Incorporation of L-Azetidine-2-carboxylic Acid into Hemoglobin in Rabbit Reticulocytes in Vitro*

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SUMMARY

L-Azetidine-2-carboxylic acid is the naturally occurring lower homologue of L-proline. Reticulocytes from anemic rabbits incubated with DL-[14C]azetidine-2-carboxylic acid synthesized radiolabeled hemoglobin, which when isolated from cell lysates co-chromatographed with unlabeled hemoglobin on Sephadex G-100 columns. Amino acid analysis of hemoglobin from reticulocytes incubated with DL-[14C]azetidine-2-carboxylic acid suggested that the homologue was incorporated into hemoglobin intact and unaltered. Alternatively, another amino acid analogue, L-aminocyclohexanepentane-[1-14C]carboxylic acid, which is purported to be a valine antagonist, was not incorporated into hemoglobin under these conditions.

Incubation of reticulocytes with 1, 5, and 10 mM L-azetidine-2-carboxylic acid reduced L-[U-14C]proline (0.10 mM) incorporation into hemoglobin by 25, 58, and 72%, respectively. Conversely, 1.45 and 145 µM L-proline reduced radiolabeled azetidine-2-carboxylic acid (0.8 mM) incorporation into hemoglobin by 45 and 92%, respectively. Incorporation of L-[U-14C]leucine and L-[U-14C]lysine (0.1 mM each) into hemoglobin was unaffected at these concentrations of L-azetidine-2-carboxylic acid. These results suggest that L-azetidine-2-carboxylic acid is incorporated into hemoglobin without reducing the rate of globin synthesis in rabbit reticulocytes in vitro.

The α and β chains of hemoglobin into which [14C]azetidine-2-carboxylic acid had been incorporated in rabbit reticulocytes in vitro were resolved electrophoretically on sodium dodecyl sulfate-polyacrylamide gels. The ratio of total radioactivity in the α and β chains separately extracted from gels was in good agreement with the known 7:4 ratio of prolyl residues in the respective chains. Autoradiograms of two dimensional tryptic peptide maps of rabbit globin into which either [14C]azetidine-2-carboxylic acid or L-[14C]proline had been incorporated showed nearly identical patterns of radioactivity. These results suggest that azetidine-2-carboxylic acid substitutes specifically for prolyl residues during in vitro hemoglobin synthesis in rabbit reticulocytes.

DL-Azetidine-2-carboxylic acid, the lower homologue of L-proline (Fig. 1), is a naturally occurring imino acid in certain species of the Liliaceae (1). Azetidine carboxylate has been reported to constitute up to 8% of the dry weight of Convallaria majalis (lily-of-the-valley) leaves, but apparently is found only in the free “imino and amino acid fraction” and not in the polypeptide backbone of plant protein (2). Although the role of azetidine carboxylate in natural selection is incompletely understood, it has been shown that azetidine carboxylate-trRNA is not produced in species where azetidine carboxylate occurs naturally but is readily synthesized in microorganisms and in higher plants (3). In Escherichia coli and in Mung bean seedlings, azetidine carboxylate administration resulted in stoichiometric replacement of prolyl residues in newly synthesized protein (4), and when given simultaneously with other amino acid analogues to HeLa cells infected with poliovirus, abnormal virus coat protein was produced (5). More recently, azetidine carboxylate has been reported to ameliorate hepatic cirrhosis in rats treated with carbon tetrachloride without affecting synthesis of non-collagenous proteins in the liver (6, 7). Azetidine carboxylate is incorporated into collagen in avian embryos (8). In preliminary communications, we have shown that azetidine carboxylate is incorporated into protein in rabbit reticulocytes (9) and into protein synthesized by the alga, Cyanidium caldarium (10).

Replacement of proline with azetidine carboxylate in protein may have effects not normally associated with amino acid analogue substitution. Due to differences in bond angles in the azetidine carboxylate and proline rings, it has been postulated that azetidine carboxylate would rotate the polypeptide chain through an angle 15° less than would proline (11). Consequently, substitution of proline with azetidine carboxylate would be expected to cause changes in protein conformation.

This communication is concerned with the kinetics of azetidine carboxylate incorporation into hemoglobin and the effects of azetidine carboxylate on the rate of incorporation of other amino acids into hemoglobin in rabbit reticulocytes. The properties of those hemoglobin molecules into which radiolabeled azetidine carboxylate had been incorporated were characterized by meas-
\[
\text{L- AZETIDINE-2-CARBOXYLIC ACID (AEC)}
\]

\[
\text{PROLINE}
\]

**Fig. 1.** Schematic diagram of L-azetidine-2-carboxylic acid and L-proline.

...uring radioactivity in the individual \(\alpha\) and \(\beta\) chains and by radioautography of tryptic peptide maps.

### EXPERIMENTAL PROCEDURES

| dl-[\text{\textsuperscript{14}C}]Azetidine-2-carboxylic acid (ring-labeled, 10 mCi per mmol) and cycloheximide were purchased from Schwarz-Mann. l-[\text{\textsuperscript{14}C}]Proline (232 mCi per mmol), l-[\text{\textsuperscript{14}C}]Cleucine (250 mCi per mmol), L-[\text{\textsuperscript{14}C}]Cystine (307 mCi per mmol), l-[\text{\textsuperscript{14}C}]Cystidine (252 mCi per mmol), l-aminocyclopentane-[\text{\textsuperscript{14}C}]Carboxylic acid (33 mCi per mmol) and Aquasol were purchased from New England Nuclear. L-Azetidine-2-carboxylic acid was obtained from Aldrich and all other unlabeled amino acids were purchased from Nutritional Biochemicals. New Zealand white rabbits were obtained from Gloucester Rabbitry, Chepachet, R.I.; acrylamide and \(N,N^\prime\)-methylenebisacrylamide, from Bio-Rad; and fluoro-n-sulfonic acid, from Fisher. Silica Gel G plates (20 × 20 cm) were obtained from Analtech, and Kodak No-Screen medical x-ray film (20 × 25 cm) was from E. M. Parker Co. TPCK-treated trypsin was supplied by Worthington. The portion of the column eluate obtained were assayed for radioactivity. The distribution of protein was obtained in a solution of 10% methanol-70% acetic acid at 37°.

**Production of Reticulocytes**

New Zealand white rabbits (2 kg body weight) were made reticulocytotic by five daily subcutaneous injections of neutralized phenylhydrazine hydrochloride (7 mg per kg body weight). The animals were bled by cardiac puncture 48 hours after the last injection. This treatment produced hematocrits of 20 to 30% and reticulocyte counts of 60 to 80%. Heparinized blood was centrifuged at 2000 \(\times\) \(g\) for 5 min. The buffy coat was removed, and the resulting packed cells were washed three times with normal saline (0.9% \(NaCl\)).

**Synthesis and Isolation of Hemoglobin**

**Incubation**—The incubation medium employed for amino acid incorporation studies was essentially the same as that described by Lodish (12). Saline-washed blood cells were incubated in 25-mL Erlenmeyer flasks at 37° in air on a reciprocating metabolic shaker in a volume of 3.0 ml containing: glucose (3.7 mm), ferrous ammonium sulfate (0.051 mM), KCl (3.3 mM), MgSO\(_4\) (1.3 mM), NaCl (84 mM), CaCl\(_2\) (0.07 mM), sodium phosphate buffer, pH 7.4 (13.0 mM), packed cells (0.05 ml), and 18 unlabeled amino acids (0.1 mCi each; minus radiolabeled amino acid employed). After preincubation for 15 min, radiolabeled amino acids were added as required by experimental protocol. At the conclusion of incubations, cells were collected by centrifugation at 2000 \(\times\) \(g\) at 4°, washed three times with 10 ml of normal saline, lysed in 4 to 8 volumes of distilled water, and centrifuged at 100,000 \(\times\) \(g\) for 1 hour. The resulting ribosome-free supernatant was further purified by gel filtration or hot CCl\(_4\)COOH precipitation.

**Amino Acid Analyses**

Hot CCl\(_4\)COOH-insoluble material was hydrolyzed in 3 N p-toluene sulphonic acid for 22 hours at 108° according to the method of Liu and Chang (15). The hydrolysates were chromatographed on a Technicon automated analyzer (type A resin, 130 × 0.6 cm column), equipped with a split stream device, using the buffer regimen described by Hamilton (16). The portion of the column eluate analyzed was diverted to a fraction collector, and an aliquot from each tube was assayed for radioactivity.

**Kinetic Experiments**

Reticulocytes were preincubated for 15 min in the medium described above (12) and 3 \(\mu\)Ci of radiolabeled proline, leucine, or lysine were added. At each time point investigated, 50-\(\mu\)l aliquots were removed and an aliquot of incubation mixture and lysed in 2 ml of distilled water. Cell debris was removed by centrifugation at 2000 \(\times\) \(g\) for 5 min, 1.0 ml of 30% CCl\(_4\)COOH was added to the resulting supernatant, and CCl\(_4\)COOH-insoluble material was collected on a Millipore filter (0.45 \(\mu\)m, 25-mm diameter) in a sampling manifold. The filters were washed twice with 3 ml of 5% CCl\(_4\)COOH, dried, and assayed for radioactivity.

This procedure proved unsatisfactory for kinetic experiments in which azetidine carboxylate was employed due to the low specific activity of the commercially available product. Consequently, reticulocytes were preincubated for 15 min in the medium described above (12), 25 \(\mu\)Ci of dl-[\text{\textsuperscript{14}C}]azetidine carboxylate was added, and at some time points studied, 1.0 ml was removed from the incubation mixture. Reticulocytes were collected by centrifugation, washed three times with 8 ml of saline, lysed in 4 to 8 volumes of distilled water, and centrifuged at 100,000 \(\times\) \(g\) for 1 hour. Hot CCl\(_4\)COOH-insoluble material was prepared from the resulting ribosome-free supernatant and assayed for radioactivity.

**Polyacrylamide Gel Electrophoresis**

Polyacrylamide gel electrophoresis was carried out on 10% gels according to the method of Weber and Osborn (17), using a ratio of \(N,N^\prime\)-methylenebisacrylamide to acrylamide of 1:18.5. Gels were cast to a length of 9 cm in gel tubes (12 × 0.5 cm). Lysoph-...
gels were separated into three parts corresponding to α chains, β chains, and the remaining unstained gel portions. Each of these was separately extracted in several changes of 0.1% sodium dodecyl sulfate, 0.05 M sodium phosphate buffer, pH 7.0, at 37°C with vigorous mechanical agitation. This procedure resulted in essentially complete removal of all Coomassie blue-stained protein and radioactivity from the gel slices. The volume of each extract was measured and aliquots were assayed for radioactivity as described above.

To evaluate the homogeneity of chain extracts, the following procedure was employed. Protein was precipitated from each chain extract by the addition of 1 M KCl to a final concentration of 0.2 M, left on ice for 15 min, and centrifuged at 39,000 X g for 15 min (19). The resulting pellets were washed once in cold acetone containing 0.1 M HCl, once in acetone alone, and dried under a stream of nitrogen. Precipitated proteins were boiled in the β-mercaptoethanol-sodium dodecyl sulfate sample mix and were subjected to electrophoresis as described above.

Tryptic Digestion of Globin

Lyophilized globin samples (3.5 mg) prepared from hemoglobins into which radiolabeled amino acids were incorporated in vitro were suspended in 0.4 ml of 0.1 M NH4HCO₃, pH 8.0. Twenty microliters of freshly prepared TPCK-treated trypsin (1 mg per ml) in 0.05 M sodium phosphate buffer, pH 8.0, were added, and the digestion allowed to proceed overnight with gentle mixing at 37°C. Following addition of a second and equal volume of freshly prepared trypsin solution to give a final enzyme to protein ratio of 1:25, the digestion was continued for an additional 2 hours. After centrifugation at 9000 X g, the resulting supernatant was lyophilized. It was necessary to suspend the dried residue in water and repeat the lyophilization several times to ensure complete removal of NH₄HCO₃.

Peptide Mapping

Lyophilized globin digests were suspended in 150 μl of water and appropriate aliquots containing approximately 300 μg of digest were spotted in the lower left-hand corner of Silica Gel G plates. Thin layer peptide mapping was performed according to the methods of Ritschard (20). Chromatography in the first dimension was carried out in an ascending system of chloroform-methanol-30% NH₄OH (2:2:1) until the front had traveled a distance of 14 cm (about 1 1/2 hours). Plates were air-dried and sprayed with pyridine-glacial acetic acid-water (1:10:489), pH 3.5. Electrohoresis in the same buffer was carried out at 1000 volts for 80 min (cathode at right) using a Shandon thin layer electrohoresis unit (SAE-3230-Mk II). The resulting peptide maps were sprayed with fluorescamine (3 mg per 20 ml of acetone) and examined under ultraviolet light (21). Autoradiography was performed using Kodak No-Screen medical x-ray film with exposure times ranging from several days to several months depending on the specific activity of the globin preparation.

RESULTS

Earlier studies have suggested that azetidine carboxylate is stable to hydrolysis in 5 N Ba(OH)₂ but is destroyed by hydrolysis in 6 N HCl (1, 22). Under those circumstances where acid hydrolysis is preferable, hydrolysis in 3 N p-toluene sulfonic acid was shown to improve recoveries of intact radiolabeled azetidine carboxylate (Table I). Azetidine carboxylate eluted 13 min after aspartic acid. Homoserine, a product of azetidine carboxylate (Table I), was obtained following dialysis against 0.05 M sodium phosphate buffer, pH 7.0. C shows the pattern obtained when rabbit hemoglobin (unlabeled) was mixed with L-[14C]azetidine carboxylate prior to chromatography. The column was calibrated with blue dextran (BD), rabbit hemoglobin (Hb), trypsin (T), cytochrome c (CytO) and L-[14C]azetidine carboxylate (AZC). Absorbance (280 nm and 407 nm) and radioactivity were determined as described under “Experimental Procedures.”

![Fig. 2. Gel filtration on Sephadex G-100 of ribosome-free supernatants prepared from reticulocytes.](http://www.jbc.org/)
Reticulocytes were preincubated for 15 min in incubation medium from which proline (Experiments 1 and 2) or leucine (Experiment 3) had been omitted. Radiolabeled amino acids were added, the reaction was stopped at the times indicated, and hot CCl₃COOH-insoluble material was prepared and assayed for radioactivity.

### Table II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Amino acid</th>
<th>Dose</th>
<th>Duration</th>
<th>Packed cell volume</th>
<th>Hot CCl₃COOH-insoluble material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DL-[¹⁴C]AZC</td>
<td>25 µCi</td>
<td>3 hrs</td>
<td>0.1 ml</td>
<td>3,750 pCi/mg</td>
</tr>
<tr>
<td>2</td>
<td>L-[¹⁴C]Proline</td>
<td>3 µCi</td>
<td>1 hrs</td>
<td>0.05 ml</td>
<td>28,700 pCi/mg</td>
</tr>
<tr>
<td>3</td>
<td>L-[¹⁴C]Leucine</td>
<td>10 µCi</td>
<td>1 hrs</td>
<td>0.05 ml</td>
<td>240,000 pCi/mg</td>
</tr>
</tbody>
</table>

**Amino Acid Incorporation Studies**—Rabbit reticulocytes were incubated with DL-[¹⁴C]azetidine carboxylate, L-[¹⁴C]proline, or L-[¹⁴C]leucine for 1 to 3 hours as described under “Experimental Procedures.” The specific activity (counts per min per mg) of hot CCl₃COOH-insoluble material into which the respective amino acids had been incorporated is shown in Table II.

**Amino Acid Analysis**—Radiolabeled hemoglobins from the experiment shown in Table II were hydrolyzed and chromatographed on the column of the amino acid analyzer. The elution profile of radioactivity in these hydrolysates is shown in Fig. 3. More than 90% of the radioactivity in the hydrolysates of hemoglobin from reticulocytes incubated with DL-[¹⁴C]azetidine carboxylate eluted from the column with the azetidine carboxylate standard. An additional 6% of the radioactivity eluted with homoserine standard. Radioactivity in hydrolysates of hemoglobins into which proline and leucine had been incorporated eluted with these amino acids. It was concluded from these data that the respective amino acids were incorporated into hemoglobin intact and unaltered.

**Incubation with 1-Aminocyclopentane-[¹⁴C]carboxylic Acid**—It was decided to examine the fate of another amino acid analogue, 1-aminocyclopentane-1-carboxylic acid in rabbit reticulocytes in vitro. This compound has been reported to inhibit amino acid transport (22, 23) and valine incorporation into protein in rat liver homogenates (24). Reticulocytes were incubated in valine-free medium with either 25 µCi of 1-aminocyclopentane-[¹⁴C]carboxylic acid or 3 µCi of L-[¹⁴C]valine as described under “Experimental Procedures.” After incubation, the reticulocytes were washed and lysed, and hot CCl₃COOH-insoluble material from ribosome-free supernatants was assayed for radioactivity. The specific activity of hot CCl₃COOH-insoluble material into which valine had been incorporated was 64,000 cpm per mg 1-Aminocyclopentane-[¹⁴C]carboxylic acid was not incorporated into protein.

**Kinetic Experiments**—The kinetics of azetidine carboxylate, proline, leucine, and lysine incorporation into hemoglobin in rabbit reticulocytes is shown in Fig. 4. The rate of incorporation of azetidine carboxylate was comparable to the rates of incorporation of the other three amino acids. Incorporation of each amino acid into hemoglobin was completely inhibited by 50% but completely abolished by 1.0 mM cycloheximide. In one experiment, it was shown that cycloheximide inhibited proline uptake by 50% but completely abolished proline incorporation into hemoglobin. The effect of cycloheximide on uptake of other amino acids was not determined.

**Competition Experiments**—Fig. 5 shows the effect of azetidine carboxylate on proline, leucine, and lysine incorporation into hemoglobin. Using the results obtained after 60 min of incubation, 1, 5, and 10 mM azetidine carboxylate inhibited proline (0.1 mM) incorporation into hemoglobin by 24, 58, and 72%, respectively (Fig. 5A). At these same concentrations, azetidine carboxylate did not alter the course of leucine or lysine incorpora-
Fig. 4. Kinetics of incorporation of radiolabeled amino acids into hemoglobin in rabbit reticulocytes. Experiments A and B, C, and D were performed in incubation mixtures from which unlabeled proline, leucine, and lysine, respectively, had been omitted. The data in Experiment A shows the counts per min per mg observed in hot CCl₄-insoluble material prepared as described under "Experimental Procedures." The data in Experiments B to D were obtained from the average of six determinations of radioactivity in cold CCl₄-insoluble material (from 0.05-ml aliquots) collected on Millipore filters as described in the text. In those experiments in which cycloheximide (cycle, 0.1 mM) was employed, reticulocytes were preincubated for 15 min with inhibitor prior to addition of the radiolabeled amino acid.

Incorporation of Amino Acids Into α and β Chains of Rabbit Hemoglobin—The α and β chains of hemoglobin into which radiolabeled azetidine carboxylate was incorporated in vitro were separated electrophoretically on sodium dodecyl sulfate-polyacrylamide gels (Fig. 6A). The densitometric scan indicated that greater than 95% of the resulting stained material was divided equally between the two chains. This electrophoretic pattern was typical of that observed for each of the purified radiolabeled hemoglobin preparations examined in this study. The more rapidly migrating band was identified as the α chain and the more slowly migrating band as the β chain, by comparison with chain standards prepared using the method of Schapira et al. (25). To determine the homogeneity of electrophoretically resolved chains and to assess the possible errors involved in gel slicing, protein was precipitated from each chain extract and subjected to electrophoresis again. The major portion of the material contained in each chain extract was observed to migrate as a single component with a mobility equal to that observed initially (Fig. 6, B and C). Some aggregation of the extracted chains resulted during the precipitation process.
FIG. 6. Electrophoretic and densitometric analyses of chromatographically purified rabbit hemoglobin and \( \alpha \) and \( \beta \) chain extracts. A, rabbit hemoglobin into which [\( ^{14} \)C]azetidine carboxylate was incorporated in vitro, 25 \( \mu \)g; B, protein obtained from the \( \alpha \) chain extract, approximately 12 \( \mu \)g; C, protein obtained from the \( \beta \) chain extract, approximately 12 \( \mu \)g. Gels were stained with 1% fast green. The identity of the electrophoretically resolved chains was established by comparison with \( \alpha \) and \( \beta \) chain standards prepared by the method of Schapira et al. (25). Details of protein precipitation from gel extracts, electrophoresis, and densitometry are given under "Experimental Procedures."

as evidenced by the presence of several high molecular weight bands which were not present prior to extraction.

The ratios of total radioactivity obtained from the isolated \( \alpha \) and \( \beta \) chains of hemoglobins into which radiolabeled azetidine carboxylate and radiolabeled proline, respectively, had been incorporated are shown in Table IV. The observed ratios correspond closely to calculated ratios based on the proline composition of rabbit hemoglobin chains, i.e. 7 prolyl residues in \( \alpha \) and, 4 prolyl residues in \( \beta \) (20).

In another series of experiments, the incorporation of radiolabeled leucine, lysine, and valine, respectively, into the \( \alpha \) and \( \beta \) chains was evaluated by this method. In each case examined, the ratio of total radioactivity in the respective chains was in good agreement with values expected from the known amino acid compositions of the chains. The latter data are also included in Table IV.

Peptide Mapping—Two-dimensional peptide maps of radiolabeled globin digests were prepared as described under "Experimental Procedures." Each of the digests displayed an identical pattern of peptides based on detection with fluorescamine. As revealed by autoradiography, the arrangement of peptides in digests containing radiolabeled azetidine carboxylate or proline were nearly identical (Fig. 7, A and B). Small variations in the two maps may be due to differences in mobility of peptides containing azetidine carboxylate versus peptides containing proline. The maps of globins into which radiolabeled leucine and lysine had been separately incorporated are shown in Fig. 7, C and D. In addition to being different from each other, each map was clearly distinct from the prolyl or azetidyl maps shown in Fig. 7, A and B.

DISCUSSION

Our interest in azetidine carboxylate as an amino acid analogue arose initially from the proposed consequences of azetidine carboxylate substitution for proline on protein conformation (11) and from the restriction of prolyl residues to interhelical regions

### Table IV

*Distribution of radioactivity in the \( \alpha \) and \( \beta \) chains of several rabbit hemoglobin preparations*

Radiolabeled hemoglobins were purified by gel filtration chromatography on Sephadex G-100 columns. Chains were resolved electrophoretically, extracted from gels, and assayed for radioactivity as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Amino Acid</th>
<th>Hemoglobin (dpm/mg)</th>
<th>Gels/Expt.</th>
<th>Total dpm Loaded</th>
<th>( \alpha ) dpm Recovered</th>
<th>( \beta ) dpm Recovered</th>
<th>Total dpm Recovered</th>
<th>% Total Recovery</th>
<th>% Recovered dpm in Chains</th>
<th>( \alpha: \beta ) (dpm) Expected</th>
<th>( \alpha: \beta ) (Residues) Expected</th>
<th>( \alpha: \beta ) (Residues) Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AZC</td>
<td>2,993</td>
<td>28</td>
<td>2,135</td>
<td>1,034</td>
<td>588</td>
<td>1,432</td>
<td>86</td>
<td>86</td>
<td>1.0:0.57</td>
<td>7.0:4.6</td>
<td>7.0:4.6</td>
</tr>
<tr>
<td>2</td>
<td>AZC</td>
<td>2,995</td>
<td>30</td>
<td>2,678</td>
<td>1,236</td>
<td>803</td>
<td>1,884</td>
<td>89</td>
<td>86</td>
<td>1.0:0.55</td>
<td>7.0:4.6</td>
<td>7.0:4.6</td>
</tr>
<tr>
<td>3</td>
<td>PRO</td>
<td>79,228</td>
<td>14</td>
<td>30,748</td>
<td>15,059</td>
<td>8,450</td>
<td>23,510</td>
<td>83</td>
<td>83</td>
<td>1.0:0.56</td>
<td>7.0:3.9</td>
<td>7.0:4.7</td>
</tr>
<tr>
<td>4</td>
<td>PRO</td>
<td>250,000</td>
<td>24</td>
<td>131,800</td>
<td>74,233</td>
<td>48,000</td>
<td>122,233</td>
<td>87</td>
<td>93</td>
<td>1.0:0.67</td>
<td>7.0:4.7</td>
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<tr>
<td>5</td>
<td>LEU</td>
<td>332,484</td>
<td>8</td>
<td>91,490</td>
<td>40,718</td>
<td>36,066</td>
<td>76,784</td>
<td>94</td>
<td>94</td>
<td>1.0:0.89</td>
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<tr>
<td>6</td>
<td>LEU</td>
<td>332,484</td>
<td>7</td>
<td>80,054</td>
<td>25,818</td>
<td>34,230</td>
<td>60,048</td>
<td>95</td>
<td>92</td>
<td>1.0:0.97</td>
<td>17.0:16.5</td>
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</tr>
<tr>
<td>7</td>
<td>LYS</td>
<td>78,500</td>
<td>14</td>
<td>27,475</td>
<td>11,566</td>
<td>11,400</td>
<td>22,966</td>
<td>83</td>
<td>83</td>
<td>1.0:0.99</td>
<td>12.0:11.0</td>
<td>12.0:11.0</td>
</tr>
<tr>
<td>8</td>
<td>LYS</td>
<td>78,500</td>
<td>13</td>
<td>27,475</td>
<td>11,566</td>
<td>11,400</td>
<td>22,966</td>
<td>83</td>
<td>83</td>
<td>1.0:0.99</td>
<td>12.0:11.0</td>
<td>12.0:11.0</td>
</tr>
<tr>
<td>9</td>
<td>VAL</td>
<td>57,430</td>
<td>7</td>
<td>6,639</td>
<td>4,434</td>
<td>3,634</td>
<td>8,068</td>
<td>96</td>
<td>96</td>
<td>1.0:1.00</td>
<td>11.0:10.2</td>
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<tr>
<td>10</td>
<td>VAL</td>
<td>57,430</td>
<td>14</td>
<td>2,635</td>
<td>1,264</td>
<td>2,370</td>
<td>3,634</td>
<td>90</td>
<td>90</td>
<td>1.0:2.10</td>
<td>11.0:23.1</td>
<td>11.0:23.1</td>
</tr>
</tbody>
</table>

*Experiments 7 and 8 were performed on globin prepared by acid-acetone precipitation of hemoglobin

\( \alpha \) is normalized to 1.0

\( [\alpha: \beta \) (dpm) observed] \times [number of the respective amino acid residues in \( \alpha \) - [\alpha: \beta \) (in residues) observed] \]

Based on 7 prolyl residues per \( \alpha \) chain

Taken from reference 26
in hemoglobin, myoglobin, and other proteins (27). This suggested the possibility that abnormal hemoglobins might be altered phenotypically at the level of protein synthesis in the intact organism. This surmise seemed particularly interesting with respect to the genetic defect in sickle cell anemia where proline immediately precedes the valine residue which has substituted for glutamic acid at position 6 in the β chains of sickle hemoglobin (28, 29). Interestingly, proline is replaced or inserted in place of other amino acids in more than 10 abnormal hemoglobins described in man (20). In certain instances, point mutations resulting in proline substitution have occurred without severe clinical consequences (30, 31).

The results of the present communication have shown that azetidine carboxylate was incorporated into material in rabbit reticulocytes which was indistinguishable from hemoglobin by gel filtration chromatography. This suggests that the principal product of protein synthesis in reticulocytes incubated with azetidine carboxylate is tetrameric hemoglobin.

The results in Fig. 3 show that azetidine carboxylate was incorporated intact into hemoglobin in rabbit reticulocytes in vivo. It is felt that this observation is noteworthy because we have found that azetidine carboxylate is metabolized to other cellular intermediates in addition to being incorporated intact into protein in Cyanidium caldarium (10).

Additionally, the kinetic data suggest that azetidine carboxylate was incorporated into hemoglobin in rabbit reticulocytes at rates comparable to those observed with proline, leucine, and lysine. It should be mentioned that under the conditions employed, the concentration of dl-[14C]azetidine carboxylate was 0.8 mM, while the concentration of the other amino acids (l-isomers) was 0.1 mM. The effects of the n isomer of azetidine carboxylate on amino acid transport was not examined in these experiments. Complete inhibition of amino acid incorporation into hemoglobin in reticulocytes preincubated with cycloheximide was presumed to result from the established effect of this compound on protein synthesis in eukaryotic cells (32). Although it was found in the present work that cycloheximide inhibited proline transport into reticulocytes by 50%, as might be expected from recent work on the effects of this inhibitor on amino acid transport (33–35), amino acid incorporation into hemoglobin was abolished (Fig. 4). It was concluded that cycloheximide reduced amino acid transport and inhibited protein synthesis completely, under the conditions employed.

Competition studies showed that azetidine carboxylate (l-isomer) inhibited proline but not leucine or lysine incorporation into hemoglobin (Fig. 5). Conversely, proline inhibited azetidine carboxylate incorporation into hemoglobin without affecting azetidine carboxylate transport (Table III). These results suggest that azetidine carboxylate substitutes for proline in hemoglobin synthesized in vitro. However, competition between azetidine carboxylate and proline did not display a 1:1 stoichiometry. This may well result from the inability of azetidine carboxylate to compete effectively with proline for activation. It has been reported that the Kₘ values for activation of azetidine carboxylate and proline in a cell-free system from rat liver were 10 and 0.1 mM, respectively (36). Competition between phenylalanine and p-fluorophenylalanine for activation in a reticulocyte cell-free system (37) suggests that analogues of other amino acids may also be activated inefficiently in the presence of the naturally occurring amino acid. It would be of interest to know, therefore, the specificity of the proline tRNA synthetase and tRNAs for azetidine carboxylate in rabbit reticulocytes. This issue is also of consequence regarding the block in azetidine carboxylate activation in Consallaria majilis (3), and the physiological role of azetidine carboxylate in species where it occurs naturally.

Gel experiments and peptide maps demonstrate that azetidine carboxylate is incorporated into both chains of rabbit hemoglobin and that the distribution of this proline analogue between the two chains is identical with the distribution of proline. These
results provide unequivocal evidence for the specificity of azetidine carboxylate replacement of proline and suggest that all prolyl sites are susceptible to substitution by azetidine carboxylate. It is recognized that these results do not indicate the number of prolyl residues replaced by azetidine carboxylate in any given hemoglobin molecule.

Although polyacrylamide gel electrophoresis represents a departure from the more traditional use of column chromatography for purposes of chain separation (38, 39), the electrophoretic method employed in this investigation affords certain advantages for purposes of chain separation (38, 39), the electrophoretic method employed in this investigation affords certain advantages over the more traditional use of column chromatography. First, the close agreement between experimentally determined and actual numbers of residues per chain in incorporation studies involving naturally occurring amino acids attests to the unambiguous separation afforded by the electrophoretic procedures. This was confirmed in experiments which showed that the resolved chains extracted from gels were homogeneous when subjected to electrophoresis again. Second, greater than 95% of the stained material was associated with the individual chains. Moreover, recovery of radioactive chains from the gels was almost quantitative. This was an absolute prerequisite for the present study since the total incorporation of radiolabeled amino acids into the chains was evaluated. Finally, although the quantity of hemoglobin was not a limiting factor in the present work, the extreme sensitivity of the electrophoretic method made it possible to determine the relative and/or total incorporation of azetidine carboxylate into the chains of rabbit hemoglobin using approximately 700 μg of protein (2993 dpm per mg) per experiment. In those instances where hemoglobin of higher specific activity was available for study, far less protein was required.

The choice of the amino acid used to evaluate the distribution of total radioactivity in the α and β chains is of considerable importance. If lysine which is equally distributed in the two chains is selected, no cross-contamination between the chains would be detected. However, if proline which has an α:β ratio of 7:4 is employed, even relatively small amounts of cross-contamination become evident. For example, a 10% contamination of the β chain fraction by α chains, or vice versa, would lead to α:β ratios for proline of 7.5:2 and 7.3:4, respectively. Similarly, if 10% contamination of each chain by the other occurred, one would obtain an α:β ratio for proline of 7:4.5. From this type of reasoning, it is evident that the use of an amino acid not equally distributed in the two chains serves as an independent criterion for assessing the extent of chain separation.

The effects of proline replacement by azetidine carboxylate in rabbit hemoglobin on the physiological function of this protein are not known at present. It is interesting to note that the kinetic properties of Escherichia coli alkaline phosphatase were not significantly altered when 5% of the proline residues of this enzyme were replaced by the proline analogue, 3,4-dehydroproline (22). Although the conformational changes resulting from azetidine carboxylate substitution (11) might be more complex than those obtained with 3,4-dehydroproline replacement, it is conceivable that azetidine carboxylate incorporation may still result in a biologically active polymer. With this in mind, it is reasonable to propose that replacement of key proline residues by azetidine carboxylate in hemoglobin S might provide sufficient change in the molecular architecture of the molecule so as to inhibit molecular stacking while at the same time not interfere with its ability to function effectively.

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Incorporation of L-azetidine-2-carboxylic acid into hemoglobin in rabbit reticulocytes in vitro.
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