Degradation of Abnormal Proteins in *Escherichia coli*

FORMATION OF PROTEIN INCLUSIONS IN CELLS EXPOSED TO AMINO ACID ANALOGS*

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SUMMARY

Cells of *Escherichia coli* selectively degrade proteins that have incorporated amino acid analogs. Within 1 hour after exposure of cells to canavanine, 50% of the analog-containing proteins were degraded to acid-soluble form. At the same time, no net loss of canavanine-containing protein occurred from the 100,000 × g supernatant. Instead, most of the proteins containing the analog, unlike normal ones, accumulated in particulate fractions sedimenting at 10,000 × g or 100,000 × g. They were then lost from these fractions concomitant with the degradation of the abnormal proteins. The loss of such proteins from particulate fractions accounted for all of the protein degraded to acid-soluble form. Similar observations were obtained after incorporation of other analogs or puromycin.

The 10,000 × g pellets correspond to amorphous dense intracellular granules visible in electron micrographs of cells exposed to canavanine. Upon removal of the analog, these granules disappeared, simultaneously with the degradation of the analog-containing proteins. These pellets do not resemble a degradative organelle, like the lysosome; they are not osmotically sensitive, do not exclude inulin, are not enclosed by a membrane, and do not show autolytic activity.

The proteins in the granules could be solubilized by sodium dodecyl sulfate but not by Triton, NaCl, dithiothreitol, RNase, DNase, or phospholipase. The proteins extracted from the pellet with sodium dodecyl sulfate tend to become particulate again upon removal of this detergent. Incorporation of canavanine caused a normally soluble polypeptide, the monomer of β-galactosidase, to be inactive and found in the sedimentable fraction. These findings suggest that (a) the presence of amino acid analogs in proteins can make them less soluble, and (b) the inclusions are formed by the spontaneous precipitation of abnormal proteins rather than by an active granule-forming process.

The concentration of a protein within bacteria (1, 2) or mammalian cells (2, 3) is determined in part by the rate of protein degradation. In growing bacteria, catabolism of most proteins occurs at a relatively low rate; however, this process increases severalfold during starvation for a carbon or nitrogen source probably to provide such cells with amino acids for protein synthesis (1, 2, 4). In addition, both growing and starving *Escherichia coli* cells degrade proteins with abnormal conformations far more rapidly than normal cell constituents (1, 2, 4, 5). Such abnormal proteins may result from mutation (6, 7), errors in gene translation (5, 8), or incorporation of puromycin (5, 8) or any of a variety of amino acid analogs (5, 8, 9). For example, *E. coli* can incorporate canavanine into proteins in place of arginine, but the resulting proteins are degraded by the cell to acid-soluble form up to 10 times faster than normal proteins containing arginine (4, 5, 9).

The present studies were undertaken to clarify the mechanism responsible for the rapid degradation of such proteins. Preliminary studies (9) from this laboratory have suggested that the proteins that have incorporated amino acid analogs accumulate selectively in a rapidly sedimentable fraction of the cell extract before their rapid degradation. The subsequent loss of proteins from this fraction occurred simultaneously with the degradation of analog-containing proteins to individual amino acids. In addition, both protein catabolism and loss of proteins from the particulate fraction require metabolic energy (9).

Although the accumulation of analog-containing polypeptides in easily sedimentable form appears closely related to the degradative process, the nature of the sedimentable fraction and its importance for protein hydrolysis are unclear. Possibly the rapidly sedimentable fraction represents an organelle specialized for protein hydrolysis and analogous to the lysosome in eukaryotic cells. Alternatively, the pellet could represent a membrane-associated step in the degradative process. Another possibility is that the sedimentable pellets could simply be proteinaceous aggregates that analog-containing proteins form as a result of their abnormal conformations. The present experiments attempt to clarify what factors influence the accumulation of proteins within this rapidly sedimentable fraction and to define further various physical properties of these pellets.

MATERIALS AND METHODS

Chemicals—L-[4,5-3H]Leucine and L-[U-14C]leucine and sodium dodecyl sulfate were purchased from Schwarz-Mann (Orangeburg, N. Y.) and L-canavanine sulfate from Nutritional Biochemical Co.

* This work was supported by grants from the National Institute of Neurological Disease and Stroke and the Air Force Office of Scientific Research. This is the third in a series on the degradation of abnormal proteins in *Escherichia coli*. The previous papers in this series are Refs. 5 and 9.

‡ Postdoctoral Fellow of the National Institute of Mental Health.

§ Career Development Awardee of the National Institute of Neurological Disease and Stroke.
Acrylamide, N,N',N'-methylenebisacrylamide, N,N',N,N'-tetramethylethylenediamine (TEMED), and β-mercaptoethanol were purchased from Eastman Organic Chemical Co. (Rochester, N.Y.) and ammonium persulfate from Fisher Scientific (Morristown, N.J.). α,β-Aminonitrile (cryotone) was generously provided by Dr. Max Rabilloud. Proteins used as markers for electrophoresis were obtained from Boehringer Mannheim.

Culture Conditions—Except where noted, E. coli ATCC 27782, an (K12) auxotroph for arginine and tryptophan, was used in these experiments. In certain experiments, strain ATCC 27798, a lysine auxotroph was used. Cells were grown on glucose minimal medium as described previously (4, 5). The cells grew with a doubling time of 2 to 80 min at 37° on a rotatory shaker.

Production of Abnormal Proteins—To produce abnormal proteins (5), growing E. coli were washed and resuspended in arginine-free medium containing glucose and canavanine (20 μg/ml) for 12 min. [3H]- or [14C]Leucine was then added to these cultures for 5 min. Control cells growing in the presence of arginine received a similar amount of label for the same period. The cells were then filtered, washed, and resuspended in a medium containing arginine (50 μg/ml) and nonradioactive L-leucine (120 μg/ml). Degradation of proteins was estimated from the appearance of radioactivity in the trichloroacetic acid-soluble fraction or from the loss of radioactivity from the trichloroacetic acid-precipitable fraction as previously described (4). Protein breakdown was expressed as the percent of acid-soluble radioactivity relative to that originally present in acid-precipitable material.

Isolation of Subcellular Fractions—Cells were disrupted by sonication (model W185, Heat Systems Ultrasonics, Inc.; sonicator power setting 4, approximately 60-80% lysis) for 2 min at 0°. The cells were sonicated for four 30-s periods separated by 30-s cooling periods to avoid excessive heating of the culture. The sonicated cells were centrifuged at 1,000 X g for 10 min to remove any unlysed cells (radioactivity in this fraction rarely exceeded 0.5% of the total). The supernatant was then centrifuged at 10,000 X g for 10 min and the pellet was resuspended in the growth medium containing nonradioactive L-leucine (120 μg/ml) (100 μl) and recentrifuged to give the 10,000 X g pellet. The 10,000 X g supernatant was centrifuged at 100,000 X g for 2 hours to yield the 100,000 X g pellet and supernatant. Radiolabeled proteins in proteins in the 100,000 X g supernatant were measured by precipitation with 5% trichloroacetic acid as described previously (4). The supernatant fraction did not reflect the marked loss of radioactive protein from the cell. Not only did no net loss of supernatant proteins occur, but some labeled protein may have actually entered that fraction during the course of protein degradation.

The only explanation for these unexpected results is that the rapidly degraded proteins must have initially accumulated in some fraction other than the 100,000 X g supernatant. Direct evidence in support of this conclusion has been presented (6). To examine this possibility more thoroughly, we compared the subcellular distribution of synthesized proteins during growth in the presence of arginine and canavanine (Fig. 1). The two cultures grew and incorporated [3H]-leucine at similar rates. Newly synthesized protein in both instances was found initially in the 100,000 X g pellet which contains the cell's polysomes. In cells exposed to canavanine and [14C]-leucine (Fig. 1), radioactive protein subsequently accumulated in a fraction sedimentable at 10,000 X g. This result is in accord with our previous findings (9) that during a short exposure to canavanine the specific activity of newly synthesized proteins in the 10,000 X g pellet was at least 24 times higher than in the 100,000 X g supernatant. In control cells, only a small percentage of total radioactive protein became associated with this rapidly sedimentable fraction (Fig. 1). Similar results were obtained when the cells

Page dimensions: 596.0x793.0

Electron Microscopy—E. coli 27872 incubated with canavanine was collected by centrifugation in 2% osmium tetroxide buffered with 0.1 M cacodylate (pH 7.4). Fixation was for 12 hours at 4°. After centrifugation at 1500 rpm, an aliquot of the supernatant from just above the pellet was mixed with buffer and stained in 0.5% aqueous uranyl acetate for 50 min at 4°. The bacteria were then dehydrated and imbedded in Epon by conventional techniques. Thin sections were stained with lead citrate.

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RESULTS

Appearance of Analog-containing Proteins in a Rapidly Sedimentable Fraction—Accumulation of analog-containing proteins within particulate fractions was not anticipated at the outset of these studies since the great majority of E. coli proteins are normally soluble (9). Evidence for the involvement of rapidly sedimentable fractions was first obtained in experiments that attempted to identify proteins selectively degraded by the cell and possible intermediates in the degradative process. To minimize experimental variability, these experiments used a double label technique (10) in which the cells were exposed to canavanine and either [3H]-leucine or [14C]-leucine for 5 min. The [3H]-labeled cells were frozen, while the [14C]-labeled cells were washed, and allowed to degrade the canavanine-containing proteins (5, 9). At various times, aliquots of [14C]-labeled cells were removed and combined with the cold [3H]-labeled cells for analysis by acrylamide gel electrophoresis (11) in the presence of sodium dodecyl sulfate.

Within 1 hour, the incubated cells degraded 50% of the canavanine-containing proteins (labeled with [14C]) to acid-soluble form (5, 9), and the average ratio of [3H]:[14C] in total protein of cell extracts increased by a factor of 2.0. However, no specific proteins were lost from the 100,000 X g supernatant during the course of degradation, and no increase in the mean [3H]:[14C] was observed. In other words the radioactive protein in the supernatant fraction did not reflect the marked loss of radioactive protein from the cell.

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Radioactive proteins must have been lost from some other particulate fraction of the cell. In addition, after the short exposure to canavanine, a smaller proportion of radioactive protein was found in the supernatant fraction than in those exposed to arginine. With continued exposure to the analog, the percentage of labeled proteins in particulate form increased, while no such increase occurred in cells growing in the arginine-supplemented medium. After 60 min, over 50% of the canavanine-containing proteins were contained in the 10,000 × g pellet as compared to only 9% of the arginine-containing proteins.

Although proteins synthesized during growth in the presence of canavanine accumulated in the 10,000 × g pellet, the exposure to arginine did not similarly affect the subcellular distribution of arginine-containing proteins within the same cells. Thus when cells were grown in the presence of [3H]leucine and arginine for two generations, 3.4% of the proteins were recovered in the 10,000 × g pellet. When such cells were resuspended in media containing canavanine and nonradioactive leucine, and permitted to grow for 1 hour, the 10,000 × g pellet contained only 5.8% of the labeled proteins. These experiments indicate that the formation of the rapidly sedimenting particles is highly selective; although such granules contain nearly 50% of the analog-containing proteins (Fig. 1), they do not contain appreciable amounts of normal proteins.

To study the turnover of the abnormal proteins in these different fractions, cells were initially exposed to canavanine and [3H]leucine for 5 min, and the fate of labeled components was measured during subsequent growth in the presence of arginine and nonradioactive leucine. In accord with earlier observations (9), the canavanine-containing proteins initially accumulated in the 10,000 × g sedimentable fraction (Fig. 2). The subsequent loss of labeled protein from this fraction could account for about 70% of the total radioactive protein degraded to acid-soluble material, as observed previously (9). Since the 100,000 × g supernatant showed no decrease in labeled protein (Fig. 2), radioactive proteins must have been lost from some other particulate fraction of the cell. In addition, after the short exposure to canavanine and [3H]leucine, the 100,000 × g pellets also showed a net loss of radioactive protein. Loss of material from the 10,000 × g and 100,000 × g particulate fractions together can account for all the labeled protein that became acid-soluble.

To characterize further the particles in which the abnormal proteins had accumulated, cells were exposed for 5 min to either arginine and [3H]leucine or canavanine and [14C]leucine, and were mixed and lysed by sonication. The distribution of labeled proteins was then measured in various fractions (Fig. 3) sedimentable at 40,000 × g or less. In the control cells, about 15% of total cell radioactivity was recovered in fractions sedimentable below 40,000 × g that probably consist of proteins associated with pieces of cell membrane. The amount of labeled protein was similar in all of the sedimentable fractions examined and did not change significantly after removal of the labeled precursor (Fig. 3). In contrast, in cells exposed to canavanine, 43% of the labeled protein was recovered in these particulate fractions, and most of these proteins sedimented below 5,000 × g (38%) and between 5,000 and 10,000 × g (37%). At a subsequent time, when 40 to 60% of the radioactivity in the particulate fractions had been lost, the relative distribution of the radioactivity in the various pellet fractions had not changed significantly. In other words, canavanine-containing proteins accumulated primarily in very rapidly sedimenting granules, although appreciable amounts were found in more slowly sedimenting particles. Subsequently, no specific centrifugal fraction appeared to lose radioactivity selectively as the canavanine-containing proteins were hydrolyzed to trichloroacetic acid-soluble form.

Results with Other Types of Abnormal Proteins—Experiments were undertaken to determine whether similar phenomena occur
successive sedimentable fractions of cell sonicates was measured. salts solution, solubilized in soluene, and counted as described before. proportionately large amounts in the 10,000 X g pellet prior to supernatant from one centrifugation was centrifuged at the next higher speed for 10 min to yield the next pellet fraction. All pellets were washed with twice the original volume of cold basal salts solution, solubilized in soluene, and counted as described under "Methods."

under other conditions that promote production of abnormal proteins. Exposure of tryptophan auxotrophs to 7-azatryptophan or 5-fluorotryptophan (9) also resulted in accumulation of the resulting proteins in the 10,000 X g pellet. After removal of these analogs, about 70% of the labeled proteins initially present in this fraction were lost concomitant with the rapid hydrolysis of the analog-containing polypeptides to acid-soluble form (Table I). Similar observations have been made with E. coli 27798, a lysine auxotroph, exposed to S-(β-aminoethyl)-cysteine (Table I) and a proline auxotroph exposed to azetidine carboxylic acid.1 Incorporation of these analogs also caused rapid breakdown of the resultant proteins after their accumulation in particulate fractions. Furthermore, those proteins initially in the 10,000 X g pellet were lost disproportionately. In fact, in an experiment analogous to that in Fig. 3, the degradation of proteins containing S-(β-aminoethyl)cysteine to acid-soluble form could be accounted for entirely by the loss of labeled proteins from both the 10,000 X g and 100,000 X g pellets. Finally, incomplete polypeptides produced as a consequence of incorporation of puromycin into the growing chains are also rapidly degraded by growing E. coli. Polypeptides synthesized in the presence of puromycin were also found in disproportionately large amounts in the 10,000 X g pellet prior to their rapid degradation (Table I).

1 Unpublished data.

Fig. 3. Radioactive proteins in various centrifugable fractions measured during degradation of abnormal proteins. This experiment attempted to compare the distribution of normal and analog-containing proteins in the particulate fractions and to see if any fraction was lost selectively during subsequent protein breakdown. Cells of Escherichia coli 27873 during exponential growth on glucose minimal medium were washed and resuspended in medium containing arginine or canavanine plus [3H]leucine as in Table III. The cells were mixed, filtered, washed, and resuspended in glucose minimal medium containing leucine (120 μg per ml). At the specified times, aliquots were removed in duplicate and radioactivity in the whole cells and in successive sedimentable fractions of cell sonicates was measured. In 40 min, 30% of the 14C and 1% of the 3H became acid-soluble. Initially, 43% and 15% of total cell radioactivity were found in the fractions sedimentable at 40,000 X g of cells exposed to canavanine and arginine, respectively. For fractionation of the sonicates, the supernatant from one centrifugation was centrifuged at the next higher speed for 10 min to yield the next pellet fraction. All pellets were washed with twice the original volume of cold basal salts solution, solubilized in soluene, and counted as described under "Methods."

In Experiment 1, cells of Escherichia coli 27873, auxotrophic for both arginine and tryptophan, were collected during logarithmic growth, washed, and resuspended in glucose minimal medium lacking the required amino acids. The culture was divided and the required amino acid or analog was added at the following concentrations: arginine and tryptophan, 60 μg per ml, and S-canavanine, 20 μg per ml. The cultures were incubated for 12 min and then [3H]leucine (1.7 nmol per ml, 6 Ci per nmol) was added to each flask for 5 min. In Experiment 2, cells of 27873 growing in minimal medium received puromycin (100 μg per ml) for 12 min prior to addition of [3H]leucine. In Experiment 3, cells of 27798, a lysine auxotroph, were collected during logarithmic growth, washed, and resuspended in glucose minimal medium containing either lysine (20 μg per ml) or S-(β-aminoethyl)cysteine (20 μg per ml). After 12 min, [3H]leucine was added for 5 min. After removal of [3H]leucine and the analogs or puromycin, the cells were resuspended on the original growth medium supplemented with nonradioactive leucine. Protein degradation was measured as described previously (5) and expressed as per cent of the radioactivity initially in protein that became acid-soluble. Aliquots were also taken for measurements of the amount of labeled protein sedimentable in 10 min at 10,000 X g.

Table I

<table>
<thead>
<tr>
<th>Proteins synthesized in presence of:</th>
<th>% Total radioactive protein in cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Degraded in 60 min</td>
</tr>
<tr>
<td><strong>Exp 1</strong></td>
<td></td>
</tr>
<tr>
<td>Arginine, Tryptophan</td>
<td>5</td>
</tr>
<tr>
<td>Arginine, 5-Fluorotryptophan</td>
<td>11</td>
</tr>
<tr>
<td>Arginine, Azatryptophan</td>
<td>23</td>
</tr>
<tr>
<td>Canavanine, Tryptophan</td>
<td>48</td>
</tr>
<tr>
<td><strong>Exp 2</strong></td>
<td></td>
</tr>
<tr>
<td>Puromycin</td>
<td>50</td>
</tr>
<tr>
<td><strong>Exp 3</strong></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>5.8</td>
</tr>
<tr>
<td>β(Aminooethyl) cysteine</td>
<td>36</td>
</tr>
</tbody>
</table>

Physical Properties of Isolated Pellets—For experimental convenience we have investigated the properties of the granules sedimenting at 10,000 X g, even though all of the canavanine-containing proteins could not be isolated at this speed. This fraction rarely contained more than 6% of the proteins synthesized in the presence of arginine, and the specific activity of canavanine-containing proteins in this fraction is at least 20-fold higher than in the 10,000 X g supernatant (9). In addition, the 10,000 X g pellets unlike the 100,000 X g pellets should not include nascent proteins bound to ribosomes.

The pellets in which the abnormal proteins accumulate prior to hydrolysis might be analogous to lysosomes in eukaryotic cells (15, 10), although E. coli cells are not known to contain such organelles. To test whether or not the 10,000 X g pellet is surrounded by an osmotically sensitive membrane, cells were exposed to canavanine and [3H]leucine, and subcellular fractions were prepared in solutions of varying osmotic strength. If the radioactivity in the pellets were retained by a membrane similar to that of the mammalian lysosome or the E. coli spheroplast (17), incubation in solutions of low osmotic strength should cause release of labeled proteins. However, upon incubation in a medium of low osmotic strength or even in distilled water, no disruption of pellets was observed (Table II). In fact, in other experiments the pellets prepared and incubated in distilled

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1 Unpublished data.
Table II

Influence of osmolarity of medium on the amount of canavanine containing proteins present in rapidly sedimentable form

Cells of *Escherichia coli* 27873 were exposed to canavanine and [lH]leucine (0.5 μCi per ml, 11 ng per ml) for 5 min as described under “Methods.” Aliquots of cells were then centrifuged, washed, and resuspended in either distilled water, 0.1 or 0.58 m sucrose (0.05 m Tris, pH 8.0) or 0.3 m NaCl (0.05 m Tris, pH 8.0). The cells were disrupted by sonication and the pellet fraction was isolated by centrifugation at 10,000 × g for 10 min following low-speed centrifugation (1,000 × g for 10 min) to remove unlysed whole cells. Pellets were dissolved in soluene (Nuclear-Chicago) and counted in the usual fashion. Values represent the average of three determinations that agreed within 10%.

<table>
<thead>
<tr>
<th>10,000 × g Pellets prepared in:</th>
<th>Counts per min in remaining 10,000 × g pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>63,000</td>
</tr>
<tr>
<td>0.1 m sucrose</td>
<td>68,200</td>
</tr>
<tr>
<td>0.58 m sucrose</td>
<td>68,500</td>
</tr>
<tr>
<td>0.3 m NaCl</td>
<td>62,750</td>
</tr>
</tbody>
</table>

Table III

Permeability of particulate fraction to inulin

Cells of *Escherichia coli* 27873 were grown in the presence of canavanine (20 μg per ml) for 17 min and 10,000 × g pellets were isolated from cell sonicates in a 20% sucrose solution containing 0.05 m Tris buffer (pH 8.0). Spheroplasts, prepared by the method of Neu and Heppel (17), and whole cells resuspended in the sucrose-Tris solution served as controls. A mixture of [H]labeled H2O and [3H]inulin was added to the three cultures. [3H]Inulin was purified by paper chromatography immediately prior to use. The specific activity of the purified inulin was not determined. After 1/2 h at 0°C, aliquots were removed in triplicate from each tube and centrifuged at 10,000 × g for 10 min. The 10,000 × g pellets were prepared from 3 ml of intact cells, spheroplasts, or cell sonicates. The sides of the tubes were carefully wiped with a cotton swab, and the pellet was dissolved in 0.5 m NaOH. Radioactivity was measured by liquid scintillation counting using the solution of Patterson and Greene (13).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>[H]inulin</th>
<th>[H]H2O</th>
<th>H3C:H</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 × g pellet</td>
<td>315</td>
<td>192</td>
<td>1.64</td>
</tr>
<tr>
<td>Whole cells</td>
<td>90</td>
<td>84</td>
<td>1.05</td>
</tr>
<tr>
<td>Spheroplasts</td>
<td>197</td>
<td>144</td>
<td>1.37</td>
</tr>
<tr>
<td>Medium</td>
<td>7600</td>
<td>4650</td>
<td>1.63</td>
</tr>
</tbody>
</table>

water frequently contained greater amounts of radioactivity than those prepared and incubated in 0.58 m (20%) sucrose solution, which stabilizes E. coli spheroplasts, or in isotonic solutions of NaCl. (This anomalous result may be due to the greater density of the sucrose solution, which probably retards the sedimentation of the pellets more than the distilled water.) In related experiments, pellet fractions prepared in 0.58 m sucrose also failed to release any label to the supernatant fraction when incubated in distilled water.

To test further for the possible existence of a membrane impermeable to macromolecules, isolated pellet fractions, prepared in 0.58 m sucrose, were exposed to a mixture of [3H]labeled inulin, and [H]H2O. Virtually all known biological membranes exclude inulin, but are freely permeable to water. As shown in Table II, the H3C:H ratio in the 10,000 × g pellets was the same as that in the medium, indicating that inulin penetrated to the same extent as water. In contrast, intact *E. coli* excluded inulin as evidenced by the lowered H3C:H ratio. Spheroplasts also excluded inulin, but not as effectively as whole cells (Table III), possibly as a consequence of damage to the membranes or outer cell envelope.

Electron Micrographic Studies—It appears likely that the rapidly sedimentable pellets are related to the intracellular inclusions observed by Schacht et al. and Rogers (18, 19) in *E. coli* killed as a consequence of exposure to 50 μg per ml of canavanine for 1 h. Such cells, as well as *E. coli* that have incorporated certain other amino acid analogs for prolonged periods (20), contain from two to eight large amorphous granules per cell. Therefore, we undertook electron micrograph studies of growing *E. coli* exposed for short periods to canavanine (20 μg per ml). Under such conditions, small osmiophilic inclusions were seen in cells exposed to the analogs but not to arginine (Fig. 4). The shape, size, and number of these granules per cell were quite variable, although they were always found in the cell periphery adjacent to the membrane. These structures appear similar to those described by Schacht et al. and Rogers but are somewhat smaller in size. Micrographs at even higher magnifications did not reveal a membrane surrounding these particles or a direct association with the cell membrane. No fine organization was apparent within these inclusions, although denser areas were evident within the granules.

In the cells grown for longer periods in the presence of canavanine, the granules appeared much more prominent (Fig. 4). After growing in the presence of the analog for 1 h, the amorphous inclusions constituted a large fraction of the intracellular mass, in accord with the earlier observations (18, 19). These observations correlate with the gradual increase in rapidly sedimentable material in cells exposed to canavanine for long periods (Fig. 4). When the cells exposed to canavanine for 20 min were resuspended for 1 h in medium containing arginine, the inclusions disappeared, and the cells were indistinguishable from controls that had never been exposed to canavanine (Fig. 4). The disappearance of these granules from the cell occurred during the same period that canavanine-containing proteins were lost from the sedimentable fractions and most of the abnormal protein in the cell was hydrolyzed to acid-soluble form.

Lack of Autolytic Activity in Isolated 10,000 × g Pellets—Since the analog-containing polypeptides were rapidly lost from the particulate fraction, apparently through protein degradation (Fig. 2), the 10,000 × g pellets were tested for proteolytic activity. A possible localization of the proteolytic activity in this fraction might explain the repeated failures of investigators to demonstrate protein breakdown in cell-free extracts, that usually exclude components sedimenting at 10,000 × g. However, no such evidence was obtained in repeated experiments. Incubation of isolated 10,000 × g pellets, containing abnormal proteins in phosphate or Tris buffers, over the pH range of 6 to 9, failed to show significant autolytic activity. Negative results were also obtained at more acidic pH (4.4) such as that optimal for lysosomal proteases (16). The continued failure to demonstrate autolytic activity further suggests that these structures do not represent degradative organelles (15, 16).

In addition, a number of unsuccessful attempts were made to reconstruct a cell-free system, capable of degrading or releasing proteins from such structures. No effect was seen when pellet fractions were added to a cell-free system for protein synthesis (32), or were recombined with 10,000 × g supernatants. Finally, cells rendered permeable with toluene (23) also failed to release...
Fig. 4. Electron microscopy of cells exposed to canavanine. Cells of *Escherichia coli* 27873 were grown on glucose minimal medium to midlog phase. The cells were centrifuged, washed, and resuspended in medium lacking arginine. Canavanine (20 μg per ml) was added to one portion and arginine to another. Aliquots from the culture containing canavanine were taken for microscopy after A, 15 min, or B, 75 min. C, in addition, after 15-min exposure to canavanine, an aliquot was washed by filtration and resuspended in the original growth medium containing arginine for 1 hour prior to electron microscopy. These aliquots were kept at 0° and then centrifuged at 5000 × g for 10 min prior to fixation by an adaptation of the method of Kellenberger et al. (21). Arrows indicate inclusions not found in control cells growing on arginine. The cells shown in C were indistinguishable from those in control cells never exposed to canavanine. ×30,000.
proteins from the pellet even when supplemented with nucleoside triphosphates.

**Chemical Studies of Rapidly Sedimentable Fraction**—To gain further information concerning the interactions holding the abnormal proteins together within these granules, the isolated 10,000 × g pellets were exposed to various hydrolytic enzymes. Growing cells were exposed to either [3H]leucine or arginine and [14C]leucine as in Table I. The cells were mixed, washed, resuspended in distilled water, and disrupted by sonication as described under "Methods." The fraction sedimentable at 10,000 × g for 10 min was isolated, washed, and finally resuspended in H2O with a Dounce homogenizer (final protein concentration about 80 µg per ml). All compounds were added as crystalline solids, except Triton X-100. The tubes were then incubated at 37° for 1 hour with occasional shaking, and 10,000 × g pellets were then isolated by centrifugation at room temperature for 10 min, the pellet washed with 2 volumes of distilled water, and air-dried, and radioactivity was measured as described under "Methods." Duplicate incubations agreed within 5%.

**Additive**

<table>
<thead>
<tr>
<th>Additive</th>
<th>Counts per min remaining in 10,000 × g pellet after 1 hour at 37°</th>
<th>H:14C ratio in pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (bovine serum albumin)</td>
<td>100/100</td>
<td>5.5</td>
</tr>
<tr>
<td>Lyszyme</td>
<td>102/103</td>
<td>5.4</td>
</tr>
<tr>
<td>Phospholipase D</td>
<td>101/100</td>
<td>5.5</td>
</tr>
<tr>
<td>Trypsin</td>
<td>30/55</td>
<td>2.9</td>
</tr>
<tr>
<td>Pronase</td>
<td>20/72</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Table IV**

**Ability of various enzymes to solubilize radioactivity in the 10,000 × g pellets**

During exponential growth, cells of *Escherichia coli* 27873 were collected by centrifugation, washed, and suspended in glucose minimal medium lacking arginine. Canavanine (20 µg per ml) and arginine (60 µg per ml) were added to different portions of the culture for 12 min. For the last 5 min, [3H]leucine (0.5 µCi per ml, 11 ngm per ml) was added to the culture containing canavanine and [14C]leucine (0.1 µCi per ml, 4.2 ngm per ml) to the culture containing arginine. Incubation was stopped by adding aliquots of both cultures to an equal volume of ice-cold basal salts solution. Each aliquot was washed and resuspended in the original volume of basal salts containing leucine (120 µg per ml). The fractions were mixed, cells disrupted by sonication, and the 10,000 × g pellet fraction isolated as described under "Methods." The pellet fraction was suspended in basal salts by mixing with a Dounce homogenizer at 4°. Aliquots (1 ml) containing approximately 11,000 cpm of [3H] and 2,000 cpm of [14C] were added to test tubes containing 100 µg of the designated protein. The control tube contained 100 µg of bovine serum albumin. These tubes were incubated at 37° for 1 hour, and 10,000 × g pellet fraction isolated and washed with basal salts solution. Radioactivity in the sedimentable fraction was measured as described under "Methods."

**Table V**

**Treatment of isolated 10,000 × g pellet with various reagents**

Cells of *Escherichia coli* 27873 were exposed to either canavanine and [3H]leucine or arginine and [14C]leucine as in Table I. The cells were mixed, washed, resuspended in distilled water, and disrupted by sonication as described under "Methods." The fraction sedimentable at 10,000 × g for 10 min was isolated, washed, and finally resuspended in H2O with a Dounce homogenizer (final protein concentration about 80 µg per ml). All compounds were added as crystalline solids, except Triton X-100. The tubes were then incubated at 37° for 1 hour with occasional shaking, and 10,000 × g pellets were then isolated by centrifugation at room temperature for 10 min, the pellet washed with 2 volumes of distilled water, and air-dried, and radioactivity was measured as described under "Methods." Duplicate incubations agreed within 5%.
TABLE VI
Effects of removal of sodium dodecyl sulfate on solubility of proteins from the sedimentable and supernatant fractions

This experiment was designed to determine whether the tendency of proteins to accumulate in rapidly sedimentable form is an inherent property of the protein. In Experiment 1, cells of *Escherichia coli* 27873 grown to midlog phase were exposed to canavanine (20 μg per ml) for 7 min and [3H]leucine (0.1 μCi per ml, 2.2 ngm per ml) for an additional 5 min. Another aliquot of cells, growing on glucose minimal medium containing arginine, was exposed to [14C]leucine (0.1 μCi per ml, 4.2 ngm per ml) for 5 min. The cells were mixed, centrifuged and resuspended in one-half their original volumes. The 20,000 × g pellets were isolated from cell sonicates following removal of any unlysed cells by centrifugation at 1,000 × g for 10 minutes. The pellet proteins were solubilized in a volume of 1% sodium dodecyl sulfate corresponding to 1/4 the original culture volume by incubation for 1 hour at 37°. Crystalline sodium dodecyl sulfate was added to the supernatant to produce a 1% solution. After removal of insoluble material from both fractions by centrifugation at 20,000 × g for 10 min, sodium dodecyl sulfate was removed from the solutions by the method of Weber and Kuter (24) which consists of ion exchange chromatography in the presence of 6 M urea to remove excessive amounts of normally insoluble components (e.g. membrane-associated polypeptides) or because the presence of the analogs in proteins changes their properties. To resolve this question, we tested whether a normally soluble protein, β-galactosidase, becomes associated with the rapidly sedimentable fractions when synthesized in the presence of canavanine. Since we were unable to detect enzymatically active β-galactosidase in induced cells growing in the presence of canavanine, it was necessary to test for this protein in different fractions by a means other than enzymatic assay. Immunological techniques were not used, since the canavanine-containing polypeptides either in supernatant or in granules might fail to react with antibodies as a result of their abnormal configurations. Consequently, we chose to analyze proteins by double label acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, in which we simultaneously compared the proteins of induced (3H) and uninduced (14C) cells (Fig. 5). This technique appeared particularly suited for identifying β-galactosidase monomers, since these unusually large polypeptides (approximately 130,000 daltons) (26) are found in a region of the gels relatively free of other proteins and since they are produced in large amounts by induced cells.

In the induced cells, the 100,000 × g supernatant contained a large peak of radioactivity, with a molecular weight corresponding to that of β-galactosidase monomers (20). No other peak specific to the induced cells was detected in the supernatant. A much smaller 3H:14C peak was evident in gels of the 100,000 × g pellet, and none in the 10,000 × g pellet (Fig. 5). These data are in accord with previous findings that β-galactosidase is a soluble enzyme, although some may be associated with unwashed ribosomes (27). (Our failure to observe peaks corresponding to the two other proteins of the lac operon is not surprising, inasmuch as the M protein and galactoside transacetylase are present in much lower amounts than β-galactosidase and since they are found in regions of the gels where most of the cell proteins migrate.)

When cells were induced in the presence of canavanine, a major peak of molecular weight approximately 130,000 was evident in the 10,000 × g pellet and a similar one in the 100,000 × g pellet (Fig. 6). In contrast to the results in cells growing on arginine (Fig. 5), no peak was evident in the 100,000 × g supernatant of cells growing on canavanine. Similar results
FIG. 5. Subcellular distribution of $\beta$-galactosidase monomers synthesized in the presence of arginine. To identify proteins produced by the $Z$-gene of the lac operon, a double label electrophoretic protocol was adopted. One culture of Escherichia coli 27873 was grown on 1% glycerol minimal medium containing arginine in the presence of isopropyl-$\beta$-thiogalactoside (IPTG) (0.5 mM) for 1.5 hours and [3H]leucine (2.0 $\mu$Ci per ml, 44 ng per ml) was added for the last 5 min. A second portion of cells was grown on the same medium but without inducer; this culture was exposed to [14C]leucine (0.25 $\mu$Ci per ml, 10.5 ng per ml) for 5 min. The cells were then mixed, poured over ice-cold basal salts, centrifuged, resuspended in Tris-glycine buffer, and disrupted by sonication. Proteins of various centrifugal fractions were analyzed by acrylamide gel electrophoresis as described under "Methods." Only $^{3}H:14C$ ratios are shown in the figure. All slices contained at least 60 cpm of each isotope after background subtraction and correction for crossover of isotopes. Marker proteins for standardization of molecular weights were subjected to electrophoresis concurrently in separate gels and stained with Coomassie brilliant blue. The molecular weight of the peaks in the 100,000 X $g$ supernatant and 100,000 X $g$ pellet corresponded to that of $\beta$-galactosidase (about 130,000) (26). Similar results were obtained when the induced culture received [14C]leucine and the control [3H]leucine.

were obtained in experiments where the induced culture received [14C]leucine and the uninduced [3H]leucine. Thus, incorporation of canavanine into a normally soluble enzyme can make it inactive and cause it to associate with the rapidly sedimentable fraction.

Greater variability was observed in the electrophoretic patterns of the proteins from canavanine-treated cells than from arginine-treated cells, presumably as a consequence of the rapid protein catabolism of analog-containing proteins. In fact, when these cells were washed and resuspended in the presence of arginine for 1 hour, under conditions where most of the canavanine-containing proteins were lost from the 10,000 X $g$ pellet (Fig. 2) and the intracellular inclusions disappeared (Fig. 4), the peak corresponding to the $\beta$-galactosidase monomers was also lost from the particulate fractions, presumably through protein degradation.²

FIG. 6. Subcellular distribution of $\beta$-galactosidase monomers synthesized in the presence of canavanine. Since no enzymatically active $\beta$-galactosidase is formed in the presence of canavanine, a double label electrophoretic protocol was adopted. Escherichia coli 27873 was grown on 1% glycerol minimal medium containing canavanine (20 $\mu$g per ml) in the presence or absence of isopropyl-$\beta$-galactoside (IPTG) (0.5 mM). After 7 min the culture with isopropyl-$\beta$-galactoside was exposed to [3H]leucine (1.8 $\mu$Ci per ml, 29 ng per ml), and the culture grown without isopropyl-$\beta$-galactoside was exposed to [14C]leucine (0.28 $\mu$Ci per ml, 49 ng per ml). The cells were then mixed and subcellular fractions prepared for electrophoresis as in Fig. 5. Marker proteins for standardization of molecular weights were run concurrently as described under "Methods." The molecular weight of peaks observed in the 10,000 X $g$ and 100,000 X $g$ pellet corresponded to that of $\beta$-galactosidase (mol wt 130,000) (26).

DISCUSSION

These studies provide further evidence that abnormal proteins accumulate in intracellular granules prior to their rapid degradation (9). For example, following incorporation of canavanine, $\beta$-galactosidase, which is normally a soluble enzyme, became enzymatically inactive and was associated with the rapidly sedimenting fractions (Figs. 5 and 6). Accumulation of proteins in such granules was also observed after incorporation of other analogs or puromycin. Similar effects may occur with other types of aberrant proteins. Several mutations have, in fact, been described where the altered protein is associated with particulate fractions (28, 29), but it is unknown whether such mutant proteins are rapidly degraded like the analog-containing ones (Fig. 2 and Table I).

Abnormal proteins continually become associated with and lost from these granules (Figs. 1 and 2), and a variety of evidence indicates that this loss of proteins involves their hydrolysis to

acid-soluble components. For example, in cells exposed to different analogs, the amount of labeled protein initially associated with the 10,000 × g pellets was directly related to the subsequent rate of proteolysis (Table I). In addition, the amount of labeled proteins lost from the 10,000 × g and 100,000 × g pellets could account for nearly all of the labeled protein hydrolyzed (Fig. 2 and Table I) (9). At the same time, no loss of labeled material occurred from the 100,000 × g supernatant (Fig. 2). Furthermore, when the canavanine-containing subunits of β-galactosidase were lost from the granule fraction, only a very small amount of protein of that size could be recovered in the soluble fraction (in preparation). It is also interesting that analog-containing proteins of large molecular weight not only tend to be preferentially degraded by the cell but also accumulate disproportionately in the 10,000 × g pellets.3

These centrifugable pellets correspond to the dense osmophilic inclusions evident in electron micrographs of cells exposed to canavanine but not of normal cells (Fig. 4). Both the size of these intracellular inclusions and the amount of labeled proteins in the 10,000 × g pellets increased upon prolonged exposure to this analog (Fig. 1). Furthermore, these inclusions disappeared during growth on arginine when the canavanine-containing proteins were hydrolyzed. In addition, the isolated 10,000 × g pellets contain dense osmophilic granules resembling the intracellular inclusions.4 Similar structures have been previously reported in cells killed as a result of prolonged growth in the presence of high concentrations of canavanine (18, 19) or certain other analogs (20). It was originally suggested that the large intracellular inclusions caused cell death (18). However, the cells studied here showed no loss of viability or decrease in growth, despite the presence of the inclusions. On the contrary, accumulation of abnormal proteins in granules and their subsequent hydrolysis may represent a mechanism protecting the cell from the lethal consequences of harboring abnormal proteins.

Although accumulation of proteins in such granules appears linked to the degradative process, such inclusions do not appear to represent a degradative organelle like the mammalian lysosome (15, 16). Electron micrographs and physicochemical studies indicate that these inclusions are not surrounded by a membrane (Fig. 4 and Table II). Instead, the pellets resemble amorphous aggregates of proteins held together by noncovalent bonds. Since neither NaCl, Triton X-100, or dithiothreitol solubilized these polyptides, it is unlikely that ionic, hydrophobic, or S—S bonds alone maintain the integrity of the pellet. The particulate proteins however, could be solubilized with sodium dodecyl sulfate or guanidine hydrochloride (Table V).

The E. coli granules thus resemble the proteinaceous inclusions observed in certain pathological conditions, where large amounts of abnormal proteins are found in cells, such as Heinz-body anemia (30), thalassemia (31), or lead poisoning (32). Possibly such inclusions might also bear some relationship to intracellular protein catabolism. Interestingly, when reticulocytes incorporate various analogs into hemoglobin, an inordinately large amount of protein also accumulates in particulate fractions and is then rapidly degraded to acid-soluble material (34).3

Since the analog-containing proteins in the 10,000 × g pellets are inherently less soluble than most normal proteins (Table VI), formation of inclusions probably results simply from their precipitation and aggregation in the cell. It thus appears unnecessary to hypothesize the existence of some complex process causing the accumulation of abnormal proteins in 10,000 × g pellets. In fact, earlier studies showed that the build-up of labeled proteins in 10,000 × g pellets is not an energy-dependent reaction, unlike the subsequent loss of proteins from this fraction (9). The tendency of the abnormal proteins to precipitate (Table VI) suggests that they are denatured. This conclusion is consistent with the finding that they are more sensitive to digestion by trypsin and pronase than normal cell proteins (Table IV) (34). In fact, the greater protease sensitivity of denatured proteins offers an attractive mechanism for the selective hydrolysis of the analog- or puromycin-containing proteins (34, 35).

Even though granule formation and protein degradation appear to be closely linked, the present observations do not prove that the granules play an essential role in the degradative process. Two findings argue against such a conclusion. (a) Certain mutations in the lac operon result in proteins that are rapidly hydrolyzed even though they are soluble (6, 7). (b) Upon incorporation of trace amounts of canavanine, degradation is accelerated without a demonstrable increase in radioactive proteins in the particulate fractions.2 Even if granule formation is not absolutely necessary for rapid proteolysis, the spontaneous aggregation of the abnormal proteins may serve to promote their recognition by the degradative system. Alternatively these inclusions may form only when the cell synthesizes abnormal proteins more rapidly than it can degrade them (e.g. upon incorporation of analogs or puromycin). If the cell’s degradative capacity is saturated, the denatured proteins may then aggregate, form inclusions, and remain in this stage until the degradative system can attack them.

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