

Canavanine Inhibits Vimentin Assembly But Not Its Synthesis in Chicken Embryo Erythroid Cells

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ABSTRACT In chicken embryo erythroid cells, newly synthesized vimentin first enters a Triton X-100 (TX-100)-soluble pool and subsequently assembles posttranslationally into TX-100-insoluble vimentin filaments (Blikstad I., and E. Lazarides, *J. Cell Biol.*, 96:1803–1808). Here we show that incubation of chicken embryo erythroid cells in a medium in which arginine has been substituted by its amino acid analogue, canavanine, results in the inhibition of the posttranslational assembly of vimentin into the TX-100-insoluble filaments. Immunoprecipitation and subsequent SDS gel electrophoresis showed that the synthesis of canavanine-vimentin is not inhibited and that it accumulates in the TX-100-soluble compartment. Pulse-chase experiments with [³⁵S]methionine demonstrated that while arginine-vimentin can be rapidly chased from the soluble to the cytoskeletal fraction, canavanine-vimentin remains in the soluble fraction, where it turns over. The effect of canavanine on the assembly of vimentin did not prevent the assembly of arginine-vimentin, as cells labeled with [³⁵S]-methionine first in the presence of canavanine and then in the presence of arginine contained labeled canavanine-vimentin only in the soluble fraction, and arginine-vimentin in both the soluble and cytoskeletal fractions. These results suggest that arginine residues play an essential role in the assembly of vimentin in vivo.

While the subunit composition of the five classes of intermediate filaments may differ in various tissues and during the differentiation of a particular cell type, all intermediate filaments have certain common properties (reviewed in reference 17). Intermediate filaments display a diameter of ~10 nm, an axial periodicity of 21 nm (12, 18), and contain α -helical regions (e.g., reference 7). Recent amino acid sequence data have demonstrated that several intermediate filament proteins contain sequence homologies in a rod-like domain of α -helical regions, and extensive variability in the non- α -helical domains at the amino-termini and carboxy-termini (8). The homologies evident in the rod-like domains are likely due to requirements for the formation of a double coiled-coil structure (8). Since protofilaments from the intermediate filament subunit desmin may form 10 nm filaments under physiological ionic conditions whereas a major 38,000 M_r rod-like domain lacking the termini of the protein does not (7), one or both terminal domains may be required for filament formation. Subsequent work has specifically implicated the amino-terminal "headpiece" domains of both desmin and vimentin as being required for the assembly of these two proteins into 10-nm filaments, since vimentin and desmin modified by limited proteolysis of their amino termini do not form filaments in vitro (19). The molecular basis for the role of the amino-terminal headpiece of desmin and vimentin in filament for-

mation is unknown. It is interesting, however, that the 7,500 M_r headpiece domain of chicken desmin is very basic and contains 10 arginine residues (7, 8).

Although these and additional (21, 22) studies are contributing to an understanding of the assembly of intermediate filaments in vitro, very little is known about the regulation of the assembly of these filaments in vivo. Recently we have investigated the formation of vimentin filaments in vivo in chicken embryo erythroid cells by labeling the cells with [³⁵S]methionine, separating them into Triton X-100 soluble and insoluble fractions, and then subjecting these fractions to two-dimensional gel electrophoresis to identify vimentin (2). The validity of this approach was based on the demonstration that at steady state, virtually all of the vimentin is present in the cytoskeleton (2). Analyzing the distribution of labeled vimentin in the soluble and cytoskeletal fractions demonstrated that newly synthesized vimentin first appears in a Triton X-100-soluble precursor pool before being assembled post-translationally into the cytoskeleton (2).

The demonstration that the arginine-rich amino-terminal domain of desmin is important for filament formation in vitro (19) raises the question of whether there is a specific requirement for arginine in filament formation. In the current study we describe the effects of several amino acid analogues on the synthesis and post-translational assembly of vimentin

in chicken embryo erythroid cells. We found that canavanine, an arginine analogue, specifically blocks the assembly of newly synthesized vimentin into the cytoskeleton, whereas two other amino acid analogues do not. The unassembled vimentin accumulates in a Triton X-100-soluble pool, where it turns over. These results suggest that arginine residues play an essential role in the assembly of vimentin from a soluble precursor to insoluble intermediate filaments *in vivo*. Replacement of arginine with canavanine thus provides the first method for blocking the *in vivo* assembly of an intermediate filament protein.

MATERIALS AND METHODS

Preparation and Labeling of Cells: Erythroid cells were obtained by removing 11–12-d-old chicken embryos from all extra-embryonic membranes, bleeding the isolated embryos in Earle's balanced salt solution, and filtering the cells through cheesecloth (2). The cells were then collected in a clinical centrifuge at 500 *g* for 2 min (4°C), and washed four times by suspension in minimal essential media (0°C) containing 0.3 μ M methionine, and depleted in either arginine, threonine, or isoleucine, followed by repelleting and removing any buffy coat. Washed cells were resuspended in 100 vol of minimal essential medium containing 0.3 μ M methionine, 5% (vol/vol) dialyzed fetal bovine serum that had been filtered through a 0.45- μ m pore size nitrocellulose filter, and 20 mM triethanolamine (pH 7.4). Amino acid analogues (Sigma Chemical Co., St. Louis, MO) were substituted at the following final concentrations (13): canavanine (0.2 mg/ml) was substituted for arginine, β -hydroxyornithine (1 mg/ml) for threonine, and *o*-methylthreonine (1 mg/ml) for isoleucine.

Erythroid cells were incubated in the presence of amino acid analogues for 2–3 h (35°C) prior to labeling, with constant gentle inversion, and were then collected in a clinical centrifuge. For the short-term continuous labeling experiments in Figs. 1, 2, 3, 5 and 6, cells were resuspended to a final concentration of 10% (vol/vol) in fresh minimal essential medium containing 5% fetal bovine serum, triethanolamine, an amino acid analogue, and no unlabeled methionine. After 10 min at 35°C, [³⁵S]methionine (1,200–1,400 Ci/mmol, 13.5 mCi/ml; Amersham Corp., Arlington Heights, IL) was added to a final concentration of 700 μ Ci/ml. For the long-term continuous labeling experiment in Fig. 4, cells were preincubated as above, washed, and resuspended to a concentration of 1% (vol/vol) in fresh minimal essential medium containing 5% fetal bovine serum, triethanolamine, an amino acid analogue, and 0.3 μ M unlabeled methionine. Emetine was then added to an aliquot of the control cells to a concentration of 0.5 μ M. After 10 min at 35°C, [³⁵S]methionine was added to 220 μ Ci/ml. For the pulse-chase experiment in Fig. 4, erythroid cells were treated as above, but after 10 min of labeling with [³⁵S]methionine, unlabeled methionine was added to a concentration of 0.24 mM.

Preparation of Soluble and Cytoskeletal Fractions: At intervals during the 1.5–6 h incubation, aliquots of the cell suspensions containing 0.5–1.0 $\times 10^8$ cells (10–20- μ l packed volume) were withdrawn and diluted into 15–60 vol of 155 mM choline chloride, 5 mM HEPES, pH 7.4 at 0°C, and collected in a clinical centrifuge. The supernatant was removed by aspiration and the cells lysed for 2.5 min by suspension in 10 vol of lysis buffer (100 mM KCl, 300 mM sucrose, 20 mM PIPES, 1 mM MgOAc, 5 mM EGTA, 0.5% (wt/vol) Triton X-100, pH 6.8 at 4°C). After transfer of the cell lysate to 1.5-ml conical centrifuge tubes, the cytoskeletons were pelleted at 12,500 *g* for 5 min (20°C) in an Eppendorf microcentrifuge. The supernatant was carefully removed and transferred to a new centrifuge tube, the detergent insoluble pellet was resuspended in lysis buffer to the same volume as the supernatant, and β -mercaptoethanol and crystalline urea were added to each fraction (2).

Immunoprecipitation and Gel Electrophoresis: Antibodies raised against embryonic chicken skeletal muscle vimentin have been described (10). Aliquots (40 μ l) of the soluble and cytoskeletal fractions were diluted 10-fold with immunoprecipitation buffer B (3), mixed with 1 μ l of antiserum, and further processed for immunoprecipitation using method B (3). Immunoprecipitated polypeptides were applied to SDS/10% polyacrylamide slab gels (15). Following impregnation with Enhance or Enlightening (New England Nuclear, Boston, MA), gels were dried and exposed to Kodak X-OMAT XAR-5 X-ray film at –80°C. The film was preflashed to an absorbance of about 0.15 at 540 nm (16), and exposed within the range of linear response. Fluorographs were scanned with a densitometer (E. C. Corporation, Fort Lauderdale, FL) and the vimentin peaks were integrated by computer.

Estimation of ³⁵S Incorporation and Total Protein Concentration: After solubilization of the cytoskeletons in urea, 2- μ l aliquots of the soluble and cytoskeletal fractions were decolorized, tRNA was hydrolyzed,

and the samples were assayed for trichloroacetic acid-insoluble ³⁵S (20). Total incorporation of ³⁵S was determined by summation of the radioactivity in the soluble and cytoskeletal fractions. The concentration of total protein in the soluble and cytoskeletal fractions was estimated according to Bradford (4) using BSA as the standard.

RESULTS

Partitioning of Newly Synthesized Vimentin into Soluble and Cytoskeletal Compartments

Chicken embryo erythroid cells preincubated for 3 h with analogues for either arginine, threonine, or isoleucine incorporated [³⁵S]methionine into total protein at rates 50–100% that of controls (Fig. 1A), which facilitates the comparison of the synthesis of specific polypeptides (Fig. 1B). Many of the Triton X-100-soluble polypeptides synthesized in the presence of canavanine had relative mobilities in SDS/polyacrylamide gels similar to those of control polypeptides, although some differences in the total pattern of polypeptides were evident (Fig. 1B, lanes 1 and 3). The most striking effect of canavanine was that the cytoskeletal fraction of cells incubated with this amino acid analogue did not contain a newly synthesized polypeptide corresponding to vimentin, even though two other major erythrocyte cytoskeletal polypeptides, α - and β -spectrin, were incorporated into the cytoskeletons of both control- and canavanine-treated cells (Fig. 1B, lanes 2 and 4). As previously noted for other proteins containing canavanine (13), the canavanine-spectrin bands were more diffuse than in controls, and had a slower relative mobility. Since spectrin was incorporated into the cytoskeletal fraction, canavanine did not inhibit nonspecifically the assembly of all cytoskeletal proteins.

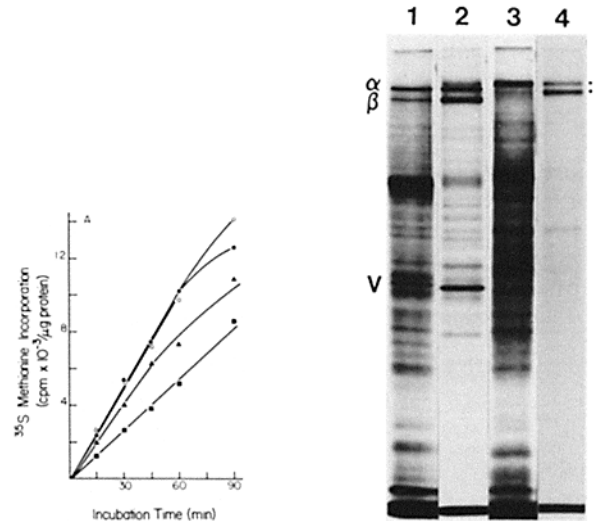


FIGURE 1 Effects of amino acid analogues on protein synthesis. (A) Erythroid cells from 11–12-d-old chicken embryos were preincubated for 3 h in an amino acid analogue, then labeled continuously with [³⁵S]methionine for the indicated incubation times in the presence of the analogues. The incorporation of [³⁵S]methionine into protein is normalized to total protein. ●, control cells; ○, canavanine-treated cells; ▲, β -hydroxyornithine; ■, *o*-methylthreonine-treated cells. (B) [³⁵S]methionine-labeled polypeptides in the soluble (lanes 1 and 3) and cytoskeletal (lanes 2 and 4) fractions of erythroid cells incubated in arginine (lanes 1 and 2) or canavanine (lanes 3 and 4). For clarity, lane 1 is from cells labeled for 30 min, and all other lanes are from cells labeled for 3 h. Vimentin is designated by v, the spectrin subunits by α and β , and canavanine-spectrin subunits by a small black dot.

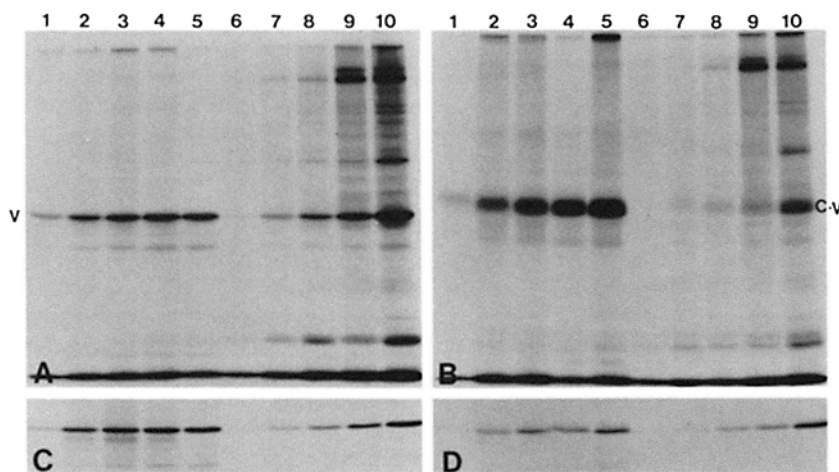
Vimentin could not be identified readily in the soluble fractions of either control- or canavanine-treated cells (Fig. 1*B*). To identify vimentin in the soluble fraction and to determine the distribution of canavanine-vimentin in the Triton X-100-soluble and insoluble fractions, we used immunoprecipitation with a vimentin-specific antiserum. As shown in Fig. 2*A* and quantified in Fig. 3*A*, control erythroid cells incubated in medium supplemented with arginine contained newly synthesized vimentin in both the soluble and cytoskeletal fractions. In agreement with previous work (2), the amount of labeled vimentin in the soluble pool quickly reached a plateau (45 min), while the amount of labeled vimentin in the cytoskeletal fraction exhibited a lag, but then increased in an approximately linear fashion. Within 60–90 min, the amount of labeled vimentin in the cytoskeletal fraction exceeded the amount in the soluble fraction. Similar to controls, cells incubated with β -hydroxynorvaline (Figs. 2*C* and 3*C*) or *o*-methylthreonine (Figs. 2*D* and 3*D*), displayed a saturation of the soluble pool, and a greater amount of newly synthesized vimentin in the cytoskeletal compared with the soluble fraction by 90 min of labeling. The amount of labeled vimentin in the cytoskeletal fraction exceeded the amount in the soluble fraction by 60 min for cells incubated in arginine (Fig. 3*A*) or *o*-methylthreonine (Fig. 3*D*), and by 90 min for those incubated in β -hydroxynorvaline (Fig. 3*C*). This suggests that β -hydroxynorvaline may have a subtle effect on vimentin assembly.

Cells incubated with canavanine also synthesized vimentin, and at a rate similar to controls (Fig. 3, *A* and *B*). However, canavanine has two effects on newly synthesized vimentin which are consistent with our inability to identify it in cell extracts (Fig. 1*B*). First, canavanine-vimentin, unlike vimentin synthesized with arginine or in the presence of the other two analogues, consistently migrated on SDS/polyacrylamide gels as a diffuse band with a slower relative mobility than arginine-vimentin (Fig. 2*B*). This is similar to what was observed with α - and β -spectrin (Fig. 1*B*) and to what has been previously found for actin and other proteins synthesized in the presence of canavanine (13). Second, the kinetic behavior of newly synthesized canavanine-vimentin differed markedly from arginine-vimentin, or vimentin synthesized in the presence of threonine or isoleucine analogues. The amount of newly synthesized canavanine-vimentin in the soluble frac-

tion did not reach a plateau during a 90-min labeling period, and even after 90 min the amount of labeled vimentin in the soluble fraction exceeded the amount of labeled canavanine-vimentin in the cytoskeletal fraction by about fourfold. The absolute amount of newly synthesized canavanine-vimentin in the soluble fraction was more than twofold greater than the soluble pool of arginine-vimentin in control cells suggesting that canavanine-vimentin accumulated in the soluble fraction. Thus canavanine specifically interfered with the cytoskeletal assembly of vimentin.

Soluble Arginine-Vimentin, but not Canavanine-Vimentin, Serves as Precursor to Cytoskeletal Vimentin

We next designed a pulse-chase experiment to compare the post-translational behavior of canavanine-vimentin versus arginine-vimentin in the soluble fraction. We labeled cells briefly with [³⁵S]methionine, then chased the labeled methionine with an excess of unlabeled methionine, thus fixing the number of labeled vimentin polypeptides early in the chase period. The results for the control cells confirmed a previous study (2) showing that arginine-vimentin is initially partitioned primarily in the soluble fraction (Fig. 4*E*). The amount of labeled vimentin in the soluble fraction then decreased rapidly while the amount of labeled vimentin in the cytoskeletal fraction increased proportionally (Fig. 4*E*). This relationship indicated that the soluble arginine-vimentin serves as a precursor for the cytoskeletal vimentin (2). A strikingly different result was obtained with pulse-labeled canavanine-vimentin (Fig. 4*F*). First, most of the labeled canavanine-vimentin remained in the soluble fraction throughout the 6-h chase period, which conclusively demonstrated that very little canavanine-vimentin is assembled into the cytoskeleton. Second, although there was no evident turnover of arginine-vimentin during the 6-h time period (Fig. 4*E*), ~50% of the canavanine-vimentin turned over during the chase period (Fig. 4*F*). This difference in vimentin stability may reflect the previously reported increased rates of degradation of proteins that contain canavanine (9, 14). Alternatively, the cytoskeletal localization of arginine-vimentin as compared with the soluble nature of canavanine-vimentin may itself lead to differential turnover.



of the gel of cells incubated with *o*-methylthreonine. *A* and *B* were processed for immunoprecipitation at the same time, and the fluorographs exposed 7 h. *C* and *D* were processed simultaneously at a later date and the fluorographs exposed 9 h.

FIGURE 2 Effect of amino acid analogues on vimentin assembly. Cells from the experiment in Fig. 1 were labeled with [³⁵S]methionine and separated into Triton X-100-soluble (lanes 1–5) and insoluble (lanes 6–10) fractions and processed for immunoprecipitation of vimentin, electrophoresis, and fluorography as in Materials and Methods. The fluorograph depicts vimentin in the soluble and cytoskeletal fractions at the following times: lanes 1 and 6, 15 min labeling; lanes 2 and 7, 30 min labeling; lanes 3 and 8, 45 min labeling; lanes 4 and 9, 60 min labeling; lanes 5 and 10, 90 min labeling. (*A*) control cells, with vimentin designated *v*; (*B*) cells incubated with canavanine, with vimentin designated *c-v*; (*C*) the vimentin region of the gel of cells incubated with β -hydroxynorvaline; (*D*) the vimentin region

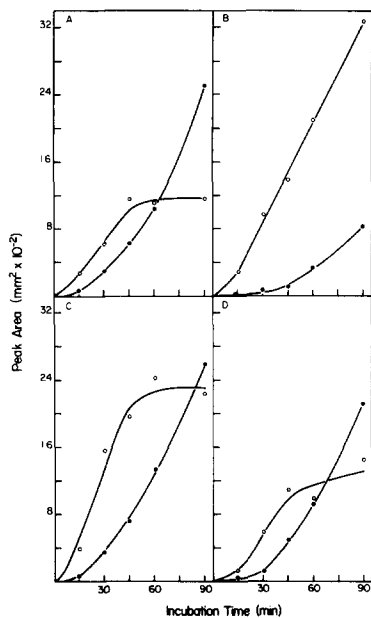


FIGURE 3 Quantification of the effect of amino acid analogues on vimentin assembly. Fluorographs from the gels used for Fig. 2, but somewhat less exposed, were scanned and the vimentin peak area integrated as in Materials and Methods to display the amount of newly synthesized vimentin in the soluble (○) and cytoskeletal (●) fractions of cells incubated as controls (A) or with canavanine (B), β -hydroxynorvaline (C) or *o*-methylthreonine (D). A and B can be compared quantitatively with each other, as can C and D, but comparisons between the two groups are qualitative since they were not processed simultaneously for immunoprecipitation and hence there may be slight variations in vimentin recovery.

The pulse-chase experiment necessarily entailed several parallel control experiments with continuous labeling of cells, to demonstrate the viability of the cells throughout the pulse-chase period, and to demonstrate that long-term continuous labeling supported the conclusions derived from Fig. 3. As shown in Fig. 4A, cells preincubated with arginine or canavanine for 2 h, then labeled continuously, incorporated increasing amounts of [35 S]methionine over a 6-h period, which establishes the viability of the cells during the period of the pulse-chase. The rate of incorporation did, however, decline somewhat after 2 h of labeling. The cells treated with canavanine incorporated $\sim 50\%$ as much [35 S]methionine as controls, which is less than in Fig. 1, but consistent with previous reports (6, 13).

To determine if the rate of incorporation of vimentin into the cytoskeleton was similar to the rate of protein synthesis during the 6-h culture, vimentin was recovered by immunoprecipitation from the soluble and cytoskeletal fractions of cells incubated with either arginine or canavanine and labeled continuously with [35 S]methionine. In control cells the soluble pool of vimentin reached a plateau value within 1 h, while the amount of labeled vimentin in the cytoskeletal fraction increased throughout the labeling period (Fig. 4C) thus indicating continuous vimentin assembly. Significantly, very little vimentin accumulated in the cytoskeletal fraction of canavanine-treated cells even upon long-term culture, while the amount of labeled vimentin in the soluble fraction continued to increase (Fig. 4D). The rate of accumulation of canavanine-vimentin in the soluble pool declined somewhat after 2 h of labeling (Fig. 4D), but this may in part reflect altered cellular metabolism upon prolonged culture, as the soluble vimentin

in the control cells declined during this same period (Fig. 4C). As in Fig. 3, the absolute level of labeled vimentin in the soluble pool was higher in canavanine- than in arginine-treated cells (Fig. 4, C and D), although precise quantification was not feasible. Since canavanine-vimentin was found almost exclusively in the soluble fraction, this further supports the existence of a soluble precursor pool of vimentin (2), and in conjunction with pulse-chase experiments (reference 2, and above), eliminates the possibility that the soluble pool arises by dissociation of vimentin from filaments during cell lysis.

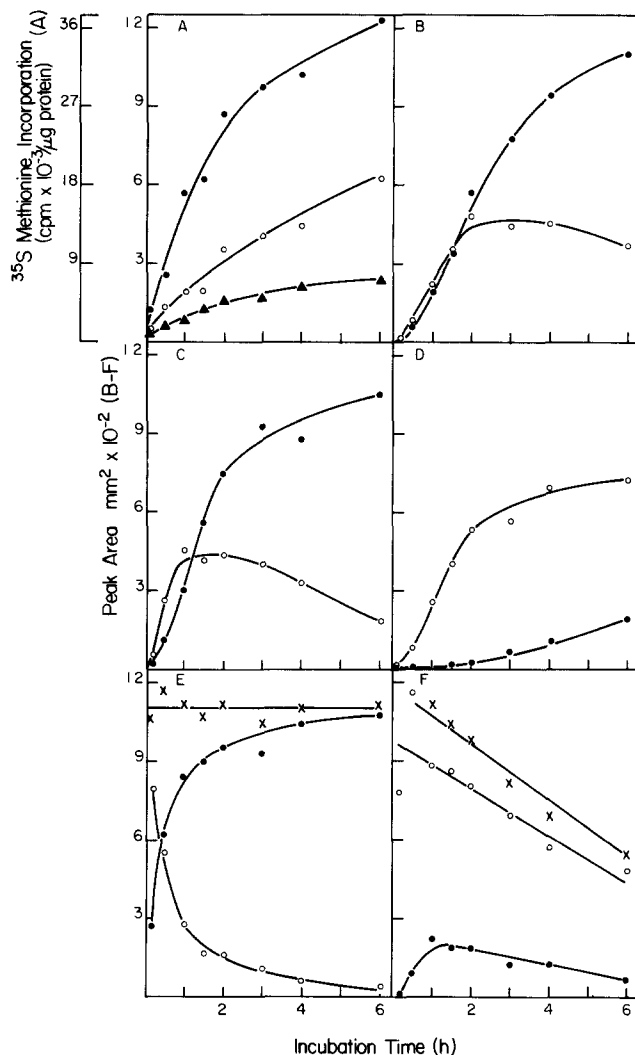


FIGURE 4 Canavanine inhibition of vimentin assembly and vimentin turnover in the soluble fraction. Chicken embryo erythroid cells were preincubated in minimal essential medium containing arginine or canavanine, then labeled with [35 S]methionine in the presence of canavanine or arginine, with or without emetine. Cells were separated into soluble or cytoskeletal fractions, and vimentin quantified by immunoprecipitation and fluorography as in Materials and Methods. (A) Incorporation of [35 S]methionine into total protein in the presence of arginine (●), canavanine (○), or arginine and 0.5 μ M emetine (▲); (B-D) distribution of labeled vimentin between the soluble (○) and cytoskeletal (●) fractions of cells labeled continuously in the presence of arginine and emetine (B), arginine (C), or canavanine (D); (E and F) distribution of labeled vimentin between the soluble and cytoskeletal fractions of cells labeled with [35 S]methionine for 10 min, then chased with unlabeled methionine, in the presence of arginine (E) or canavanine (F). The amount of total labeled vimentin is indicated (x). Fluorographs were exposed for 75 h (B), 13 h (C-E), and 37 h (F).

As noted above, cells incubated in canavanine during long-term culture had lower rates of protein synthesis than control cells (Fig. 4A). The lower rate of protein synthesis in cells incubated in canavanine cannot itself lead to the preferential partitioning of newly synthesized vimentin into the soluble fraction. This conclusion is based on data showing that cells incubated in 0.5 μ m emetine, an inhibitor of ribosome elongation, had lower rates of protein synthesis than either control cells or cells incubated in canavanine (Fig. 4A), and yet the partitioning and kinetic behavior of newly synthesized vimentin resembled that in controls (Fig. 4B). It is, however, interesting to note that the soluble pool of vimentin saturated after 1 h in control cells, but not for 2 h in emetine-treated cells (Fig. 4, B and C).

Centrifugation of Soluble Vimentin

Triton X-100-soluble fractions from cells incubated with arginine or canavanine, and labeled with [³⁵S]methionine, were centrifuged for up to 1 h at 150,000 g in an air-driven microcentrifuge to determine if soluble canavanine- and arginine-vimentin displayed similar sedimentation properties. Immunoprecipitation of vimentin from the supernatant (lanes 1, 3, 5, and 7) and pellet (lanes 2, 4, 6, and 8) demonstrated that both canavanine- and arginine-vimentin remained exclusively in the supernatant (Fig. 5). As previously discussed for arginine-vimentin (2), these data argue against the possibility that the soluble vimentin represents large filaments that are soluble in Triton X-100. These data, however, do not rule out the possibility that soluble vimentin forms protofilaments consisting of a small number of subunits (8).

Arginine Reverses the Canavanine Block to Vimentin Assembly

If canavanine blocks the assembly of vimentin into the cytoskeletal fraction due to its incorporation into and alteration of the charge and conformation of vimentin, then the effect of canavanine on erythroid cells should be reversible. To test this hypothesis, we preincubated cells in arginine or canavanine prior to a 1-h labeling with [³⁵S]methionine. The results show that about half of the arginine-vimentin (Fig. 6, lanes 1 and 2), but no detectable canavanine-vimentin (Fig. 6, lanes 3 and 4) was present in the cytoskeleton. One half of the cells in canavanine were then washed and labeled with [³⁵S]methionine in the presence of arginine. Vimentin in cells labeled continuously in canavanine remained primarily in the soluble fraction (Fig. 6, lanes 5 and 6), whereas cells labeled in canavanine and then arginine yielded a different distribution of vimentin. About half of the arginine-vimentin, which migrated as a discrete band, was present in the cytoskeletal fraction (Fig. 6, lane 8), and about half was present in the soluble fraction (lane 7), similar to cells labeled continuously in arginine (lanes 1 and 2). Virtually all of the canavanine-vimentin was present in the soluble fraction as a diffuse band with a slower relative mobility than the discrete band of arginine-vimentin (Fig. 6, lane 7), and no canavanine-vimentin was detectable in the cytoskeletal fraction (lane 8). We interpret these data to indicate that the diffuse band of soluble vimentin represents canavanine-vimentin that cannot form filaments. The discrete bands of vimentin in both the soluble and cytoskeletal fractions represent arginine-vimentin which, as shown in Figs. 2, 3, and 4, and reference 2, enters into a saturable soluble pool prior to its post-translational assembly.

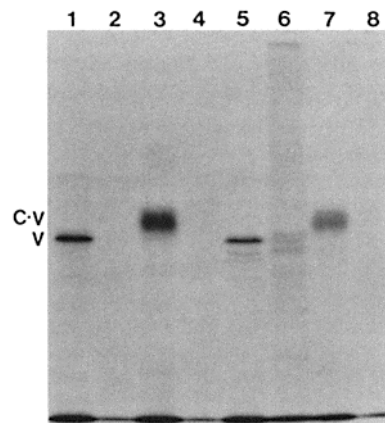


FIGURE 5 High speed centrifugation of soluble vimentin. Aliquots of 50 μ l of the Triton X-100-soluble fractions of cells labeled with [³⁵S]methionine for 1 h in the presence of arginine (lanes 1, 2, 4, and 5) or canavanine (lanes 3, 4, 7, and 8) were centrifuged in a Beckman airfuge at 150,000 g for 30 min (lanes 1-4) or 60 min (lanes 5-8) as previously described (2). The pellet was solubilized and suspended to the same volume as the supernatant, and arginine-vimentin (v) and canavanine-vimentin (c-v) recovered from the supernatant (lanes 1, 3, 5, and 7) and pellets (lanes 2, 4, 6, and 8) by immunoprecipitation.

We conclude that the incorporation of canavanine into vimentin irreversibly prevents the assembly of canavanine-vimentin, while treatment of erythroid cells with canavanine does not interfere with the assembly of arginine-vimentin. This conclusion thus argues against an indirect effect of canavanine on vimentin assembly, such as may occur in response to induction of the heat shock response by canavanine (13). Finally, it is interesting to note that the small amount of canavanine-vimentin that is incorporated into the cytoskeleton (Fig. 6, lane 6) has a more rapid electrophoretic mobility than the soluble canavanine-vimentin (lane 5). The increased mobility of this vimentin may be due to the incorporation of lesser amounts of canavanine, and hence this vimentin is more competent to assemble.

DISCUSSION

We have used the arginine analogue, canavanine, to demonstrate for the first time a means for blocking the *in vivo* assembly of vimentin, the major intermediate filament protein in avian erythroid cells (11). Substitution of other amino acid analogues such as β -hydroxynorvaline for threonine, or o-methylthreonine for isoleucine did not block vimentin assembly in chicken embryo erythroid cells, suggesting that arginine residues are important for the assembly of vimentin. That canavanine does not nonspecifically prevent the assembly of cytoskeletal polypeptides is evident since α - and β -spectrin still assemble into the cytoskeleton in the presence of canavanine.

Our demonstration of the potential significance of arginine residues for the assembly of vimentin is compatible with two related yet independent lines of research from other laboratories. Geisler and co-workers (7) have demonstrated that there are 10 arginine residues in the positively-charged, 73 residue non- α -helical headpiece of chicken desmin, which has a very similar amino acid sequence to vimentin. Second, Nelson and Traub (19) have demonstrated that the headpiece of both vimentin and desmin is required for formation of 10-

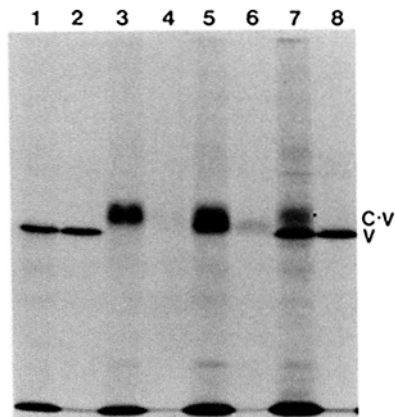


FIGURE 6 Arginine reverses the canavanine block to vimentin assembly. Erythroid cells were preincubated for 2 h in minimal essential medium containing arginine (lanes 1 and 2) or canavanine (lanes 3 and 4) prior to a 1 h continuous labeling with [³⁵S]methionine in the presence of arginine (lanes 1 and 2) or canavanine (lanes 3 and 4). At this time, aliquots of cells were separated into soluble (lanes 1 and 3) or cytoskeletal (lanes 2 and 4) fractions. One half of the remaining cells in canavanine were washed once by centrifugation in minimal essential medium containing 0.4 mM arginine, then suspended in minimal essential medium containing arginine and [³⁵S]methionine. The erythroid cells were incubated for an additional 1.5 h, at which time the cells labeled continuously in the presence of canavanine (lanes 5 and 6), or in canavanine then arginine (lanes 7 and 8), were separated into soluble (lanes 5 and 7) and cytoskeletal (lanes 6 and 8) fractions. Arginine-vimentin (v) and canavanine - vimentin (c-v) were immunoprecipitated from the fractions and subjected to gel electrophoresis, then fluorography for 60 h.

nm filaments *in vitro*. Therefore, substitution of canavanine in the headpiece of vimentin may sufficiently alter its distribution of charges or its conformation to prevent filament assembly. Since the head region of desmin also contains ten threonine residues (8), it is interesting to note that substitution with β -hydroxynorvaline did not prevent the assembly of vimentin into the cytoskeleton, although it did slightly retard this process. It is also possible that the disruptive effect that canavanine has on filament formation is due to changes in the charge or conformation of a rod domain in vimentin which may be homologous to the 38,000 *M_r* rod domain of desmin (7, 8). In desmin, the 38,000 *M_r* rod domain contains a substantial number of arginine residues and has been postulated to be involved in protofilament formation (8). Although our data are consistent with the above hypothesis on the role of arginine in filament formation, it is not yet possible to determine the exact mechanism by which canavanine blocks vimentin assembly *in vivo*.

In many cells, the level of tubulin mRNA (5), and hence tubulin synthesis (1) decreases rapidly in response to increases in the monomer pool. Since vimentin synthesis did not specifically decline upon canavanine-treatment, which may slightly increase the size of the soluble vimentin pool, it is possible that the regulation of vimentin synthesis differs from

tubulin synthesis. However, the decreased level of tubulin mRNA occurred in response to the depolymerization of existing microtubules, so the change in the size of the soluble pool of vimentin indeed affects vimentin synthesis may, therefore, require larger changes in soluble pool size than occurred in the present study.

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