vitro* (Figs. 4 and 5; Table I). The data obtained for cytosolic proteins, on the other hand, indicate that these residues do not accumulate at a rate that we can distinguish from the background racemization that occurs during the hydrolysis step. Differences in the rate of racemization of intrinsic and extrinsic membrane proteins and cytosolic proteins in intact cells may represent either differences in their intrinsic susceptibility to racemization or the differential activity of a repair or degradation system (5). It is clear that the fraction of aspartyl/asparaginyl residues in intrinsic membrane proteins exposed on the extracellular surface would not be accessible to cytosolic enzymes of such a system.

Our failure to detect a difference in the rates of *in vivo* versus *in vitro* racemization may indicate that an efficient racemization repair mechanism does not function in erythrocytes (5). However, there are two major limitations on these experiments that may make the detection of a racemization repair system difficult. In the first place, D-aspartic acid found in hydrolysates of erythrocyte proteins can be derived from D-aspartic acid, D-asparagine, D-isospartic acid, and other modifications of these amino acids in the D-configuration. There is no evidence that protein carboxymethyltransferases can recognize D-asparaginyl or D-isospartyl residues. If one compares the steady-state number of protein methyl esters with the number of racemized residues, it is clear that only a small fraction can be esterified at a given time. For the membrane fraction of an erythrocyte of average age (60 days), containing about $2.3 \times 10^6$ aspartyl and asparaginyl residues (26), the steady-state number of protein methyl esters has been estimated at 154,000 (9). From the data in Table I, we can calculate that this cell would contain $5,500,000$ D-aspartyl, -isospartyl, or -asparaginyl residues. Thus only 4.3% of the potential D-sites appear to be methylated at a given time. A similar calculation shows that less than 1% of the potential cytosolic D-residues are methylated at steady state. This disparity is amplified when one considers that the number of methyl esters may reflect methylation at L-isospartyl residues as well as at D-aspartyl residues (7, 8).

A second major limitation involved in comparing the rates of racemization *in vitro* and *in vivo* is whether the *in vitro* conditions provide an appropriate match for the environment of an intact cell. In the bloodstream, the erythrocyte is subjected to great shear forces and is required to undergo rapid changes in shape in order to flow through the smallest of capillaries (27, 28). If the rate of asparaginyl/isospartyl racemization were increased as a consequence of shape change then we would expect to see a faster rate of D-aspartate accumulation in the proteins most involved in the shape change (the membrane proteins) *in vivo*. Another difference

**Table II**

<table>
<thead>
<tr>
<th>Tissue/protein source (Ref)</th>
<th>k&lt;sub&gt;rec&lt;/sub&gt; (×10&lt;sup&gt;-8&lt;/sup&gt;) day&lt;sup&gt;-1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vivo</strong></td>
<td><strong>In vitro</strong></td>
</tr>
<tr>
<td>Erythrocyte proteins</td>
<td></td>
</tr>
<tr>
<td>(this study)</td>
<td></td>
</tr>
<tr>
<td>Whole membranes</td>
<td>25.9</td>
</tr>
<tr>
<td>Intrinsic membrane fraction</td>
<td>29.4</td>
</tr>
<tr>
<td>Extrinsic membrane fraction</td>
<td>7.15</td>
</tr>
<tr>
<td>Cytosol</td>
<td>1.61&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lenses tissue (3)</td>
<td>0.336</td>
</tr>
<tr>
<td>Tooth enamel (1)</td>
<td>0.228</td>
</tr>
<tr>
<td>Dentin (2)</td>
<td>0.216</td>
</tr>
<tr>
<td>Casein (30)</td>
<td>533 (140.1) pH 8.0</td>
</tr>
<tr>
<td>Collagen (31)</td>
<td>265 (20.5) pH 8.0</td>
</tr>
<tr>
<td>Calmodulin (32)</td>
<td>113 (7.4) pH 6.0</td>
</tr>
<tr>
<td>Ribonuclease (33)</td>
<td>153 (10.0) pH 5.4</td>
</tr>
<tr>
<td>Polyaspartic acid (33)</td>
<td>151 (9.9) pH 7.65</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values for calmodulin, ribonuclease, and polyaspartic acid were determined for single values at 100 °C, casein from a single value at 65 °C, and collagen from four determinations in the range of 95.5-140.5 °C. The pH of the measurement is indicated. All values were corrected to a temperature of 37 °C by the Arrhenius equation assuming that all of these proteins have the same activation energy shown by collagen at pH 8.0 (20.8 kcal/mol, Ref. 31). This value is similar to that determined for casein in 0.1 M NaOH (20.8 kcal/mol, Ref. 30). Alternative values in parentheses were calculated using an activation energy of 30 kcal/mol (J. L. Bada, personal communication).

<sup>a</sup>Value not statistically different from zero.

**Fig. 5.** *In vitro* accumulation of D residues in erythrocyte membrane and cytosolic proteins. Whole membranes and cytosol were prepared under sterile conditions and incubated at 37 °C for the indicated time as described under “Materials and Methods.” The contents of each tube were then hydrolyzed, the aspartate isolated, and the D/L-aspartate ratio determined as described. **Top**, change in D/L-aspartate ratio as a function of incubation time for whole membranes. **C**, trial 1; **O**, trial 2. **Bottom**, change in D/L-aspartate ratio as a function of time for cytosol (D) trial 3. Error bars indicate the range of duplicate or triplicate samples analyzed in duplicate runs on the HPLC. Slopes indicate the least squares best fit of all data points.
is that some protein denaturation was noted after 30 days of \textit{in vitro} incubation. This denaturation was evidenced by the appearance of fibrous and particulate matter and a change from a red to a reddish-brown color of the cytosolic proteins. Finally, it is possible that buffer salts and metabolites may alter the rate of racemization (cf. Refs. 4 and 20), and that there may be differing levels of these compounds in intact cells and in the \textit{in vitro} incubation mixtures.

Previous experiments designed to test the racemization repair hypothesis have involved measuring the D-aspartic acid content of erythrocyte membranes from cells incubated for 20 h in the presence and absence of inhibitors of protein methylation (29). A small, but statistically significant increase in D-aspartate content was found in the cells without an active methylation system. If this increase represented the rate of spontaneous racemization that was corrected by repair, one could estimate that the racemization rate would be about 1.6 days. Because both the \textit{in vitro} and \textit{in vivo} rates measured in the present work are much slower than this, the former results may reflect nonphysiological conditions during the incubation, or a combination of a repair step and an artifactual slow racemization rate in the \textit{in vitro} system (see above).

\textbf{Comparison of Racemization Rates of Erythrocyte and Nonerythrocyte Proteins}—Table II compares the \textit{in vivo} and \textit{in vitro} rates of D-aspartyl residue accumulation that we have measured for red cell proteins with published values for other proteins. \textit{In vivo}, erythrocyte protein D-aspartyl residues appear to accumulate 10–100 times faster than they do in lens, tooth enamel, and dentin. Proteins in these latter cells are potentially much longer lived than those in red cells (50–80 years \textit{versus} 120 days) and these tissues may have found it necessary to evolve proteins which are more resistant to racemization or to increase the efficiency of repair mechanisms in order to limit the accumulation of racemized residues during their life time. Indeed, if these tissues accumulated D-aspartyl residues at a rate similar to that seen in red cell membranes, we would predict that their proteins would contain 39% D-aspartate in only 6 years.

Racemization leading to D-aspartic acid in hydrolysates has been measured in the neutral pH range for several proteins. These rates, generally obtained at elevated temperatures, can be compared to those measured here for erythrocyte proteins by direct extrapolation to 37°C (as with collagen) or by assuming that the other proteins have the same energy of activation for racemization as collagen (31). The data in Table II indicate that red cell protein aspartyl/asparaginyl residues appear to racemize more slowly than those in these other species. These data suggest that caution should be used in interpreting data from tissue and fossil dating methods which are based on aspartate racemization rates (20, 34) since not all types of proteins may accumulate D-aspartyl/D-asparaginyl residues at the same rate.

\textit{Acknowledgments}—We would like to thank Dr. E. David Murray for his assistance in the HPLC analysis, Daisy Wang and Pablo Villanueva of the Medical Immunology Laboratory at UCLA for supplying us with blood samples, and Dr. Jeffrey L. Bada for helpful comments on the manuscript.

\textbf{REFERENCES}

Preparation of Erythrocyte Membranes and Cytosol—Fresh human erythrocytes from healthy volunteers were obtained by venipuncture into heparinized vacutainers. The cells were fractionated within a maximum of 24-30 hours after isolation. After removing leukocytes on a nylon column, cells were separated into age fractions on the basis of density using a modification of the self-forming gradient technique of Van Kampen and Zijlstra (13) as described previously (14). Percoll was obtained from Sigma and Aquoplate 282 (60% w/v) diatrizoate meglumine (Hoechst Laboratories) was substituted for the Percoll-Ficoll (1:1) mixture in the age fractions. The red cells were then washed four times by centrifugation at 1000 g for 5 minutes at 0-4°C in at least 10 volumes of isotonic phosphate buffer, pH 7.4. Care was taken to remove all visible traces of the buffy coat and density gradient material during aspiration of the wash supernatants. The approximate average age of each cell fraction was calculated from deviations of cytoplasmic pyruvate kinase specific activity from that of unfractionated cells (14), assuming that the average age of unfractionated cells was 60 days and that pyruvate kinase decays with a first order halftime of 29 days (14, 15). Pyruvate kinase specific activity was measured in duplicate by the coupled assay of Deen et al. (15). Hemoglobin was measured by the method of Van Kampen and Buitkaitis (16).

Preparation of Intrinsically and Extrinsic Membrane Proteins—Extrinsic membrane proteins were extracted from erythrocyte membranes by incubation in 10 volumes of 1% (w/v) acetic acid for 15 minutes at room temperature (18). The resulting solution was then centrifuged at 30,000 g for 15 minutes. The supernatant, containing the extrinsic membrane proteins, was removed by aspiration. The pellet, containing the intrinsic membrane proteins and lipids, was washed twice by centrifugation in 1% acetic acid to remove any remaining contaminating extrinsic proteins. Extraction of red cell membranes under hypotonic conditions results in the separation of approximately 30% of the steady state membrane asparagine ester (extrinsic fraction) from the remaining 70% of the aspartyl esters (intrinsic fraction) (19).

Determination of Optimal Acid Hydrolysis Time for Erythrocyte Proteins—In order to minimize acid racemization under conditions for acid hydrolysis of protein samples (20-22), we determined the minimum time necessary to quantitatively release aspartate in erythrocyte proteins in 6M HCl at 120°C. The results of a control experiment measuring aspartate release from red cell membranes as a function of hydrolysis time at 120°C are shown in Fig. 1. Membranes, aged with trace amounts of time [14C]-L-aspartate, were mixed with an equal volume of 1M HCl, sealed in vacuo, and then allowed to hydrolyze at 120°C for various times. Erythrocyte acid released was detected by amino acid analysis on a Beckman 120C amino acid analyzer. The results were corrected for the recovery of the [14C]-L-aspartate standard. These experiments show that the release of aspartate from unfractionated red cell membranes at age fraction 2 (55-70 days old) is complete in approximately 3-4 hours. In the experiments below, we used a 5 hour hydrolysis time.

**Preparation of Intrinsic and Extrinsic Membrane Proteins**—Extrinsic membrane proteins were extracted from erythrocyte membranes by incubation in 10 volumes of 1% (w/v) acetic acid for 15 minutes at room temperature (18). The resulting solution was then centrifuged at 30,000 g for 15 minutes. The supernatant, containing the extrinsic membrane proteins, was removed by aspiration. The pellet, containing the intrinsic membrane proteins and lipids, was washed twice by centrifugation in 1% acetic acid to remove any remaining contaminating extrinsic proteins. Extraction of red cell membranes under hypotonic conditions results in the separation of approximately 30% of the steady state membrane asparagine ester (extrinsic fraction) from the remaining 70% of the aspartyl esters (intrinsic fraction) (19).

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**Preparation of Intrinsically and Extrinsic Membrane Proteins**—Extrinsic membrane proteins were extracted from erythrocyte membranes by incubation in 10 volumes of 1% (w/v) acetic acid for 15 minutes at room temperature (18). The resulting solution was then centrifuged at 30,000 g for 15 minutes. The supernatant, containing the extrinsic membrane proteins, was removed by aspiration. The pellet, containing the intrinsic membrane proteins and lipids, was washed twice by centrifugation in 1% acetic acid to remove any remaining contaminating extrinsic proteins. Extraction of red cell membranes under hypotonic conditions results in the separation of approximately 30% of the steady state membrane asparagine ester (extrinsic fraction) from the remaining 70% of the aspartyl esters (intrinsic fraction) (19).

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Fig. 1 shows a typical elution profile obtained when the standards L-glutamate and [14C]-L-aspartate are co-chromatographed. Basic and hydrophobic amino acids are not retained by this resin and the amino acid mixture, threonine, and tyrosine were found to bind only slightly to the column, eluting prior to glutamate (data not shown). Greater than 95% of the glutamate is eluted in the first 0.5 M acetic acid wash step. Aspartate is then eluted from the column with 1.0 M acetic acid. The recovery of aspartate loaded onto the column is greater than 90%.

The abbreviations used are: L-aspartyl and D-asparaginyl and D-isoasparaginyl derivatives of synthetic D,L-aspartate are co-chromatographed. Basic and hydrophobic amino acids are not retained by this resin and the amino acid mixture, threonine, and tyrosine were found to bind only slightly to the column, eluting prior to glutamate (data not shown). Greater than 95% of the glutamate is eluted in the first 0.5 M acetic acid wash step. Aspartate is then eluted from the column with 1.0 M acetic acid. The recovery of aspartate loaded onto the column is greater than 90%.

Analysis of D- and L-Aspartate in Erythrocyte Proteins---The relative amounts of D- and L-aspartate present in purified aspartate fractions of hydrolysates of erythrocyte proteins were determined by high performance liquid chromatography. Dried samples were dissolved in water. Aliquots of each sample, containing between 10 and 1000 pmol of aspartate, purified from 20-1000 mg of red cell protein, were converted to diastereomeric derivatives with o-phthalaldehyde (sigma) and N-acetyl-L-cysteine (Sigma) and analyzed on a Hitachi model 550 fluorometer equipped with a 95% acetic acid wash step. The eluting solvent mixture of 0.03 M sodium acetate, pH 5.90, and 1 mM EDTA, pH 8.2, was observed at Ex 340 nm and Em 410 nm. Each sample was dissolved in 2 ml 1 N hydrochloric acid, 0.1 M sodium phosphate, pH 7.0, and 0.5 M sodium chloride, pH 7.4, and brought up in 4 volumes of 118 mM sodium phosphate, pH 7.0, containing 0.1 M sodium chloride and 0.1 M sodium chloride, pH 7.4, containing sodium chloride and sodium perchlorate as above. The resulting creamy white membranes were then washed twice with 5 mM sodium phosphate, pH 6.5, and 1 M sodium phosphate, pH 7.4, containing 0.1 M sodium chloride and 0.1 M sodium chloride, pH 7.4, containing sodium chloride and sodium perchlorate as above. Samples of the membrane and cytosol preparations were then eluted into sterile glass dialysis tubes. The ends of the tubes were sealed and placed in a 37°C water bath. As appropriate intervals, triplicate samples were removed from the water bath and frozen at -20°C for later HPLC analysis as described above. Each sample was streaked on rich agar plates, after incubating in the water bath, to screen for any bacterial contamination.