*Proc. Natl. Acad. Sci. USA*
Vol. 84, pp. 1829–1833, April 1987
Biochemistry

# Ubiquitin-aldehyde: A general inhibitor of ubiquitin-recycling processes

**(protein breakdown/isopeptidase/C-terminal hydrolase)**

AVRAM HERSHKO* AND IRWIN A. ROSE†

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111

*Contributed by Irwin A. Rose, October 24, 1986*

ABSTRACT **The generation and characterization of ubiquitin (Ub)-aldehyde, a potent inhibitor of Ub-C-terminal hydrolase, has previously been reported. We now examine the action of this compound on the Ub-mediated proteolytic pathway using the system derived from rabbit reticulocytes. Addition of Ub-aldehyde was found to strongly inhibit breakdown of added $^{125}$I-labeled lysozyme, but inhibition was overcome by increasing concentrations of Ub. The following evidence shows the effect of Ub-aldehyde on protein breakdown to be indirectly caused by its interference with the recycling of Ub, leading to exhaustion of the supply of free Ub: (*i*) Ub-aldehyde markedly increased the accumulation of Ub-protein conjugates coincident with a much decreased rate of conjugate breakdown. (*ii*) release of Ub from isolated Ub-protein conjugates in the absence of ATP (and therefore not coupled to protein degradation) is markedly inhibited by Ub-aldehyde. On the other hand, the ATP-dependent degradation of the protein moiety of Ub conjugates, which is an integral part of the proteolytic process, is not inhibited by this agent. (*iii*) Direct measurement of levels of free Ub showed a rapid disappearance caused by the inhibitor. The Ub is found to be distributed in derivatives of a wide range of molecular weight classes. It thus seems that Ub-aldehyde, previously demonstrated to inhibit the hydrolysis of Ub conjugates of small molecules, also inhibits the activity of a series of enzymes that regenerate free Ub from adducts with proteins and intermediates in protein breakdown.**

Current studies indicate that the polypeptide ubiquitin (Ub) has at least two types of cellular function. Ub plays a role in intracellular protein breakdown, in which conjugation with Ub commits proteins for degradation. The second role is modification of protein function, as seems to be the case in the attachment of Ub to certain histones. In both cases, Ub is linked to amino groups of proteins by way of its C terminus (for reviews, see refs. 1–3).

An essential feature in both processes is the necessity to regenerate free Ub. In the Ub-mediated proteolytic pathway, it is assumed that Ub is recycled following proteolysis, even though this step has not yet been investigated primarily because the intermediates in the process have not been available. In functions of protein modification, the release of Ub is required for reversal of modification.

The enzymes that carry out the recycling of Ub are not well characterized. The best studied of these is a Ub-C-terminal hydrolase, which cleaves the linkage of Ub to a variety of compounds such as thiols, amines, and acyl phosphates (4–6). The hydrolase acts only on adducts of Ub with small compounds and not on Ub-protein conjugates (5). It has been proposed that the function of this enzyme is to release Ub from adducts generated by the attack of thiols or amines on enzyme-Ub thiolesters, which are intermediates in the formation of Ub-protein conjugates (7), and that it may also be responsible for the liberation of Ub from end products of the proteolytic pathway (4, 5). Other enzymes that release Ub from proteins (termed isopeptidases or Ub-protein lyases)‡ seem to be abundant in crude extracts (8). An enzyme able to release Ub from its conjugate with histone has been described and partially purified (9–12). It is not clear whether or not this enzyme is specific for the histone conjugate. At least three other Ub-protein lyases were observed in reticulocyte extracts, with apparent molecular weights of 100,000, 300,000 and 450,000 (E. Leshinsky and A.H., unpublished results). It seems, therefore, that many enzymes, probably of differing specificities, may be necessary for Ub regeneration from its various adducts.

One approach to define the processes of Ub recycling is to use specific inhibitors. During studies on the mechanism of Ub-C-terminal hydrolase it was found that borohydride inactivates the enzyme in the presence of Ub (6). This is due to the generation of Ub-aldehyde, in which the C terminus of Ub is reduced to the aldehyde oxidation state. Ub-aldehyde inhibits the hydrolase by the formation of an extremely tight complex, in which the inhibitor is presumably bound to the active site in a manner analogous to the tetrahedral intermediate of catalysis (6). In the present investigation we have examined the effects of Ub-aldehyde on the proteolytic system of rabbit reticulocytes and find that it inhibits most avenues of Ub recycling.

## METHODS

Fraction II from rabbit reticulocytes was prepared and subjected to affinity chromatography on Ub-Sepharose as described (7). The fraction not adsorbed to Ub-Sepharose (the "affinity-unadsorbed" fraction) was passed again though the affinity column for a more complete removal of Ub-conjugating enzymes. Ub-C-terminal hydrolase was prepared from human erythrocytes by DEAE-cellulose chromatography, treatment with trichloroacetic acid, and affinity chromatography on Ub-Sepharose as described (large scale method of ref. 5). In some preparations, adsorption of the enzyme to Ub-Sepharose was poor due to residual free Ub. In such cases, the trichloroacetic acid-treated preparation (≈900 mg of protein) was applied again to a 200-ml column of DE-52 (Whatman) equilibrated with 3 mM potassium phosphate, pH 7.0/20 mM KCl/1 mM dithiothreitol, and free Ub

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Ub, ubiquitin; $^{125}$I-Ub, $^{125}$I-labeled ubiquitin; $^{125}$I-lysozyme, $^{125}$I-labeled lysozyme; Ox-RNase, performic acid-oxidized RNase; Me-Ub, reductively methylated ubiquitin; MetO-RNase, RNase whose methionine residues had been oxidized to sulfoxides.

*Present address: Unit of Biochemistry, Faculty of Medicine, Technion–Israel Institute of Technology, Hafia, Israel.
†To whom reprint requests should be addressed at: Institute for Cancer Research, 7701 Burholme Avenue, Philadelphia, PA 19111.
‡These are, of course, also Ub-C-terminal hydrolases, but the terminology in usage will continue to be used.

was removed by washing the column with 1 liter of the above buffer. The enzyme was eluted under conditions identical to those described for the preparation of fraction II (7). Following this procedure, essentially all Ub-C-terminal hydrolase was bound to Ub-Sepharose and isolated by the procedure described earlier (5).

**Preparation of Ub-Aldehyde.** Ub-aldehyde was prepared by borohydride reduction of Ub in the presence of Ub-C-terminal hydrolase, a modification of the previously described procedure (6). The reaction mixture contained (in a volume of 1 ml) 25 mM Tris·HCl (pH 8.5), 0.2 mM EDTA, 2 mg of ovalbumin, 5.0 nmol of hydrolase, and 30.0 nmol of Ub. $^{125}$I-labeled Ub ($^{125}$I-Ub) (450,000 cpm) was added to facilitate the detection of reaction products. The mixture was incubated at 37°C while five 30-μl portions of 0.1 M sodium borohydride were added at 30-min intervals. The mixture was diluted with 2 ml of water and was applied to a column (1 × 9 ml) of DE-52 equilibrated with 10 mM potassium phosphate, pH 7.4/50 mM KCl/1 mM 2-mercaptoethanol. The column was eluted with a 240-ml gradient of 50–350 mM KCl in the above buffer. Fractions of 3 ml were collected, and the positions of free Ub and of the Ub-aldehyde·hydrolase complex were determined by gamma counting. Free Ub is not adsorbed to the resin and is removed in the first 10 fractions while Ub-aldehyde elutes with the hydrolase at ≈170 mM KCl. The peak fractions of the Ub-aldehyde·hydrolase complex were collected and concentrated by a CF-25 Centriflo cone (Amicon) in the presence of 2 mg of ovalbumin. Salt was removed by two 10-fold dilutions with 10 mM potassium phosphate (pH 7.4) followed by concentration in the same cone. The yield of the preparation was ≈80% of the amount of hydrolase used. The concentration of Ub-aldehyde was calculated by the known specific radioactivity of $^{125}$I-Ub used for its preparation. The Ub-aldehyde·hydrolase complex was kept at −70°C and was stable for a period of several months. Before use, the preparation was heated at 75°C for 10 min to inactivate the enzyme and thus to liberate free Ub-aldehyde. Control experiments showed that all the effects of Ub-aldehyde reported in this paper required the above heat treatment.

**Determination of Ub.** Levels of free and active Ub were determined as described (13), by the formation of [$^3$H]AMP-Ub from [$^3$H]ATP, catalyzed by the Ub-activating enzyme $E_1$. The assay mixture was (in a volume of 20 μl) 50 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/0.3 mM EDTA containing bovine serum albumin (2 mg/ml), yeast inorganic pyrophosphatase (0.06 unit, Sigma), 10 pmol of [2,8-$^3$H]ATP (ICN; 30 Ci/mmol; 1 Ci = 37 GBq), 2 pmol of $E_1$, and up to 1 pmol of Ub. $E_1$ was isolated by covalent affinity chromatography (14), and contaminating Ub was removed by a small column of DEAE-cellulose as described for another enzyme, $E_3$ (15). Prior to use, $E_1$ was treated with 5 mM iodoacetamide (37°C for 10 min) to inactivate its thiol site (16). Iodoacetamide treatment was terminated with 5 mM dithiothreitol. The assay mixtures were incubated at 37°C for 15 min. Then AMP-Ub was precipitated with 1 ml of 12% (vol/vol) trichloroacetic acid to which 100 μg of bovine serum albumin was then added. The samples were allowed to stay on ice for 5 min and then were centrifuged at 12,000 × *g* for 5 min. The supernatant was thoroughly removed, and the tubes were rinsed with 1 ml of 2% (vol/vol) trichloroacetic acid without resuspending the pellet. The pellets were dissolved in 0.1 ml of 0.1 M NaOH, and their radioactivity was determined in a liquid scintillation counter. Blank values (incubations without added Ub) were subtracted, and the amount of Ub was calculated by the known specific radioactivity of [$^3$H]ATP (usually, 10–12 × $10^3$ cpm/pmol). Under these conditions, the linear range of the assay was 0.05–1 pmol of Ub. All dilutions of Ub-containing samples were done with bovine serum albumin (2 mg/ml) to prevent nonspecific adsorption.

Since the assay relies on the specific radioactivity of [$^3$H]ATP, it was important to remove unlabeled ATP from samples prior to the assay. This was achieved by precipitation of the sample with 12% (vol/vol) trichloroacetic acid in the presence of 200 μg of bovine serum albumin and rinsing with 2% (vol/vol) trichloroacetic acid, as described above for the isolation of [$^3$H]AMP-Ub. The samples were dissolved in 100 μl of 50 mM Tris·HCl (pH 9.0), and the trichloroacetic precipitation procedure was repeated. Recovery of Ub in this procedure was 80–90%.

## RESULTS AND DISCUSSION

**Influence of Ub-Aldehyde on Protein Breakdown and Accumulation of Ub-Protein Conjugates.** In the experiment shown in Fig. 1, the effect of Ub-aldehyde on the ATP/Ub-dependent degradation of $^{125}$I-labeled lysozyme ($^{125}$I-lysozyme) was examined. Ub-aldehyde strongly inhibited protein breakdown when the reaction was carried out at low concentrations of Ub (0.5–1 μM). However, inhibition was overcome by increasing the concentration of Ub. In different experiments, there was some variation with regard to the concentrations of Ub required to abolish the inhibition of Ub-aldehyde; the effective concentrations were in the range of 3–10 μM Ub.

The above observations may be explained by two hypotheses. One possibility is that at high concentrations, Ub displaces Ub-aldehyde from a common site on an inhibited enzyme. Another explanation is that Ub-aldehyde inhibits some enzyme(s) that releases Ub from adducts with proteins or smaller compounds. In the latter case, lysozyme degradation would be inhibited as the supply of free Ub is exhausted. Trying to distinguish between these possibilities, the effects of Ub-aldehyde on the formation and breakdown of Ub-protein conjugates, was examined.

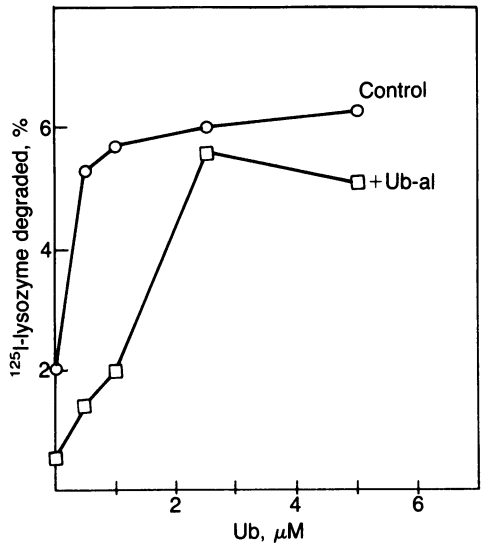


FIG. 1. Inhibition of protein breakdown by Ub-aldehyde and its reversal by increased concentrations of Ub. The reaction mixture contained (in a volume of 15 μl) 50 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/2 mM dithiothreitol/4 mM ATP, approximately 50 μg of protein of fraction II from reticulocytes, and Ub as indicated. Where indicated, Ub-aldehyde (+Ub-al) was supplemented at 1 μM. The mixtures were incubated at 37°C for 20 min; then the reaction was initiated by the addition of $^{125}$I-lysozyme (2 × $10^5$ cpm, ≈0.5 μg). Following an additional incubation for 60 min at 37°C, the amount of radioactivity soluble in 15% (vol/vol) trichloroacetic acid was determined. The amount of acid-soluble radioactivity determined in the absence of ATP (1.3% of the total radioactivity) was subtracted from all results.

The effect of Ub-aldehyde on the conjugation of $^{125}$I-Ub to endogenous reticulocyte proteins or to exogenous protein substrates is shown in Fig. 2. The enzyme source used was crude fraction II from reticulocytes (rather than purified Ub-conjugating enzymes) to allow comparison with experimental conditions used for estimation of protein breakdown. A dramatic increase in the concentration of Ub-protein conjugates was observed. Without added exogenous proteins, the main derivatives of $^{125}$I-Ub that accumulated under the influence of Ub-aldehyde were a 14-kDa adduct and some higher molecular weight conjugates. With some added protein substrates, such as performic acid-oxidized RNase (Ox-RNase) or RNase whose methionine residues had been oxidized to sulfoxides (MetO-RNase) (17), Ub-aldehyde caused a similar pronounced increase in the accumulation of their respective derivatives of Ub. On the other hand, much less effect of Ub-aldehyde on the accumulation of Ub conjugates of lysozyme was observed. In the latter case, the main effect of Ub-aldehyde was a shift to higher molecular weight products (Fig. 2, lanes D).

One possible explanation of these results is that Ub-aldehyde inhibits the action of some Ub-releasing enzyme(s), thus causing the accumulation of Ub-protein conjugates. In such a case, it will have to be further assumed that conjugates of lysozyme with Ub are less subject to the action of these enzymes than conjugates of RNase derivatives. That the effect of Ub-aldehyde on the accumulation of Ub-protein conjugates may indeed be due to the inhibition of Ub-releasing enzymes is suggested by the time course of accumulation of Ub-protein conjugates in the presence or absence of this compound (Fig. 3). Without Ub-aldehyde, conjugate accumulation ceases at a low steady-state level, while in the presence of the inhibitor, conjugate accumulation proceeds at a nearly linear fashion. The concentrations of Ub-aldehyde required for the effect on conjugate accumulation were rather low. Half-maximal effect was obtained at ≈0.2 μM, and maximal effect occurred at 0.5–1 μM Ub-aldehyde (data not shown).

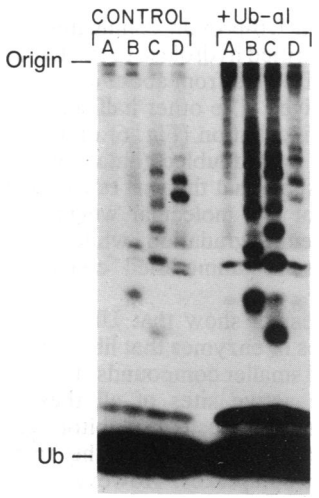


FIG. 2. Influence of Ub-aldehyde on the formation of Ub-protein conjugates. The reaction mixture contained (in a volume of 20 μl) 50 mM Tris·HCl, pH 7.6/2.5 mM $MgCl_2$/2 mM dithiothreitol/1 mM ATP/3 μM $^{125}$I-Ub (approximately 2.5 × $10^5$ cpm) and 15 μg of protein from fraction II, which had been previously incubated at 37°C for 20 min in the absence (CONTROL) or presence (+Ub-al) of 7.5 pmol of Ub-aldehyde. The following protein substrates (2 μg) were added: none (lanes A), Ox-RNase (lanes B), MetO-RNase (lanes C), lysozyme (lanes D). Following incubation at 37°C for 30 min, the samples were electrophoresed on a 12.5% $NaDodSO_4$/polyacrylamide gel.

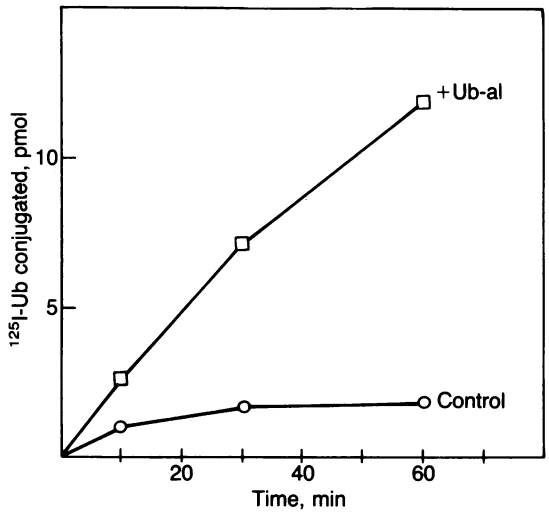


FIG. 3. Time course of accumulation of Ub-protein conjugates in the presence of Ub-aldehyde (+Ub-al). Reaction conditions were similar to those described for Fig. 2, except that the reaction was carried out in the presence of 10 μg of Ox-RNase (10 μg) and was stopped at the times indicated. Following polyacrylamide gel electrophoresis, the amount of Ub-protein conjugates formed was estimated by cutting out the entire length of the gel and subjecting it to gamma counting.

**Ub-Aldehyde Inhibits ATP-Independent, But Not ATP-Dependent, Pathways of Breakdown of Ub-Protein Conjugates.** Previous studies have shown that Ub-protein conjugates are degraded by two alternative pathways in reticulocyte extracts: an ATP-dependent pathway, which attacks the protein substrate moiety, and ATP-independent Ub-protein lyases (presumably, mostly isopeptidases), which cleave the amide linkage and release undegraded protein and free Ub (8, 18, 19). To examine which of these is inhibited by Ub-aldehyde, isolated conjugates of $^{125}$I-lysozyme were incubated in the presence or absence of ATP with the fraction of reticulocyte proteins that is retained by DE-52 and not retained by Ub-Sepharose. This fraction is capable of both ATP-dependent breakdown of conjugates and the hydrolysis of conjugates at the isopeptide bond. For the preparation of the conjugates, we used reductively methylated Ub (Me-Ub), in which all amino groups are blocked (15). Conjugates of Me-Ub are simpler to analyze, as they do not contain poly-Ub chains, yet they are subject to the action of the conjugate-degrading enzyme systems (15). These conjugates were derived from an incubation containing the enzymes required for conjugation but lacking the degradative enzymes (15). Analysis of the reaction products by $NaDodSO_4$/polyacrylamide gel electrophoresis is shown in Fig. 4, and quantitation of the results is shown in Fig. 5. Without the inhibitor, the previously observed pattern is seen (8)—i.e., rapid decay of high molecular weight conjugates in the presence or absence of ATP, increase of free $^{125}$I-lysozyme (mainly in the absence of ATP), and stimulation of the formation of acid-soluble products by ATP. The degradation occurring in the absence of ATP was attributed to the action of Ub-protein lyases, while that taking place in the presence of ATP was interpreted to represent the latter part of the proteolytic pathway as well as the lyase pathway (8). In the presence of Ub-aldehyde, the decay of high molecular weight conjugates in the absence of ATP is nearly completely prevented (Figs. 4 and 5). Correspondingly, there is much less of an increase in the levels of free $^{125}$I-lysozyme. On the other hand, the ATP-dependent degradation of high molecular weight conjugates to acid-soluble products is not affected significantly by Ub-aldehyde (Fig. 5). These results show that Ub-

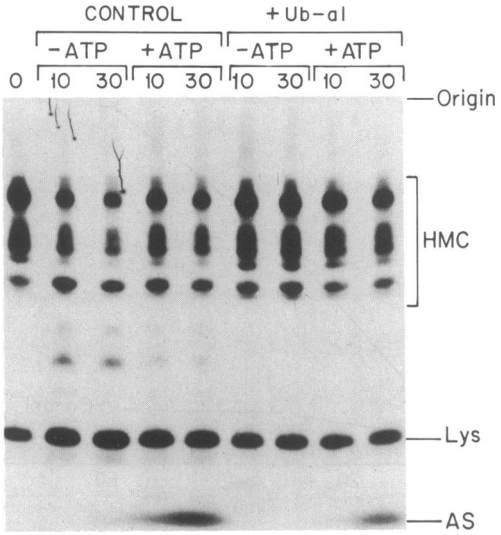


FIG. 4. Influence of Ub-aldehyde on the breakdown of $^{125}$I-lysozyme-Me-Ub conjugates in the presence or absence of ATP. Reaction mixtures contained (in a volume of 20 μl) 50 mM Tris·HCl pH 7.6/5 mM $MgCl_2$/2 mM dithiothreitol, 12,800 cpm of $^{125}$I-lysozyme-Me-Ub conjugates, and 17 μg of the affinity-unadsorbed fraction. $^{125}$I-lysozyme-Me-Ub conjugates were prepared as described (15) by the reaction of $^{125}$I-lysozyme with unlabeled Me-Ub. The affinity-unadsorbed fraction is the part of reticulocyte fraction II, which does adsorb to Ub-Sepharose and which contains all enzymes which carry out the degradation of Ub-protein conjugates (8, 15). The affinity-unadsorbed fraction was pretreated with 10 pmol of Ub-aldehyde (+Ub-al) for 20 min at 37°C. Where indicated, ATP was supplemented at 4 mM. Numbers at the top indicate the incubation times (min) at 37°C. Following gel electrophoresis (12.5% polyacrylamide) the gel was radioautographed without staining or fixation to prevent the loss of acid-soluble products. HMC, high molecular weight conjugates; Lys, free lysozyme; AS, acid-soluble degradation products.

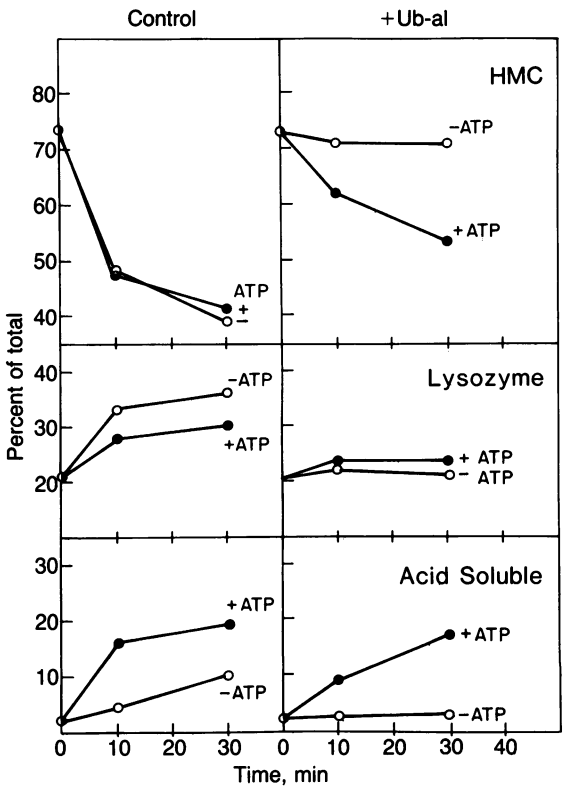


FIG. 5. Ub-aldehyde inhibits ATP-independent but not ATP-dependent degradation of Ub-protein conjugates. The gel regions marked in Fig. 4 were cut out and quantitated by gamma counting. The results are expressed as the percentage of total radioactivity. (*Left*) Control (without Ub-aldehyde). (*Right*) +Ub-al (with Ub-aldehyde). (*Top*) HMC (high-molecular-weight conjugates). (*Middle*) Lysozyme (free lysozyme). (*Bottom*) Acid-Soluble (acid-soluble degradation products).

aldehyde specifically inhibits Ub-protein lyases, while ATP-dependent proteolysis, acting on the same conjugates, is not affected by this agent. The immediate source of the acid-soluble radioactivity in the presence and absence of ATP remains to be determined.

**Inhibition of Ub-Recycling Processes by Ub-Aldehyde.** Since neither the formation of Ub-protein conjugates nor their ATP-dependent degradation is inhibited by Ub-aldehyde, the above results suggest that the inhibition of protein breakdown by this compound (Fig. 1) is not due to a direct effect on the enzymes responsible for the ATP/Ub-dependent conversion of protein to amino acids. Rather, it seems to be the consequence of interference with processes responsible for the regeneration of free Ub from protein conjugates. This interpretation is supported by the direct measurement of free Ub levels (Fig. 6). The supplementation of reticulocyte fraction II with ATP caused some decrease of free Ub in the initial 30 min. Thereafter, free Ub levels remained essentially constant, presumably due to a steady state between processes of Ub utilization and regeneration. Further addition of Ub-aldehyde caused a drastic decline in free Ub levels within 30 min and a continuing disappearance with prolonged incubation. This shows a strong and generalized inhibition of Ub-recycling processes.

We also asked what classes of Ub derivatives accumulate under these conditions. It seemed reasonable to assume that in addition to Ub-protein conjugates, adducts of Ub with smaller compounds (such as amines or thiols) may accumulate in extracts treated with Ub-aldehyde. In addition, since proteolytic breakdown of Ub-protein conjugates should continue without free Ub, putative intermediary products of Ub-dependent proteolysis (possibly small peptides still linked to Ub by lysine residues) may also accumulate under these conditions. The enzyme Ub-C-terminal hydrolase acts on Ub derivatives with low molecular weight compounds, but it does not act on Ub-protein conjugates (5). Addition of a large excess of purified hydrolase to the Ub-aldehyde-treated extract released free Ub from about one-half of the accumulated Ub derivatives. The other half was not released even upon prolonged incubation (Fig. 6) or when the amount of added hydrolase was doubled (data not shown). It seems reasonable to assume that the part released by the hydrolase is a population of low molecular weight adducts, possibly products of protein degradation, while the part resistant to hydrolase represents unmodified conjugates of Ub with proteins.

The present results show that Ub-aldehyde inhibits the activity of a series of enzymes that liberate Ub from linkages with proteins and smaller compounds. It seems reasonable to assume that the active sites of all these enzymes share common features, and thus the inhibitor acts in all cases by a common mechanism involving the formation of tight enzyme–inhibitor complexes. However, not all Ub-protein lyases are inhibited by Ub-aldehyde. When isolated $^{125}$I-Ub-Ox-RNase conjugates were incubated with reticulocyte fraction II in the presence of increasing concentrations of Ub-aldehyde and the release of free $^{125}$I-Ub was monitored, a small fraction of lyase activity remained insensitive to even high concentrations of Ub-aldehyde (data not shown). An indication for Ub-aldehyde-insensitive enzyme in preparations of the hydrolase was observed in studies of recycling of Ub from Ub-dithiothreitol thiolester (6).

It is noteworthy that in spite of the large increase in the concentration of Ub-protein conjugates caused by Ub-alde-

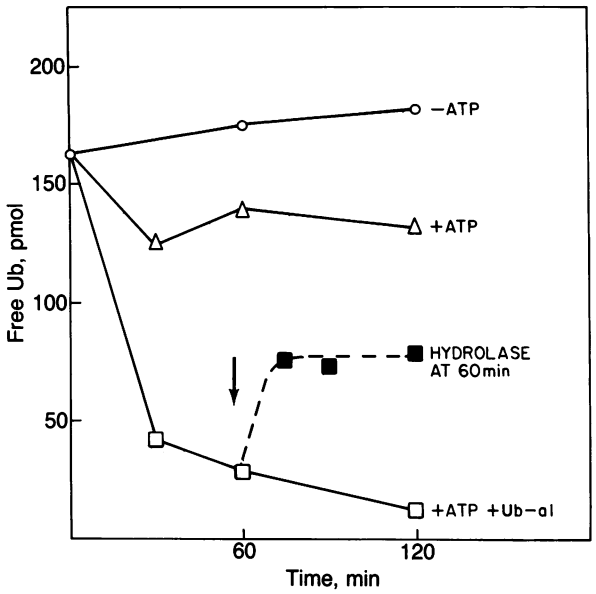


FIG. 6. Effects of Ub-aldehyde and of Ub-C-terminal hydrolase on levels of free Ub. Reaction conditions were similar to those described for Fig. 1 except that Ub was supplemented at 200 pmol (13.3 μM) in all cases. Where indicated, ATP (0.2 mM) was added together with creatine phosphate (10 mM) and creatine phosphokinase (5 μg/ml). Ub-aldehyde (Ub-al) was supplemented at 1.3 μM. Where indicated, purified Ub-C-terminal hydrolase (hydrolase) was added at 4.3 μM, following incubation with Ub-aldehyde for 60 min (arrow). Levels of free Ub were determined as described in *Methods*.

hyde under contitions of excess Ub (Figs. 2 and 3), the rate of protein breakdown is not accelerated. This suggests that the proteolysis of Ub-conjugated proteins is strongly rate-limiting or that the Ub-protein conjugates that accumulate under the influence of the inhibitor are either not correct substrates for proteolysis or that they have already been diminished by proteolysis to limit structures. These limit structures may have high molecular weights due to poly(Ub) chains known to be present in protein conjugates that are well degraded (15). It has been proposed that Ub-protein lyases have a correction role to undo unproductive conjugations that occur during the processing of proteins for degradation (20). According to this notion, the Ub-protein conjugates that accumulate when correction is inhibited are incorrectly conjugated for processing by the Ub-dependent protease system. Reports of alternative mechanisms for Ub-protein conjugate formation (21, 22) provide avenues in addition to the one originally envisaged. It remains to be seen what distinguishes "correct" and "incorrect" Ub-protein conjugates. In addition, enzymes that are important for recycling of proteins that are "futilely" conjugated to Ub remain to be identified.

The specificity of action of Ub-aldehyde renders it a valuable tool not only for the investigation of Ub-recycling processes but also for studies on the mechanisms of protein breakdown and other functions of Ub. Studies on the mode of degradation of Ub-conjugated proteins and on the role of ATP in this process (8, 19) have been hampered by the presence of Ub-protein lyases in the partially purified enzyme preparations. Ub conjugates of some specific proteins may have been difficult to detect due to their low steady-state levels. The use of Ub-aldehyde may facilitate such studies.

This study was performed during the stay of A.H. at the Institute for Cancer Research, Philadelphia. This work was supported by American Cancer Society Grant BC-414 to I.A.R., National Institutes of Health Grants CA-06927 and RR-05539, and appropriations from the Commonwealth of Pennsylvania to the Institute for Cancer Research. A.H. also acknowledges support of United States Public Health Service Grant AM-25614 and a grant from the United States–Israel Binational Science Foundation.

1. Hershko, A. & Ciechanover, A. (1982) *Annu. Rev. Biochem.* **51**, 335–364.
2. Finley, D. & Varshavsky, A. (1985) *Trends Biochem. Sci.* **10**, 343–347.
3. Hershko, A. & Ciechanover, A. (1986) *Prog. Nucleic Acid Res. Mol. Biol.* **33**, 19–59.
4. Rose, I. A. & Warms, J. V. B. (1983) *Biochemistry* **22**, 4234–4237.
5. Pickart, C. M. & Rose, I. A. (1985) *J. Biol. Chem.* **260**, 7903–7910.
6. Pickart, C. M. & Rose, I. A. (1986) *J. Biol. Chem.* **261**, 10210–10217.
7. Hershko, A., Heller, H., Elias, S. & Ciechanover, A. (1983) *J. Biol. Chem.* **258**, 8206–8214.
8. Hershko, A., Leshinsky, E., Ganoth, D. & Heller, H. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1619–1623.
9. Andersen, M. W., Ballal, N. R., Goldknopf, I. L. & Busch, H. (1981) *Biochemistry* **20**, 1100–1104.
10. Andersen, M. W., Goldknopf, I. L. & Busch, H. (1981) *FEBS Lett.* **132**, 210–214.
11. Matsui, S. I., Sandberg, A., Negoro, S., Seon, B. K. & Goldstein, G. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1535–1539.
12. Kanda, F., Matsui, W. I., Sykes, D. E. & Sandberg, A. A. (1984) *Biochem. Biophys. Res. Commun.* **122**, 1296–1306.
13. Rose, I. A. & Warms, J. V. B. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1477–1481.
14. Ciechanover, A., Elias, S., Heller, H. & Hershko, A. (1982) *J. Biol. Chem.* **257**, 2537–2542.
15. Hershko, A. & Heller, H. (1985) *Biochem. Biophys. Res. Commun.* **128**, 1079–1086.
16. Haas, A. L., Warms, J. V. B., Hershko, A. & Rose, I. A. (1982) *J. Biol. Chem.* **257**, 2543–2548.
17. Hershko, A., Heller, H., Eytan, E. & Reiss, Y. (1986) *J. Biol. Chem.* **261**, 11992–11999.
18. Hough, R. & Rechsteiner, M. (1986) *J. Biol. Chem.* **261**, 2391–2399.
19. Hough, R., Pratt, G. & Rechsteiner, M. (1986) *J. Biol. Chem.* **261**, 2400–2408.
20. Hershko, A., Ciechanover, A., Heller, H., Haas, A. L. & Rose, I. A. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1783–1786.
21. Pickart, C. M. & Rose, I. A. (1985) *J. Biol. Chem.* **260**, 1573–1581.
22. Lee, P. L., Midelfort, C. F., Murakami, K. & Hatcher, V. B. (1986) *Biochemistry* **25**, 3134–3138.