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Biochemistry

A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes

(protein turnover/abnormal hemoglobins/lysosomes/intracellular proteases/energy-requirement)

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ABSTRACT **Reticulocytes, like other cells, selectively degrade certain abnormal proteins by an energy-dependent process. When isolated rabbit reticulocytes incorporate the valine analog 2-amino-3-chlorobutyric acid (ClAbu) in place of valine, they produce an abnormal globin that is degraded with a half-life of 15 min. Normal hemoglobin, in contrast, undergoes little or no breakdown within these cells. Cell-free extracts from reticulocytes have been shown to rapidly hydrolyze these abnormal proteins. The degradative system is located in the 100,000 × *g* supernatant, has a pH optimum of 7.8, and does not appear to be of lysosomal origin. This breakdown of analog-containing protein was stimulated severalfold by ATP, and slightly by ADP. AMP and adenosine-3′:5′-cyclic monophosphate had no significant effect on proteolysis. Experiments with ATP analogs suggest that the terminal high energy phosphate is important in the degradative process.**

Proteolysis in the cell-free system and in intact reticulocytes was inhibited by the same agents (L-1-tosylamido-2-phenylethylchloromethyl ketone, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, *N*-ethylmaleimide, iodoacetamide, and *o*-phenanthroline). In addition, the relative rates of degradation of several polypeptides in the cell-free extracts paralleled degradative rates within cells. Thus, a soluble nonlysosomal proteolytic system appears responsible for the energy-dependent degradation of abnormal proteins in reticulocytes.

Proteins within animal and bacterial cells are continuously being degraded to amino acids, and their rates of degradation in part determine their intracellular concentrations (1–4). Proteins with abnormal structures are degraded especially rapidly (1–3, 5–8) and this process prevents the accumulation of potentially harmful polypeptides (1, 2). Despite the physiological significance of protein catabolism, the responsible enzymes and degradative pathways are still unknown. In animal cells, most workers have assumed that the lysosome is the site of protein degradation, because of the high concentration of proteases within this organelle and because the lysosome is involved in the degradation of endocytosed proteins (9, 10). However, various nonlysosomal proteases also exist and may well be involved in the breakdown of cell proteins (2, 11, 12). One reason for the lack of knowledge of the degradative pathways is that cell-free preparations have generally failed to display important characteristics of intracellular proteolysis. For example, a puzzling feature of this process is that inhibitors of energy metabolism which severely reduce cellular ATP levels also inhibit protein degradation (2, 7). To explain this effect, previous workers have suggested an energy requirement for transport of substrate into the lysosome (2, 10) or for maintaining an acid milieu within this organelle (2, 13). However, an energy requirement for protein degradation also has been demonstrated in bacteria which lack lysosomes (2, 7). In addition, cell-free preparations have often failed to degrade proteins that have very short half-lives in intact rat liver cells, such as tyrosine amino transferase (2, 4), and analog-containing proteins in bacterial cells (*Escherichia coli*) (2).

In an attempt to establish a cell-free system which displays the apparent energy-requirement and specificity of protein degradation in intact cells, we have studied the rapid degradation of abnormal proteins in reticulocytes (refs. 5 and 14–16, K. Olden, J. D. Etlinger, and A. L. Goldberg, manuscript in preparation). These cells offer several advantages for the study of protein degradation. Preparations of reticulocytes can be obtained quite easily and these cells synthesize one protein, hemoglobin, almost exclusively. In addition, because the structures of normal hemoglobin and of many hemoglobin variants are known, it may be possible to define what features of this protein can influence its rate of catabolism. Although normal tetrameric hemoglobin undergoes little if any intracellular degradation, there is evidence that several human hemoglobin variants resulting from missense mutations and the excess free α-chains that are found in β-thalassemia are rapidly degraded within reticulocytes (2). In addition, Rabinowitz and Fisher showed that incorporation of valine and lysine analogs in reticulocytes leads to polypeptides which undergo rapid intracellular degradation (5, 14). Other groups have shown that prematurely terminated polypeptides, resulting from incorporation of puromycin, are also rapidly degraded within reticulocytes (refs. 15 and 16; K. Olden, J. D. Etlinger, and A. L. Goldberg, manuscript in preparation) as in other cells. The present report describes a new soluble proteolytic system that appears responsible for the energy-dependent breakdown of analog- and puromycin-containing polypeptides in reticulocytes.

MATERIALS AND METHODS

Preparation of Reticulocytes and Radioactive Protein. Reticulocytosis was induced in adult rabbits (weighing approximately 6 lbs) by daily subcutaneous injections of 2.5% phenylhydrazine hydrochloride. About 0.8 ml of this solution was injected daily for 4 days, and 0.5 ml was injected on the fifth day. The rabbit was bled through the marginal ear vein on the eighth day, and the cells were collected by centrifugation at 800 × *g* for 5 min. Such blood preparations showed reticulocyte counts of about 90%. The white buffy coat on the top of the pellets was discarded, and the cells were suspended in 10 volumes of Krebs-Ringer phosphate buffer containing NaCl (0.12 M), KCl (0.005 M), $CaCl_2$ (0.003 M), $MgSO_4$ (0.001 M), Na_2HPO_4 (0.016 M), and glucose (2 mg/ml) and washed five times in the same buffer.

For synthesis of labeled proteins, reticulocytes were first suspended in 3 to 4 volumes of Krebs-Ringer phosphate buffer containing glucose (2 mg/ml), $FeSO_4$ (30 μg/ml), and plasma concentrations of the amino acids (17). For experiments in-

Abbreviations: ClAbu, 2-amino-3-chlorobutyric acid; cAMP, adenosine 3′:5′-cyclic monophosphate.

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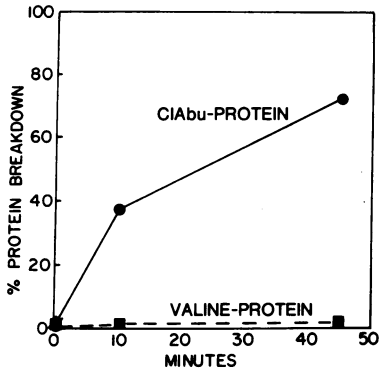

FIG. 1. Degradation of analog-containing (●) and valine-containing (■) proteins in reticulocytes. Proteins were synthesized in the presence of the valine analog, ClAbu or valine, and their hydrolysis measured as described in the *text*. Similar results were obtained with five independent preparations.

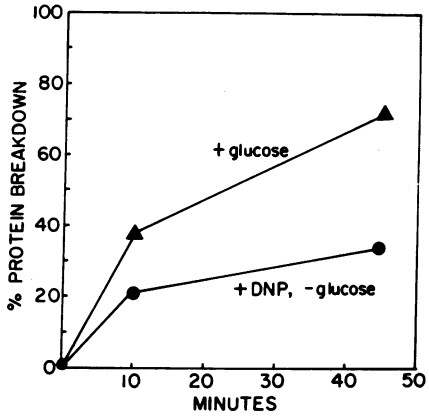

FIG. 2. Effects of inhibition of energy metabolism on the degradation of ClAbu-containing protein in reticulocytes. Breakdown of protein synthesized in the presence of ClAbu was measured in the presence of glucose (▲) and in the presence of 2,4-dinitrophenol (0.1 mM) and absence of glucose (●). Similar results were obtained with three independent preparations.

volving amino acid analogs, the corresponding amino acid was omitted from the media. The cells were incubated with shaking for 10 min at 37°, and then either the valine analog, 2-amino-3-chlorobutyric acid (ClAbu) (1 mM) (kindly provided by W. Prouty), the lysine analog, *S*-(3-aminoethyl)cysteine (1 mM) (kindly provided by M. Rabinowitz), or puromycin (10 μg/ml) were added. After incubation for 5 min, 5–10 μCi/ml of [^{3}H]leucine (6C/mmol) or 0.1–1.0 μCi/ml of [^{14}C]leucine (312 μCi/mmol) was added, and the incubation was continued for 15 min. The cells were then washed three times in the Krebs-Ringer phosphate buffer containing glucose (2 mg/ml), $FeSO_4$ (30 μg/ml), plasma amino acids, and 10 mM nonradioactive leucine.

Preparation of the Cell-Free System. Reticulocytes containing labeled proteins were prepared as described above. All subsequent steps were carried out on ice. Cells were centrifuged at 800 × *g* for 10 min, and then lysed by suspension in 1.6 volumes of glass distilled water at 0–4°. Preliminary experiments were carried out to establish conditions for maximal rates of hydrolysis of ClAbu-containing protein. The lysate was centrifuged at 100,000 × *g* for 1 hr, and the supernatant was dialyzed for 20 hr against 500 volumes of 8 mM KCl, 4 mM $MgCl_2$, and 0.5 mM dithiothreitol with one change of the dialysis solution. After dialysis, 0.2 M Tris·HCl buffer containing the above salts was usually added to give a final concentration of 0.05 M Tris·HCl, and a final pH of 7.8. For studies of pH dependence, several buffers were used.

Measurement of Protein Breakdown. Protein breakdown was determined as the amount of acid-soluble radioactivity relative to that initially found in protein (i.e., acid-precipitable form). For studies of intact reticulocytes, the cells were suspended in 5 volumes of Krebs-Ringer phosphate buffer with glucose, $FeSO_4$, plasma level amino acids, and 10 mM nonradioactive leucine, and incubated at 37°. Fifty microliter aliquots of cells in media were added to 1 ml of 10% trichloroacetic acid (TCA). Cell-free extracts were incubated at 37°, 20 μl aliquots were removed, and added to 1 ml of 10% TCA. TCA-precipitable and soluble radioactivity were counted as previously described (6). An external standard was used to correct for differences in quenching. Each point represents the average of two determinations which agreed within 5%.

RESULTS

Degradation of Protein in Reticulocytes. Initial studies compared the effects of various amino acid analogs on rates of protein degradation (K. Olden, J. D. Etlinger, and A. L. Goldberg, in preparation). Incorporation of the lysine analog, *S*-(2-aminoethyl)cysteine, and the valine analog, ClAbu, were most effective in producing proteins that were rapidly degraded. For example, the ClAbu-containing protein was hydrolyzed to acid-soluble material with a half-life of 15–20 min (Fig. 1). In contrast, little or no degradation of protein containing valine occurs within these cells (Fig. 1). The dramatic effects of these analogs presumably are a consequence of the large amounts of valine and lysine found in hemoglobin, or the particular importance that these residues play in its structure.

Because metabolic energy appears essential for proteolysis in a variety of cells (2, 7), we examined whether a similar requirement obtained for degradation in reticulocytes. When ATP production was inhibited by incubating cells without glucose to reduce glycolysis and with 2,4-dinitrophenol to prevent oxidative phosphorylation, proteolysis was markedly reduced (Fig. 2). The inhibition of proteolysis by ATP depletion in these cells is not secondary to a requirement for protein synthesis, because the rate of degradation of ClAbu-containing protein was not affected by addition of 0.5 mM cycloheximide.

Degradation of Protein in Cell-Free Extracts. We have attempted to establish conditions for the degradation of these abnormal proteins in cell-free lysates. A highly active degradative system was found in the soluble dialyzed fraction of reticulocyte lysates when proteolysis was measured in the presence of ATP (Fig. 3). Such preparations rapidly hydrolyzed analog-containing proteins synthesized in the cells prior to their disruption. Centrifugation at 100,000 × *g* for even 10 hr left most of the ATP-dependent activity in the supernatant fraction. Approximately 50% of the analog-containing protein was recovered in the 100,000 × *g* supernatant; the remainder accumulated in rapidly sedimenting fractions of cell lysates as do abnormal proteins in bacteria (1, 2, 6). Thus, both the substrate and proteolytic system were contained in these cell-free extracts.

The specificity of the cell-free system resembled that of the intact cells, since valine-containing globin underwent little degradation, while ClAbu-containing globin was rapidly hydrolyzed (Fig. 3). Because protein catabolism in intact cells appears to require metabolic energy, we examined in detail the effect of ATP in these cell-free preparations (Fig. 4). The presence of ATP (0.1–1 mM) consistently stimulated the

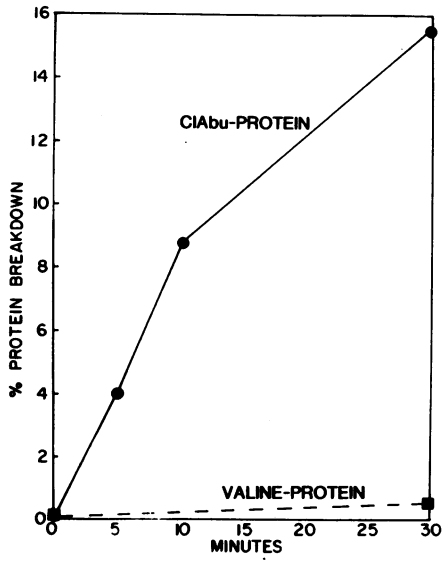

FIG. 3. Cell-free degradation of proteins containing ClAbu (●) or valine (■). Prior to lysis, reticulocytes were incubated in the presence of [^{14}C]leucine and either valine or ClAbu. ATP (1 mM) was present in these incubations. Similar results were obtained with five independent preparations.

breakdown of abnormal protein. The degree of stimulation varied from about 2- to 5-fold in different preparations. Furthermore, neither AMP adenosine-3′:5′-cyclic monophosphate or (cAMP) caused significant stimulation of protein degradation (Table 1). However, ADP had a small but significant stimulatory effect (Table 1). Additional experiments with ATP analogs (18) examined whether cleavage of one or the other high energy phosphates might be important for this effect (Table 1). α,β-methylene-ATP, which can be hydrolyzed to yield the γ-phosphate, promoted proteolysis significantly, although less extensively than ATP. The β,γ-methylene-ATP which can only be hydrolyzed at the α–β bond to release pyrophosphate had much less effect. These observations are direct evidence that high energy phosphates influence the degradative process and can account for the apparent energy requirement for proteolysis in intact cells (Fig. 2).

To characterize this degradative system further, we exam-

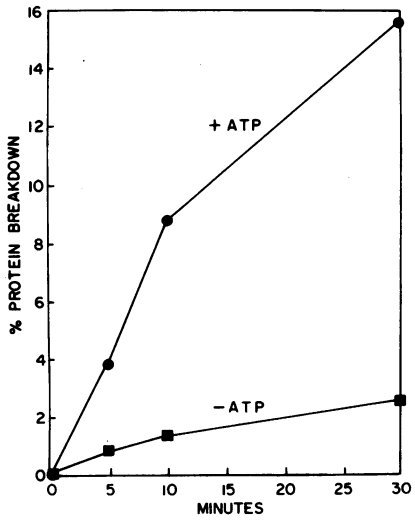

FIG. 4. Effect of ATP on the cell-free degradation of ClAbu-containing protein. Protein breakdown was measured in the presence (●) or absence (■) of ATP (1 mM). Similar results were obtained with five independent preparations.

Table 1. Effects of nucleotides and ATP analogs on the hydrolysis of ClAbu-containing protein in cell-free extracts

Experiment	Nucleotide (1 mM)	% Stimulation
1	ATP	189
	ADP	21
	AMP	8
	cAMP	5
	cAMP + ATP	195
2	ATP	384
	α,β-methylene ATP	57
	β,γ-methylene ATP	13

Cell-free incubations were carried out for 30 min as described in the *text*. The % stimulation equals the increase in protein degradation beyond that seen in the absence of added nucleotide. ATP analogs were obtained from P-L Biochemicals (Milwaukee, Wis.). Similar results were obtained with four independent preparations.

ined the hydrolysis of abnormal protein as a function of pH (Fig. 5). The ATP-dependent activity was only evident in the neutral and alkaline range with a sharp pH optimum of about 7.8. Almost no detectable proteolysis occurred in the acid range where most of the known lysosomal proteases are active (9, 10).

Substrate Specificity and Sensitivity to Inhibitors. To test whether this proteolytic activity is responsible for the degradation of abnormal protein in intact reticulocytes, we compared the relative rates of breakdown of several polypeptides in cells and in extracts (Table 2). The intact cells degraded puromycyl-polypeptides to acid soluble material more rapidly than ClAbu-containing protein. Proteins containing *S*-(2-aminoethyl)cysteine were hydrolyzed more slowly while normal hemoglobin was most stable. Cell-free preparations hydrolyzed these various proteins in the same relative order as intact cells. Generally, rates of hydrolysis in the extracts averaged ⅓ to ½ those in intact cells.

Finally, to help define the responsible enzymes, we compared the effects of various inhibitors on the hydrolysis of ClAbu-containing protein in reticulocytes and in cell-free preparations

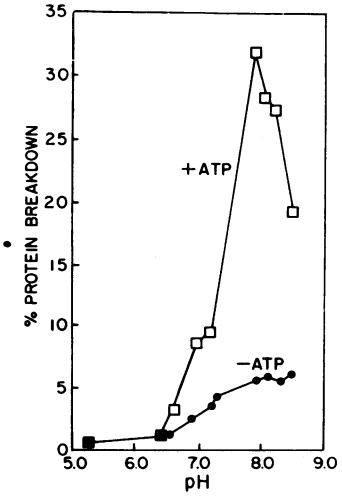

FIG. 5. pH dependence of the degradation of ClAbu-containing protein in reticulocyte lysates in the presence (□) or absence (●) of ATP (1 mM). Buffers were present at a concentration of 0.05 M. Acetate was used below pH 6.5, phosphate was used between pH 6.5 and 7.0, and Tris·HCl was used above pH 7.0. All incubations were for 30 min. Similar results were obtained with three independent preparations.

Table 2. Relative susceptibility of different polypeptides to degradation in reticulocytes and in cell-free extracts

Substrate	Reticulocytes (% protein breakdown/ 45 min)	Cell-free extracts (% protein breakdown/ 30 min)
Puromycyl-polypeptides	81	19
Protein containing ClAbu	70	18
Protein containing *S*-(β-aminoethyl)cysteine	16	3
Protein containing valine	3	1

Similar results were obtained with four independent preparations. Cell-free incubations included ATP (1 mM).

Table 3. Effects of various inhibitors on the breakdown of ClAbu-containing protein in reticulocytes and extracts

Inhibitor	Reticulocytes (% inhibition/ 45 min)	Cell-free extracts (% inhibition/30 min)
N-ethylmaleimide (5 mM)	90	66
Iodoacetamide (5 mM)	43	46
TPCK (0.13 mM)	80	51
TLCK (1 mM)	52	55
o-Phenanthroline (1 mM)	61	60

Similar results were obtained with five independent preparations. TPCK and TLCK refer to L-1-tosylamido-2-phenylethyl chloromethyl ketone and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, respectively. Cell-free incubations included ATP.

(Table 3). The chloromethyl ketones, TPCK and TLCK, both inhibited degradation in the cells and the extracts. In addition, the sulfhydral blocking reagents, *N*-ethylmaleimide and iodoacetamide, effectively inhibited proteolysis in both the cells and extracts. In addition, *o*-phenanthroline, a heavy metal chelator with a high affinity for zinc, inhibited protein breakdown in the cells and cell-free extracts. These results suggest the involvement of sulfhydryl-dependent enzyme(s) and metalloenzyme(s) in the degradative process.

DISCUSSION

A variety of observations indicate that the novel proteolytic system described here is responsible for the rapid degradation of abnormal proteins in reticulocytes: (*i*) the cell-free extracts, like intact cells, rapidly degrade analog-containing proteins and puromycyl-polypeptides but do not hydrolyze normal hemoglobin. (*ii*) Proteolysis in the cells and cell-free extracts is inhibited by the same agents. The fact that both chloromethyl ketones (TPCK and TLCK) and sulfhydryl blocking reagents (*N*-ethylmaleimide and iodoacetamide) inhibit this activity is consistent with a role for sulfhydryl protease(s) in the degradative process while the inhibition by *o*-phenanthroline also suggests a role of metalloenzyme(s). (*iii*) The breakdown of abnormal proteins in the extracts is stimulated by physiological concentrations of ATP which can account for the energy-dependence of degradation in reticulocytes. This ATP-dependent proteolytic system appears to be derived from the reticulocytes and not from contaminating cells, because varying the amount of white cell contamination did not alter the amount of ATP-dependent activity obtained, and lysates from mature red cells do not exhibit this activity (J. D. Etlinger & A. L. Goldberg, manuscript in preparation).

This degradative system differs in a number of respects from membrane-bound organelles, such as the lysosome. The ATP-dependent proteolytic activity is soluble at 100,000 × *g* even after prolonged centrifugation. Furthermore, no evidence for latency of this proteolytic activity was obtained with detergents which caused release of lysosomal enzymes in the same extracts (J. D. Etlinger & A. L. Goldberg, manuscript in preparation). In addition, the degradation of analog-containing proteins showed a pH optimum of 7.8, which is considerably higher than the acid optima characteristic of most lysosomal proteases (9, 19). In our studies, relatively little proteolytic activity was observed in the acidic range, although some activity could be detected in the presence of Triton X-100 (unpublished observation). Rapoport and coworkers (20) described a lysosomal proteolytic activity which decreases during the maturation of reticulocytes into erythrocytes, and suggested that this activity may be involved in the degradation of ribosomes, mitochondria, and other proteins which disappear during red cell development. Whether or not the degradative system described here may also be involved in such maturational changes remains to be established.

The nature of the apparent requirement for energy for intracellular protein breakdown is an important unsolved question. We have found that ATP, at physiological concentrations, can stimulate protein degradation severalfold in a dialyzed extract (Fig. 4). Other nucleoside triphosphates also stimulated proteolysis although the magnitude of their effects were consistently less than those of ATP (21). AMP and cAMP did not enhance protein breakdown (Table 1). The small stimulatory effects noted with ADP may be due to conversion of ADP to ATP in the extract. The experiments with ATP analogs suggested that cleavage of the terminal phosphate of ATP may be essential for the stimulation of protein degradation (Table 1).

Most previous hypotheses regarding the energy requirement have focused on some role of ATP in lysosomal functioning. However, because *E. coli* lacks lysosomes but also requires high energy phosphates for proteolysis (7), a more general role of ATP in protein degradation appears likely. It was also suggested that this ATP requirement might be secondary to an energy requirement for protein synthesis (2). However, our cell-free preparation lacks ribosomes and inhibition of protein synthesis with cyclohexamide did not affect the degradation of abnormal protein in reticulocytes. Because this degradative system appears soluble, the biochemical basis for the energy requirement is particularly intriguing. It seems unlikely that ATP is directly involved in proteolysis because no known protease requires high energy cofactors, although two unusual ATP-dependent reactions involving cleavage of peptide bonds have been described (22, 23). High energy phosphates may be involved in activation of the crucial protease(s). Alternatively, phosphorylation or some other chemical modification of the peptide substrates may serve to enhance their susceptibility to the degradative system although such abnormal proteins are already quite sensitive to proteolytic enzymes (24).

Preliminary studies suggest that a similar ATP-dependent system is present in *E. coli* and other mammalian cells (K. Murakami, R. Voellmy & A. L. Goldberg; J. D. Etlinger & A. L. Goldberg, manuscripts in preparation). We have utilized highly abnormal polypeptides for studying the degradative pathways because of the technical advantages which these short-lived proteins afford. However, this soluble degradative system may also be involved in hydrolysis of normal cell proteins. For example, the rate-limiting step in the degradation of

most proteins may be their intracellular denaturation or chemical modification (2, 4). If true, then once a protein becomes denatured, it may be hydrolyzed by the same degradative system which is involved in the breakdown of analog-containing proteins. If normal hemoglobin is stripped of its heme moieties, then the resulting globin becomes susceptible to the ATP-dependent degradative system (J. D. Etlinger & A. L. Goldberg, in preparation).

Studies in *E. coli* (2, 25) have suggested the existence of at least two proteolytic systems: one that degrades abnormal protein and certain normal proteins during growth; and another system responsible for the increased degradation of normal cell protein during poor nutritional conditions (2, 25). Multiple proteolytic systems which serve distinct physiological functions may also exist in mammalian cells (26). For example, there is growing evidence that lysosomes may be involved in the increased protein degradation (2), which occurs in response to lack of nutrients or insulin (27, 28), or to the presence of glucagon (29). Thus, the ATP-dependent degradative system described may serve physiological functions different from those of the lysosomal proteases.

Note Added in Proof. Preliminary reports of some of these findings have appeared previously (21) and in Goldberg, A. L., Kowit, J. D. & Etlinger, J. D. (1976) in *Proteolysis and Physiological Regulation*, eds. Ribbons, D. W. & Brew, K. (Academic Press, New York), pp. 313–337.

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