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Ubiquitin metabolism in *Chlamydomonas reinhardtii*

A Thesis

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Abstract

This work characterizes parameters of ubiquitin metabolism in *Chlamydomonas reinhardtii* Dangeard growing under constant conditions and after an exposure to cold shock. Ratio of free and conjugated ubiquitin to total protein, and rate constant of ubiquitin synthesis and conjugation increased about two-fold during first 4 hours after cold treatment, whereas rate constant of ubiquitin degradation reached its maximum 9 hours after treatment. Half-life of ubiquitin calculated from the constant of degradation decreased from 6 hours to 3.5 hours during first four hours after the cold treatment. Rate constant of ubiquitin deconjugation did not change after cold treatment. Ratio of free to conjugated ubiquitin decreased temporarily to approx. 8 immediately after cold treatment and raised back to its original value at 2 h after cold treatment. These observations raise questions regarding the regulatory mechanisms of ubiquitin synthesis and hydrolysis.

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I would like to thank professor Malek for his help with this project and professors Omeljaniuk and Weir for valuable suggestions during writing the final copy of my thesis. I also thank Tina McIntosh and Karen Watt for keeping me a company during the two years I spent in this lab. Taky musím poděkovat rodičům za to že to beze mě ty dva roky vydrželi.

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List of Abbreviations

AA	amino acid(s)
ATP	adenosine 5'-(tetrahydrogen triphosphate)
BSA	bovine serum albumin
CA	carbonic anhydrase
cpm	counts per minute
EDTA	N,N'-1,2-ethanediylbis[N-(carboxy-methyl) glycine]
EM	electron microscopy
EMI	N-ethyl-maleimide
FIA	fluorescence immunoassay
GF	gel filtration
HS medium	Sueoka high-salt medium
IgG	immunoglobulin G
k_C	rate constant of ubiquitin conjugation
k_D	rate constant of ubiquitin degradation
k_{DC}	rate constant of ubiquitin deconjugation
k_S	rate constant of ubiquitin synthesis
MW	molecular weight
NSB	non-specific binding
PBS	phosphate-buffered saline
PMSF	phenylmethylsulfonyl fluoride

probit	percentage points of the probability distribution
R_C	rate of ubiquitin conjugation
R_D	rate of ubiquitin degradation
R_{DC}	rate of ubiquitin deconjugation
R_S	rate of ubiquitin synthesis
SDS	n-dodecyl sulfate sodium salt
Staph-A	*Staphylococcus aureus* Cowan I strain
TBS	Tris-buffered saline
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Ub	ubiquitin
Ub-C	ubiquitin-protein conjugate
Ub-F	ubiquitin-fluorescein conjugate
v/v	volume per volume
w/w	weight per weight
w/v	weight per volume

1. Introduction

It has been known since the early 1940's that proteins in living cells are in a state of constant turnover (Vickery *et al.* 1940, Schoenheimer 1942). Protein turnover has two components: synthesis of protein molecules and their degradation into constituent amino acids. While there is a considerable body of knowledge about processes leading from information stored in nucleic acids to protein molecules, less attention has been paid to mechanisms of protein degradation. Perhaps the best studied cytosolic pathway responsible for dismemberment of polypeptides is the ubiquitin pathway (Ciechanover 1994), in which protein substrates are first covalently linked to one or many molecules of 76 amino acid protein ubiquitin in an ATP-dependent manner. Proteins tagged with multiubiquitin chain(s) are then specifically degraded by an ATP-dependent 26S proteasome complex.

Proteolysis plays an essential role in the cell response to stress conditions such as extreme temperature and pH, UV radiation (Hilt & Wolf 1992), microinjection of denaturated proteins (Ananthan *et al.* 1986), introduction into bacteria of a vector generating large amounts of foreign protein (Goff & Goldberg 1985), and 'poisoning' with heavy metals or amino acid analogues (Goldberg 1972). Abnormal proteins arising as a result of stress conditions (Finley *et al.* 1984, Kabakov & Gabai 1993) can be channelled into ubiquitin pathway for protein degradation (Hershko & Ciechanover 1982, Parag *et al.* 1987). Ubiquitin itself has been shown to be one of the heat shock proteins, a group of proteins that are synthesized as a part of the cellular response to stress (Parsell & Lindquist 1993).

One of the possible stressful conditions a cell can encounter is a temperature shock, an exposure to extreme temperature. Involvement of the ubiquitin system in cellular responses to heat shock has been well established in yeast (Finley *et al.* 1987), plants (Ferguson *et al.* 1990)

and animals (Niedzwiecki & Fleming 1993). Enzymes of the ubiquitin-conjugating system have been identified as responsible for cell survival at elevated temperature (Ciechanover *et al.* 1984, Finley *et al.* 1984, Seufert & Jentsch 1990, Gropper *et al.* 1991). One of the endogenous inhibitors of the 20S proteasome has been recognised as heat shock protein HSP90 (Tsubuki *et al.* 1994) and this proteolytic complex has been found essential for stress-induced proteolysis (Hilt *et al.* 1993). Increased transcription of ubiquitin genes as a reaction to heat shock has been observed (Finley *et al.* 1987, Burke *et al.* 1988, Shimogawara & Muto 1989, Ferguson *et al.* 1990, Binet *et al.* 1991, Christensen *et al.* 1992, Garbarino *et al.* 1992, Genschik *et al.* 1992, Niedzwiecki & Fleming 1993, Schiedlmeier & Schmitt 1994), as well as changes in cellular content of ubiquitin and ubiquitin conjugation to protein substrates (Shimogawara & Muto 1989, Ferguson *et al.* 1990, Wettern *et al.* 1990, Niedzwiecki & Fleming 1993). However, less attention has been paid to changes of ubiquitin system following cold shock (Gindin & Borochov 1992).

To investigate low temperature function of the ubiquitin system, *Chlamydomonas reinhardtii* Dangeard (a unicellular aquatic photosynthetic eukaryote) was chosen as a model organism. Availability of mutants of this organism defective in cell cycle control (Harris 1989) makes it a suitable model for study of role of ubiquitin system in cell cycle regulation and disruption of the cell cycle by changes in the environment. This work is a first step in such an effort.

The purpose of this study is to determine whether ubiquitin turnover in *Chlamydomonas reinhardtii* Dangeard changes after an exposure to a cold shock. Rate constants of ubiquitin synthesis, degradation, conjugation, and deconjugation have been determined under constant conditions and after an exposure of cells to low non-freezing temperature.

1.1. Ubiquitin system for protein degradation

1.1.1. Ubiquitin

Ubiquitin was first isolated (Goldstein *et al.* 1975) and sequenced (Schlessinger *et al.* 1975) as a lymphocyte differentiation promoting factor. Thereafter a histone H24A was found to be a covalent complex of histone H2A and a ubiquitin molecule (Goldknopf & Busch 1975, Goldknopf *et al.* 1975), joined through an isopeptide linkage between ε-amino group of histone's Lys and carboxyl-terminal Gly of ubiquitin (Goldknopf & Busch 1977). In another line of work, rabbit reticulocytes were found to contain an ATP-dependent proteolytic system (Etlinger & Goldberg 1977), which required an 'ATP-Dependent Proteolysis Factor I' - APF-I (Ciechanover *et al.* 1978, Hershko *et al.* 1979). APF-I formed covalent compounds with proteolytic substrates in an ATP-dependent manner (Ciechanover *et al.* 1980, Hershko *et al.* 1980) and was subsequently identified as ubiquitin (Wilkinson *et al.* 1980).

Ubiquitin and ubiquitin genes have been found in all eukaryotic cells examined to date (Vierstra 1993) and in several viruses (Dunigan *et al.* 1988, Guarino 1990, Russell & Rohrmann 1993, Tautz *et al.* 1993). Ubiquitin, but not its coding sequence, has recently been reported in an archaebacterium (Wolf *et al.* 1993b). Early reports of presence of ubiquitin in eubacteria (Goldstein *et al.* 1975) have not been confirmed yet.

When first sequenced, ubiquitin was determined to consist of 74 amino acids, with arginyl as the carboxyl-terminal residue (Schlessinger *et al.* 1975). In contrast, the sequence of ubiquitinated histone H2A contained an intervening glycine dipeptide between lysyl residue of the histone and arginyl of the ubiquitin (Goldknopf & Busch 1977). Physiologically active ubiquitin contains the carboxyl-terminal sequence Arg-Gly-Gly (Wilkinson & Audhya 1981) and that the terminal glycine dipeptide is sensitive to proteolytic cleavage by an *in vivo* present protease (Haas *et al.* 1985, Vierstra *et al.* 1985). Ubiquitin is perhaps the most conserved protein detected to date. Its amino acid sequence is identical among all higher-plant species examined and is

distinguished from the *Chlamydomonas*, the yeast, and the invariant mammalian ubiquitin sequence by only one-, two-, and three-amino acid substitutions respectively (Vierstra 1993).

X-ray studies revealed ubiquitin having a simple architecture rich in secondary structure, including a five-strand β-sheet with three antiparallel and one parallel pairs of strands, an α-helix (residues 23-34), a short 3_{10} helix (residues 56-59), and seven reverse turns (Vijay-Kumar *et al.* 1987). Ubiquitin shares an unusual crossover motif of the outer strands of the β-sheet and the central α-helix with G-protein (Kraulis 1991). The curved β-sheet and the flanking α-helix enclose a single core of densely packed hydrophobic side chains, which is likely to contribute to the high stability of ubiquitin toward denaturation by heat, extremes of pH, and denaturing agents (Lenkinski *et al.* 1977, Briggs & Roder 1992). Its thermal stability has been exploited for its isolation (Goldstein *et al.* 1975, Vierstra *et al.* 1985). In spite of its small size, ubiquitin can fold into a very stable globular structure without relying on disulphide bonds, metal binding sites, or prosthetic groups for structural stabilization (Khorasanizadeh *et al.* 1993), which makes it an attractive model system for protein folding studies (Pan & Briggs 1992, Katta & Chait 1993, Khorasanizadeh *et al.* 1993, Woolfson *et al.* 1993).

Presence of ubiquitin and a complex pattern of ubiquitin conjugates has been revealed in the cytosol and the nucleus of mammalian (Haas & Bright 1985), plant (Beers *et al.* 1992), and algal (Wettern *et al.* 1990) cells and ubiquitin is apparently a component of mammalian cytoskeleton (Murti *et al.* 1988) and insect muscle fibres (Ball *et al.* 1987). Ubiquitinated cell surface proteins have been localized in mammalian (Siegelman *et al.* 1986, Yarden *et al.* 1986, Spencer *et al.* 1988), plant (Schulz *et al.* 1994), and algal (Wettern *et al.* 1990) cells. Although ubiquitinated proteins have been found in lysosomal system of mouse fibroblasts (Laszlo *et al.* 1990) and in higher plant vacuoles (Beers *et al.* 1992), they were absent in algal vacuoles (Wettern *et al.* 1990). Free and conjugated ubiquitin has also been reported in mitochondria and

endoplasmic reticulum of rabbit brain (Magnani *et al.* 1991) and in chloroplast of *Chlamydomonas reinhardtii* (Wettern *et al.* 1990). Neither free nor conjugated ubiquitin was detected in plant apoplast (i.e. plant extracellular space) by Beers *et al.* (1992), but free ubiquitin has been found in human seminal plasma (Lippert *et al.* 1993).

In addition to ubiquitin's covalent conjugation with protein substrates, it has also been suggested that it can act as a 'chaotropic' agent forming stable noncovalent complexes with oxidant-damaged proteins, rendering them susceptible to degradation by 20S proteasomes (Wenzel & Baumeister 1993). A report of ubiquitin having intrinsic proteolytic activity (Fried *et al.* 1987) has not been confirmed.

In most of the eukaryotes studied to-date, ubiquitin is encoded by 3 multigene families (Schlesinger & Bond 1987, Callis & Vierstra 1989): polyubiquitin genes, ubiquitin extension (or monoubiquitin) genes and ubiquitin-like genes. There is only one organism (*Giardia lamblia*) in which ubiquitin coding sequence has been found to be present only in a single copy per cell (Krebber *et al.* 1994).

POLYUBIQUITIN GENES consist of 3 to 52 tandem head-to-tail repeats of 228 bp with various carboxyl-terminal extensions of 1 to 3 amino acids, that may function to block ligation of polyubiquitin chains to their protein targets prior to their processing into ubiquitin monomers (Schlesinger & Bond 1987, Callis & Vierstra 1989). The polyubiquitin precursor is cleaved by cytoplasmic proteases to yield monomers of ubiquitin (Wilkinson *et* al. 1989, Tobias & Varshavsky 1991, Baker *et al.* 1992).

MONOUBIQUITIN GENES consist of single monoubiquitin coding units. Translated proteins are flanked by C-terminal extensions of 52 or 76-80 amino-acid residues (Finley *et al.* 1989, Redman & Rechsteiner 1989, Cabrera *et al.* 1992). These extensions have been well conserved in evolution and have been identified as components of 40S and 60S ribosomal subunits in yeast

(Finley *et al.* 1989), mammals (Redman & Rechsteiner 1989), and *Drosophila* (Redman 1994). Several gene clusters coding 5S rRNA in *Tetrahymena pyriformis* are flanked by ubiquitin genes (Neves *et al.* 1988, Neves *et al.* 1991, Guerreiro *et al.* 1993), suggesting a common mechanism of transcription regulation and thus the possibility of involvement of ubiquitin in biogenesis of ribosomes (Guerreiro *et al.* 1993).

THE CLASS OF UBIQUITIN-LIKE GENES includes both pseudogenes and genes that encode proteins with amino acid substitutions from the conserved ubiquitin sequence (Jones & Candido 1993, Linnen *et al.* 1993, Michiels *et al.* 1993, Sun & Callis 1993). The function of these proteins is not completely understood. It has been hypothesized that there are parallel pathways of protein conjugation involving ubiquitin-like proteins and that these conjugates may have entirely different functions from those involving ubiquitin (Loeb & Haas 1992).

1.1.2. Ubiquitin conjugating machinery

The set of reactions and corresponding enzymes leading to ubiquitin conjugation to a protein substrate was first described in rabbit reticulocyte lysates (Hershko *et al.* 1983). Since then elements of the pathway have been found in yeast, mammals and plants (Sullivan *et al.* 1990), illustrating the evolutionary conservation of the system among eukaryotes (Hershko & Ciechanover 1992).

UBIQUITIN ACTIVATING ENZYMES (E1s) initiate the ubiquitin conjugation pathway (Ciechanover *et al.* 1981) by adenylating the carboxyl-terminal glycine of ubiquitin using ATP. Activated ubiquitin is then attached via a thiol-ester linkage to a cysteine in a catalytic site of the same molecule of E1 with a concomitant release of AMP (Haas & Rose 1982).

E1s have been found in the nucleus (Cook & Chock 1991, McGrath *et al.* 1991, Trausch *et al.* 1993), cytoplasm (McGrath *et al.* 1991) and they co-localise with cytoskeleton (Trausch *et*

al. 1993). They form homodimers (Ciechanover *et al.* 1982) and can form complexes with individual ubiquitin conjugating enzymes (Jentsch 1992). Genes coding E1s have been cloned from various organisms (Jentsch 1992). E1s predicted from published sequences are around 100 kD long, with two Gly-X-Gly-X-X-Gly motifs characteristic of nucleotide binding domains and nuclear targeting signals (McGrath *et al.* 1991). Nucleotide sequences of characterized E1s appear to be highly conserved among mammals (Imai *et al.* 1992).

UBIQUITIN CONJUGATING ENZYMES (E2s) are defined as a family of related proteins able to form a thiol-ester with ubiquitin accepted from E1 (Hershko *et al.* 1983, Pickart & Rose 1985). Purified E2s have been shown being capable of catalyzing isopeptide linkages between ε-amino groups of lysines of model substrates and the carboxyl-terminal Gly of ubiquitin (Haas & Bright 1988) as well as Lys^{48} -dependent ubiquitin-ubiquitin linkages on a free polyubiquitin chain (Chen & Pickart 1990) and during autoubiquitination of E2 molecule (Banerjee *et al.* 1993). Ligation occurs with or without the help of family of ubiquitin protein ligases (Bartel *et al.* 1990). Although ubiquitinated proteins may carry single ubiquitin units or multiubiquitin chains, only the latter serves as a degradation signal (Chau *et al.* 1989); monoubiquitinated proteins such as histones (Wu *et al.* 1981) and cytoskeleton subunits (Murti *et al.* 1988) are stable molecules with long half-lives. Multiubiquitin chains may be attached to target substrates either by a one-step transfer of a presynthesized multiubiquitin chain (van Nocker & Vierstra 1991) or processively by adding additional ubiquitin molecules to ubiquitins already conjugated to a target molecule (Bamezai *et al.* 1989). Recent evidence seems to support the former mechanism (van Nocker & Vierstra 1993).

E2s have been localized in yeast nucleus (Goebl *et al.* 1994), cytoplasm of various eukaryotic cells (reviewed in Jentsch 1992), and plasma membrane of *Arabidopsis thaliana* cells (Bartling *et al.* 1993). Ubiquitin-conjugating activity has been detected in chloroplasts of *Avena*

sativa (Veierskov & Ferguson 1991). Integral membrane E2 has been found in peroxisomes (Wiebel & Kunau 1992) and the endoplasmatic reticulum (Sommer & Jentsch 1993) of yeast cells.

E2s comprise a heterogenous family of mostly small molecular mass (16 to 26 kDa in wheat germ, Sullivan *et al.* 1990) isoenzymes found in yeast, mammals and plants (Jentsch *et al.* 1990, Sullivan *et al.* 1990, Sullivan & Vierstra 1991). All E2s contain a conserved catalytic core (UBC domain, Jentsch *et al.* 1990) of approximately 150 amino acids surrounding the active site cysteine required for thiol-ester formation (Sullivan & Vierstra 1991, Sullivan & Vierstra 1993). The three-dimensional structure of the E2 encoded by the *Arabidopsis At*UBC1 gene has been determined (Cook *et al.* 1992b) and a model of interaction between E2 and ubiquitin has been proposed (Cook *et al.* 1992a, Sullivan & Vierstra 1993).

E2s can be structurally divided into three classes (Jentsch *et al.* 1990): Class I enzymes consist almost entirely of the conserved UBC domain (Jentsch *et al.* 1990). They are barely able to transfer ubiquitin from E1 to test proteins *in vitro* which suggests that they may need the presence of E3s for substrate recognition (Jentsch 1992). Class II enzymes possess C-terminal extensions to the UBC domain which are in part responsible for substrate specificity (Jentsch *et al.* 1990, Sullivan & Vierstra 1991) and for cellular localization of the enzymes (High *et al.* 1991). Class III enzymes have N-terminal amino acid extensions in addition to the UBC domain, but no C-terminal extensions. The functional significance of their N-terminal extensions is unknown (Jentsch 1992).

As ubiquitin, E2s are among the most conserved proteins identified to date (Jentsch 1992). *UbcD1* gene of *Drosophila* and *ubc-2* gene of *Caenorhabditis elegans*, homologues of the *UBC4* and *UBC5* genes of yeast, have been cloned and found to exhibit a strong similarity to yeast genes, and their expression in yeast cells rescues the phenotypic defects of *ubc4 ubc5* double

mutants (Treier *et al.* 1992, Zhen *et al.* 1993). A homologue of the yeast *UBC4* and *UBC5* gene products have been also isolated from wheat germ (Girod & Vierstra 1993).

UBIQUITIN PROTEIN LIGASES (E3s) interact specifically with different E2s in recognizing various types of protein substrates (Girod & Vierstra 1993). Only few E3s have been isolated to-date (Bartel *et al.* 1990, Huibregtse *et al.* 1993, Parag *et al.* 1993). Various heat shock proteins which have a role in protein repair (Ellis & van der Vies 1991) may also have E3-like functions. Heat shock proteins have affinity to misfolded proteins and could be therefore utilized for the ubiquitin-dependent degradation of partially unfolded proteins (Jentsch 1992). They can also function both in protein repair and protein degradation pathways in *Escherichia coli* (Sherman & Goldberg 1992, Sherman & Goldberg 1993).

The E6 protein of human papillomavirus (HPV) has been found to stimulate the ubiquitin-dependent degradation of the tumor suppressor protein p53 (Scheffner *et al.* 1990) by forming a complex with cellular protein E6-AP (E6- associated protein) which then serves as an p53 specific E3 (Huibregtse *et al.* 1993, Scheffner *et al.* 1993). E6-AP also possesses an E3 activity in the absence of E6 (Scheffner *et al.* 1993). E6-AP has been found to form thioester complexes with ubiquitin accepted from E2, and the thioester formation was found necessary for E6-AP's function as an E3 (Scheffner *et al.* 1995). These results suggest that E3s do not act only as molecules recognizing specific substrates but that they have a ubiquitin-ligase activity and are a part of an E1-E2-E3 ubiquitin-thioester cascade (Scheffner *et al.* 1995).

UBIQUITIN-PROTEIN ISOPEPTIDASES. Apart from being degraded by 26S proteasome, ubiquitin-protein conjugates may be disassembled by isopeptidases (or ubiquitin carboxyl-terminal esterases) which cleave only the Lys-Gly bond between ubiquitin and the target protein, releasing both target protein and multiubiquitin chain intact (Mayer & Wilkinson 1989). A deconjugating activity present in reticulocytes has been observed to compete with ubiquitin-dependent

proteolysis (Hershko *et al.* 1980, Hershko *et al.* 1984, Hough *et al.* 1986). Similar activity has been also detected in wheat germ extracts (Sullivan *et al.* 1990). The purpose of this process may be to correct errors made by ubiquitin-conjugating system (release of ubiquitinated proteins not ready for hydrolysis), regulate ubiquitination level of specific proteins or to remove proteolytic fragments generated during conjugate digestion from ubiquitin's carboxyl-terminus (Vierstra 1989).

1.1.3. Degradation of ubiquitin-protein conjugates: Proteasome

Although the ATP requirement of intracellular proteolysis has been known since 1953 (Simpson 1953) it took 25 years since then until an *in vitro* ATP-dependent proteolytic system has been described (Etlinger & Goldberg 1977). Cytoplasmic particles called prosomes were identified in the early 70's (Shelton *et al.* 1970), but it was not until 1987 that prosomes, or 20S proteasomes, were found to be associated with the ATP-dependent protein degradation pathway as a component of 26S proteasome (Hough *et al.* 1987, Waxman *et al.* 1987).

The 20S proteasome has been isolated from many eukaryotic organisms, including rabbit reticulocytes (Hough *et al.* 1987), plant leaves (Ozaki *et al.* 1992) and plant seeds (Yang & Malek 1991, Skoda & Malek 1992). It is also present in Archaebacterium *Thermoplasma acidophilum* (Dahlmann *et al.* 1992). The 20S proteasome complex is resolved as 8-10 proteins on one-dimensional SDS gels, and 15-20 proteins in the range of 22-34 kDa on a two-dimensional gel (Rechsteiner *et al.* 1993). Its three-dimensional structure has been described as a cylindrical barrel built up from two juxtaposed rings and two disks closing off the tripartite compartment (Hegerl *et al.* 1991). Relative positions of subunits of 20S proteasome suggest that this structure itself is a complex dimer of two identical halves, composed of rings designated α and β (Kopp *et al.* 1993). Outer rings of the *T. acidophilum* proteasome exhibit 7-fold symmetry (Pühler *et al.* 1992), whereas human proteasome appear to have 6-fold symmetry (Rechsteiner *et al.* 1993).

Transfer RNA was reported as an essential part of the ubiquitin system (Ciechanover *et al.* 1985) and of the 20S proteasome itself (Coux *et al.* 1992), but it was not detected in 20S proteasome preparations from *T. acidophilum* (Pühler *et al.* 1992).

Five distinct proteolytic activities, each associated with a different component of the 20S proteasome, have been identified (Orlowski 1993): Three activities, trypsin-like, chymotrypsin-like, and peptidyl-glutamyl-peptide hydrolysing, cleave peptide bonds on the carboxyl side of basic, hydrophobic, and acidic amino acid residues, respectively (Cardozo *et al.* 1992). A fourth component cleaves bonds preferentially on the carboxyl side of branched chain amino acids (Pacifici *et al.* 1993) and the fifth cleaves bonds between small neutral amino acids (Orlowski 1993). However, purified 20S proteasomes do not catalyze ATP-dependent breakdown of ubiquitin-protein conjugates (Tanaka & Ichihara 1988, Driscoll & Goldberg 1989, Matthews *et al.* 1989).

The 26S proteasome has been found in rabbit reticulocyte lysates in (Hough *et al.* 1986) and only recently in plants (Fujinami *et al.* in press). It is thought to be a functional homologue of bacterial Clp ATP-dependent protease (Dubiel *et al.* 1992). Its assembly from multiple subunits, one of which is 20S proteasome, requires ATP (Eytan *et al.* 1989, Driscoll & Goldberg 1990, Kanayama *et al.* 1992). The 26S complex has a significant ATPase activity (Armon *et al.* 1990). Orino *et al.* (1991) confirmed that the 20S proteasomes associate reversibly and ATP-dependently with multiple protein components to form the 26S proteasome that degrades ubiquitinated proteins in an ATP-dependent manner (Orino *et al.* 1991). The 20S proteasome probably serves as a catalytic core of 26S complex, with other subunits acting as regulators and/or conferring additional enzymatic activities (Rechsteiner *et al.* 1993). These include a ubiquitin carboxyl-terminal hydrolase/isopeptidase activity releasing polyubiquitin chain from substrate protein (Eytan *et al.* 1993) and another ubiquitin hydrolase activity which disassembles polyubiquitin chains (Hadari

et al. 1992). Joint action of these 26S proteasome associated activities perhaps causes liberation of ubiquitin at the end of the proteolytic process (Eytan *et al.* 1993). Apart from its ATP- and ubiquitin dependent proteolytic activity it also seems to possess an ATP-dependent proteolytic activity independent of ubiquitin (Murakami *et al.* 1992), cleaving its substrate into oligopeptides 5-10 amino acid long (Tokunaga *et al.* 1994).

In addition to 22-34 kDa polypeptides common with the 20S proteasome, the 26S complex contains about 10 additional subunits between 40-60 kDa and two 100 and 110 kDa subunits (Rechsteiner *et al.* 1993). Three dimensional E.M. image analysis revealed two highly asymmetric masses of approximately 19S attached to both ends of a dimeric 20S proteasome in both animal (Peters *et al.* 1993) and plant (Fujinami *et al.* in press) 26S proteasome complexes. Another group used E.M. to visualize a complex of the 20S proteasome with a PA28 proteasome activator. This complex forms, in contrast to 26S proteasome, highly symmetrical caps on proteasome stacked rings (Gray *et al.* 1994).

1.1.4. Functions of the ubiquitin-dependent proteolytic pathway

STRESS RESPONSE. The involvement of ubiquitin pathway in cell responses to stress were revealed in 1984 by Ciechanover, Finley and Varshavsky when they described a mouse mutant cell line ts85 expressing a thermolabile E1 (Ciechanover *et al.* 1984, Finley *et al.* 1984); similar cell line mutants from other mammals with thermolabile E1s have been reported since (Kulka *et al.* 1988). It has number of phenotypic abnormalities at the non-permissive temperature, including inhibition of degradation of short-lived and abnormal proteins, defects in DNA synthesis, nucleoside transport and stress-induced protein degradation (Ciechanover *et al.* 1984, Gropper *et al.* 1991). *UBC1*, *UBC4*, and *UBC5* genes of *Saccharomyces cerevisiae* encode ubiquitin-conjugating enzymes essential for the cell survival, demonstrated by nonviability of triple mutants

(Seufert *et al.* 1990). In *ubc4 ubc5* double mutants the cells grow poorly at normal temperatures, and they are nonviable at 37°C and in the presence of amino acid analogues (Seufert & Jentsch 1990). Pulse-chase studies have shown that these mutants are deficient mainly in degradation of short-lived and abnormal proteins (Seufert & Jentsch 1990). In yeast, ubiquitin mutants are also hypersensitive to desiccation and starvation (Finley *et al.* 1987). The fact that *ubc4 ubc5* double mutants constitutively express major heat shock proteins (Seufert & Jentsch 1990) suggests that defects in degradation of abnormal proteins leading to their accumulation can trigger the cellular stress response (Morimoto *et al.* 1992).

Specific ubiquitin genes are heat inducible in chicken cells (Bond *et al.* 1988), yeast (Finley *et al.* 1987), *Drosophila melanogaster* (Niedzwiecki & Fleming 1993), *Arabidopsis thaliana* (Burke *et al.* 1988), wheat (Ferguson *et al.* 1990), tobacco (Genschik *et al.* 1992), sunflower (Binet *et al.* 1991), potato (Garbarino *et al.* 1992), maize (Christensen *et al.* 1992), *Volvox carteri* (Schiedlmeier & Schmitt 1994), and *Chlamydomonas reinhardtii* (Shimogawara & Muto 1989). The levels of ubiquitin and ubiquitin conjugates change during heat shock (Carlson *et al.* 1987, Parag *et al.* 1987, Niedzwiecki & Fleming 1993), omnilateral gravistimulation (Hunte *et al.* 1993, Wolf *et al.* 1993a), mechanical stress (Schulz *et al.* 1994), γ-irradiation (Delic *et al.* 1993), development (Pan *et al.* 1993, Shimbara *et al.* 1993, Callis & Bedinger 1994), and chilling (Gindin & Borochov 1992). Resistance to cadmium poisoning in yeast is mediated by ubiquitin-dependent proteolysis (Jungmann *et al.* 1993) as well. The association of ubiquitin with coat proteins of tobacco mosaic virus (Dunigan *et al.* 1988), as well as involvement of the ubiquitin system in the hypersensitive response in tobacco plants (Becker *et al.* 1993) suggest that the pathway may be connected to the defense against viral infection in plants (Vierstra 1989).

DNA METABOLISM AND CELL CYCLE CONTROL. It has been shown that yeast *RAD6* mediates E3-dependent protein degradation (Dohmen *et al.* 1991). UBC2 (*RAD6*) is involved in DNA repair,

induced mutagenesis and sporulation (Kang *et al.* 1992). *UBC3* (*CDC34*) gene responsible for transition from G1 to S phase of the cell cycle in yeast has been identified as ubiquitin-conjugating enzyme (Goebl *et al.* 1988), acting independently of E3 (Haas *et al.* 1991). Mutations in *UBC2* (*RAD6*) affect the target site preferences of the yeast retrotransposon Ty1 (Liebman & Newnam 1993). The human E2 *UBCh1* is involved in the repair of UV-damaged, alkylated and cross-linked DNA (Kaiser *et al.* 1994).

CONTROL OF TURNOVER OF REGULATORY PROTEINS. Yeast *UBC4* E2 and a putative E3 are responsible for mono-ubiquitination of calmodulin in yeast (Parag *et al.* 1993). Tryptophan decarboxylase of a plant *Catharantus roseus* is ubiquitinated *in vivo* (Fernandez & De Luca 1994).

NERVOUS SYSTEM. Members of ubiquitin-dependent pathway have been implicated in various aspects of nervous system function: The *Drosophila melanogaster* gene *ben* encodes a neural protein which is a member of E2 family. Mutation in this gene alters synaptic connectivity between a subset of central nervous system neurons and morphological abnormalities within the visual system (Muralidhar & Thomas 1993). In *Aplysia*, long-term synaptic plasticity is facilitated by selective degradation of regulatory subunit of cAMP-dependent protein kinase, causing its persistent activation. Hedge *et al.* (1993) have found that the subunit degradation requires ATP, ubiquitin and a particulate element (possibly proteasome). Authors suggest that the the 26S proteasome and the ubiquitin pathway may be involved in the acquisition of memory.

HUMAN DISEASE. The first indication that ubiquitin has a significant clinical role came from studies on neurodegenerative diseases (Mayer *et al.* 1991). Abundance of ubiquitin-protein conjugates in pathologically changed neurons in Alzenheimer's disease (He *et al.* 1993ab, Shin *et al.* 1993), schizophrenia (Horton *et al.* 1993) and motor neuron disease/amyotrophic lateral sclerosis (Bergmann 1993) is explained either as a part of cytoprotective processes or as a cause of the neuronal death (Mayer *et al.* 1991). Ubiquitin is also present in inclusions characteristic of

skeletal muscle disease inclusion body myositis (Albrecht & Bilbao 1993). Involvement of ubiquitin-dependent pathway in many kinds of cancer is well established. For example tumor suppressor protein p53 is a target of this pathway (Scheffner *et al.* 1993), and ubiquitin-ribosomal protein S27a gene appears to be an early growth response gene in human colorectal carcinoma (Wong *et al.* 1993). Glycosylation of long lived proteins such as crystalin, myelin and collagen inhibits their degradation in ubiquitin-dependent pathway, suggesting its involvement in the changes of protein catabolism in diabetes (Takizawa *et al.* 1993).

DEVELOPMENT. A mouse spermatogenesis gene *Spy*, which is required for the survival and proliferation of spermatogonia during spermatogenesis, has been found to be an E1 enzyme (Kay *et al.* 1991, Mitchell *et al.* 1991). Changes in protein levels and activity of 26S proteasome have been observed during oocyte maturation (Tokumoto *et al.* 1993). The yeast E2 gene *UBC10* (*Pas2*) is required for peroxisome biogenesis (Wiebel & Kunau 1992).

PROTEIN TRANSPORT. Yeast E2 gene *UBC6*, which encodes an integral membrane protein of the endoplasmatic reticulum, causes a protein translocation defect by mediating proteolysis of subunits of mutant translocation apparatus in *sec61* yeast strain (Sommer & Jentsch 1993). Yeast polyubiquitin gene *UBI4* can rescue otherwise nonviable Chc$^-$ yeast strain lacking clathrin heavy chain. Increased levels of ubiquitin caused by overexpression of *UBI4* may be required for turnover of mislocalized or improperly processed proteins accumulating in the absence of clathrin. On the bases of these findings it has been suggested that ubiquitin may play a general role in turnover of proteins in the secretory or endocytic pathways (Nelson & Lemmon 1993).

1.1.5. Regulation of ubiquitin pathway

Knowledge about mechanisms regulating ubiquitin conjugation system is still limited. The yeast Ubc3 (Cdc34) ubiquitin-conjugating enzyme E2 responsible for transition from G_1 to S

phase of the cell cycle is ubiquitinated and phosphorylated *in vivo* (Goebl *et al.* 1994). Banerjee *et al.* (1993) have demonstrated that when expressed in bacteria the Ubc3 (Cdc34) can multiubiquitinate itself *in vitro*, possibly regulating its own degradation.

Chicken polyubiquitin gene UbII is located 1.9kb downstream of the tRNA gene cluster (Mezquita & Mezquita 1992). The presence of this cluster containing RNA polymerase III promoter sequence may exert a positive enhancer effect on the downstream adjacent RNA polymerase II promoters; the proximity of the tRNA cluster and the polyubiquiitn gene UbII suggests the possibility of a coordinate expression of these genes (Mezquita *et al.* 1993).

2. Methods

2.1. Plant material and cultivation conditions

2.1.1. Plant material and media

Chlamydomonas reinhardtii Dangeard, wild type CC-125 mt^+ (carrying *nit-1* and *nit-2* mutations, blocking utilization of nitrate) was procured from *Chlamydomonas* Genetic Center, Duke University, Durham (NC). This strain is a widely used model in biochemical and physiological studies (Harris 1989). Sueoka high-salt medium (Sueoka 1960), with addition of microelements (Hutner *et al.* 1950) was used for autotrophic cultivation. Final medium was prepared from 9.35 mM NH_4^+, 22.12 mM K^+, 0.27 mM Na^+, 0.068 mM Ca^{2+}, 0.081 mM Mg^{2+}, 9.55 mM Cl^-, 0.182 mM SO_4^{2-}, 13.6 mM PO_4^{3-}, 17.9 mM Fe^{2+}, 76.5 mM Zn^{2+}, 6.3 mM Cu^{2+}, 6.8 mM Co^{2+}, 25.6 mM Mn^{2+}, 6.2 mM Mo^{6+}, 184 mM BO_3^{3-} and 134 mM EDTA, pH 6.8. Deionized water (NANOpure, Barnstead) was used for preparation of the media, as well as for preparation of other solutions described bellow.

2.1.2. Methods of culture characterization

Cultures of various ages were sampled, cells immobilized with a drop of IKI (1 g I_2 and 0.5 g KI per 100 cm^3 of water) and cell density (expressed as number of cells per cm^3) determined using a haemocytometer (Bright-Line hemacytometer, American Optical). An absorbance of an identical sample at 750 nm (A_{750}) was also measured using DU-50 spectrophotometer (Beckman). A standard curve was established linking cell density and A_{750} (Figure 1); it was used in all the following measurements of cell densities of *Chlamydomonas* cultures (Harris 1989).

The culture viability was expressed as a fraction of dead cells from whole cell population. Non-motility and intake of Evan's blue pigment (Gaff & Okong'o-Ogola 1971) were chosen as indicators of cell death. Samples were treated with a drop of Evan's blue (Sigma) solution (20% w/v in HS medium) and blue coloured non-motile cells were counted using a hemocytometer.

2.1.3 Cultivation regimes

Agar (Difco Laboratories) cultures were grown at variable temperature in range of 20-30°C, illuminated by cool white fluorescent bulbs (General Electrics), yielding irradiation of 80 $mE.m^{-2}s^{-1}$. Liquid pregrowth cultures were grown in 125 cm^3 Erlenmeyer flasks with an inserted Pasteur pipette as a bubbler. Medium with cells was incubated in a Conviron EF7 chamber at 25°C and 250 $mE.m^{-2}s^{-1}$ for 2 days. When the cell density reached approximately 1.10^6 cells per cm^3, 15 cm^3 of pregrowth culture was used to inoculate 1.5 dm^3 of HS medium in 2.8 dm^3 Fernbach flask. The flask was plugged by a foam stopper with an inserted bubbler. The culture was synchronized in a Conviron chamber at 25°C and 250 $mE.m^{-2}s^{-1}$ under 12h/12h dark/light cycle and aerated by filtered air (Schlösser 1966). After 2 dark/light cycles the culture was subjected to 24 hours of constant illumination (Rollins *et al.* 1983) with other conditions unchanged. Experimental treatments were conducted during this period.

2.2. Labelling of cells with ^3H and treatment conditions

The entire culture (1.5 dm^3 of approx. 2.10^6 cells/cm^3) was centrifuged for 30 min at 10,000.g and 20°C. The pelleted cells were resuspended in 20 cm^3 of fresh HS medium containing 1 $mC.cm^{-3}$ of ^3H_2O (Amersham). The suspension was transferred into 125 cm^3

Erlenmeyer flask and then aerated and vigorously shaken for 5 hours under conditions identical to cultivation of pregrowth cultures.

After the end of a labelling period, the suspension was centrifuged in screw-capped 40 cm^3 Nalgene tubes (10,000.g at 20°C for 15 min) and washed once with fresh HS medium. Resuspended cells were then transferred to a 2,800 cm^3 Fernbach flask containing 1,500 cm^3 of either 4°C or 20°C HS medium, depending on the experiment. The culture was then incubated under constant illumination of 250 $mE.m^{-2}s^{-1}$ and aerated, either in the Conviron chamber at 25°C or in the freezer at 4°C . The length of cold treatment was 60 min.

For non-radioactive experiments the flask with the original culture was placed directly to the freezer and its temperature was monitored. Remaining conditions were identical to those described above.

2.2. Protein extraction and quantification

Samples were centrifuged at 10,000 g at the temperature corresponding to the temperature of the sample and pellets were resuspended in extraction buffer (0.1 M PO_4^{3-} [Na_2HPO_4/NaH_2PO_4], 0.1% NaN_3, 10% glycerol, 1 mM iodoacetamide, 10 mM EDTA, 5 mM EMI). PMSF (100 mM PMSF solution in 95% ethanol and 5% 2-propanol, pH 7.4) was added to final concentration of 1 mM and the sample was sonicated using Sonifier with a microtip (Branson Sonic Power) at setting 3 for 10 min while cooled on an NaCl/ice bath (approx. -5°C). Samples were stored at -20°C for subsequent processing.

Protein concentration in an extract was determined according to Bradford (1976), using Biorad Protein Assay Kit. BSA (Sigma) was used as a standard, A_{595} was measured using DU-50 spectrophotometer (Beckman).

2.3. Gel filtration separation of free and conjugated ubiquitin

BioGel P-10 Fine (Biorad) with nominal exclusion limit of 20 kDa was packed into 1.5x100 cm chromatography column (Econo-Column, Biorad). Flow of GF buffer (the composition identical to the extraction buffer except for the addition of 10% glycerol) was facilitated by EP-1 Econo Pump (Biorad), placed at the output of the column. Final bed height was 90-100 cm. Crude extracts and standards were applied on top of the gel using a flow adaptor (Biorad) and separated at flow rate of 5.08 $cm.h^{-1}$ (0.15 $cm^3.min^{-1}$). Eluate absorbance at 280 nm was monitored and registered by UA-5 absorbance/fluorescence detector (ISCO) and fractions were collected using 2111 Multirack fraction collector (LKB Bromma).

Column was calibrated using carbonic anhydrase (MW 29 kDa, Sigma) and ubiquitin (MW 8.5 kDa, Sigma) as standards. Eluate fractions with elution time lower than A_{280} minimum between A_{280} peaks of CA and Ub standards were pooled as Ub-C fraction. Fractions with higher elution time were pooled and Ub fraction.

Ub and Ub-C fractions were concentrated using stirred ultrafiltration cell 8010 (Amicon)

Lyophilized powder of *Staphylococcus aureus* Cowan I (Sigma) cells (Staph A) was resuspended in PBS buffer (0.15M NaCl, 0.01M NaH_2PO_4, pH 7.4 at 25°C) with 0.02% NaN_3 to form 10% w/v suspension. The cells were fixed by 1.5% formaldehyde (Sigma) and heat treated at 80°C (Harlow & Lane 1988).

2.4.2. Preadsorption of Staph A and ^3H labelled cell extracts

Fixed Staph A was transferred to TETN250 immunoadsorption buffer (25 mM Tris-HCl, 5 mM EDTA, 250 mM NaCl, 1% Triton X-100, pH 7.5), maintaining 10% w/v concentration. Aliquots (500 mm^3) of ^3H labelled Ub and Ub-C fractions were transferred to 1.5 cm^3 Eppendorf tubes, mixed by vortexing with 10 mm^3 of Staph A/TETN250 suspension and incubated for 15 min at room temperature. The Staph A was pelleted by centrifuging in microcentrifuge at room temperature for 3 min at 12,800.g. The supernatant constituted preadsorbed ^3H labelled Ub and Ub-C fractions.

Staph A/TETN250 suspension was mixed with equal volume of an unlabelled cell extract. Mixture was incubated at room temperature for 15 min and centrifuged in a microcentrifuge at room temperature for 1 min at 12,800.g. The pellet was resuspended in such a volume of TETN250 so that 10% suspension was formed. This suspension constituted preadsorbed Staph A.

2.4.3. Formation of ubiquitin-antibody-Staph A complexes

500 mm^3 aliquots of preadsorbed ^3H labelled Ub and Ub-C fractions were added to 1.5 cm^3 Eppendorf tubes containing 100 mm^3 of 50 $mg.cm^{-3}$ BSA (Sigma) in TETN250 and 50 mm^3 of rabbit anti-ubiquitin antibody (Sigma). Reaction mixtures were briefly vortexed and

incubated for 10 min at room temperature. After that 50 mm^3 of preadsorbed Staph A was added, vortexed and incubated for 5 min at room temperature with mixing every 2 min.

Each of the immunoadsorption reactions was aspirated and layered over 600 mm^3 sucrose cushions (1M sucrose in TETN250) in Eppendorf 1.5 cm^3 microtubes. Immune complexes were centrifuged through the sucrose 3 min at 12,800.g at room temperature. The upper layer was aspirated down to the sucrose interface and sucrose cushions were overlayed with 2M urea in TETN500 (25 mM Tris-HCl, 5 mM EDTA, 500 mM NaCl, 1% Triton X-100, pH 7.5). After 5 min of incubation urea and sucrose layers were aspirated, leaving Staph A pellets. These were washed twice with excess of TETN250 buffer and final pellet resuspended in 200 mm^3 of TETN250.

2.4.4. Counting of ^3H incorporated to immunoprecipitates

Resuspended antigen-antibody-Staph A complexes were transferred to 10 cm^3 plastic scintillation counting vials and mixed with 5 cm^3 of Scinti Verse Bio-HP scintillation counting cocktail (Fisher). ß-radiation (as cpm's) was measured in 1211 RackBeta liquid scintillation counter (LKB Wallac) with open energy window (8-250 keV).

2.5. Fluorescence immunoassay for ubiquitin

Fluorescence immunoassay for ubiquitin was performed according to manufacturer's instructions [Sigma, Anonymous (1992)] with certain modifications:

2.5.1. Immunoreactions and measurement of fluorescence

Standards and samples were always run in triplicates. Standards were prepared diluting ubiquitin (Ub-F, Sigma) in FIA assay buffer (0.1 M PO_4^{3-} [Na_2HPO_4/NaH_2PO_4], 0.1% NaN_3, 0.01% bovine g-globulins [Sigma], pH 7.4).

A mixture of ubiquitin-fluorescein conjugate (Sigma), ubiquitin standards or samples and rabbit anti-ubiquitin antibody (Sigma; FIA assay buffer was added to NSB tubes instead of standards and the antibody) incubated in dark for 60 min at room temperature. After incubation, antigen-antibody complexes were precipitated by goat anti-rabbit IgG (Sigma) and centrifuged at 2,000.g for 15 min at 4°C. The pellets were washed once with 2 cm^3 of FIA assay buffer and resuspended in 1.5 cm^3 of SDS/NaOH (0.1 M NaOH, 2% w/v SDS).

Fluorescence was measured using LS50B luminescence spectrometer (Perkin-Elmer), with excitation wavelength 495 nm, analytical wavelength 525 nm and integration time 5 sec.

2.5.2. Data reduction

$$F / F_0 = \frac{F_0 - NSB}{F - NSB} \qquad 1$$

Equation 1 was used to calculate values F/F_0 for individual replicates of standards (Figure 3) and samples. F is fluorescence of a standard or of an unknown, F_0 is maximal fluorescence of standard 0 (no ubiquitin added), *NSB* represents an average fluorescence signal caused by nonspecific binding of Ub-F. Probit values corresponding to F/F_0 of individual replicates of unknown samples and averaged F/F_0 of standards were calculated using an algorithm of Odeh & Evans (1974). A standard curve was constructed using probit values of standards through which a line was fitted using a linear least-square regression (Figure 3). An average concentration of ubiquitin in individual samples was determined using probit values of individual replicates, and a standard deviation of the mean was calculated.

2.7. Analysis of data

An assumption has been made that ubiquitin molecules in *Chlamydomonas* cells are subjected to processes outlined in Figure 4. According to this model, ubiquitin molecules can be found in two pools: Ub pool of free ubiquitin and Ub-C pool of conjugated ubiquitin. Free amino acids (AA pool) are considered to be immediate precursors for synthesis of Ub. Two amino acids pools AA demonstrate the process by which the ^3H label is removed from amino acid molecules during the chase period by transaminases, so that AA pool remains unlabeled (Humphrey & Davies 1975). By virtue of labelling method used, following assumptions can be made: a) there is no recycling of the ^3H label taking place, b) the only labelled pools are Ub and Ub-C, and c) there is no label present in AA pool during the chase period (Humphrey & Davies 1975). Processes taking place in the model (synthesis, degradation, conjugation and deconjugation) are quantitatively described by their respective rate constants (k_S, k_D, k_C, k_{DC}, respectively). K_{DC} is actually an apparent constant which takes into account both the 26S deconjugation activity and the ubiquitin-protein isopeptidase activity. All the constants are considered to be of first order (units of h^{-1}), except for the rate constant of ubiquitin synthesis (units of $ng.h^{-1}$), which is defined as being of zero order (Davies 1980).

Transformations taking place in the model (Figure 4) are expressed in Equation 2:

$$\frac{dU}{dt} = (R_S - R_D) + (R_{DC} - R_C) \qquad 2$$

where dU/dt is rate of accumulation of Ub (R_A) in a cell culture. All rates are in units of $ng.h^{-1}$. If $dU/dt = 0$, a simple steady state kinetic analysis could be applied. However, *Chlamydomonas* culture in logarithmic phase was used for experiments and thus cells are in a state of

continuous growth. To consider effects of label dilution caused by synthesis in excess of degradation, non-steady state approaches have to be employed.

The rate constants were calculated for each two consecutive measurements and considered constant for the time period between those measurements. Therefore the use of the term constant is justified, even if the values of the constants change in the course of the experiment (reflecting changes in the cell metabolism). The rate constant of conjugation k_C can be calculated as described by Reiner (1953):

$$\frac{dC^*}{dt} = (U^* - C^*)k_C \qquad 3$$

where C* and U* is specific radioactivity of Ub-C and Ub pools respectively. To obtain a value of k_C , Equation 3 has to be restated:

$$k_C = \frac{dC^*}{dt}\frac{1}{U_t^* - C_t^*} \qquad 4$$

An exponential function was fitted to data points C^* and first derivative of this function was used to calculate a slope at time t.

According to Zak *et al.* (1979), if fractional rate of turnover and growth of a protein pool are considered constant,

$$k_{DC} = k_G - k_C \qquad 5$$

where k_G is a rate constant of fractional growth of a protein pool, defined as

$$k_G = \frac{dC}{dt}\frac{1}{C_t} \qquad 6$$

Rate of ubiquitin degradation is defined as

$$-\frac{d[U^*]}{dt} = k_D[U^*] - k_C[U^*] + k_{DC}[C^*] \quad 7$$

where [C*] and [U*] are total radioactivity in Ub-C and Ub pools, respectively. After solving Equation 7 one can calculate k_D as follows:

$$k_D = \frac{\left(\frac{d[U^*]}{dt} + k_C[U^*]_t - k_{DC}[C^*]_t\right)}{[U^*]_t} \quad 8$$

From Equation 2 follows that

$$\frac{dU}{dt} = k_S - k_D U - k_C U + k_{DC} C \quad 9$$

$$k_S = \frac{dU}{dt} + U_t(k_D + k_C) - k_{DC} C_t \quad 10$$

Finally, given the values of rate constants k_D and k_{DC}, half-life of molecules in Ub and Ub-C pools, respectively, can be calculated using Equation 11;

$$t_{\frac{1}{2}} = \frac{\ln 2}{k_X} \quad 11$$

where k_X is either k_D or k_{DC} .

3. Results

The growth of a *Chlamydomonas reinhardtii* culture similar to those used in pulse-chase experiments is shown in Figure 5. Synchronization, manifested by constant cell density during light phase and division taking place presumably in dark, occured after the first dark/light cycle. A total of three synchronized cycles was observed before the growth of the culture arrested. Cell density after the third dark/light cycle reached $7x10^6$ cells/cm^3 and culminated in essentially nongrowing culture after five days at value of $9x10^6$ cells/cm^3.

To assess survival of cell cultures after a cold chock, they were incubated at 4°C for various periods of time (Figure 6). 0.31% and 1.27% of cells were observed dead after 1 h and 24 h treatments, respectively.

Ratio of free and conjugated ubiquitin to total extracted protein (Figure 7) remained constant during growth of the cells under constant environment conditions. It was approximately 2500 ppm of free and 200 ppm of conjugated ubiquitin of total extracted protein. After a 60 min cold treatment both free and conjugated ubiquitin ratio to total protein increased until 4 h after a treatment, culminating in both cases at about 2.5-fold of the initial values. After that both ratios decreased, reaching values similar to those obtained at constant conditions at 16 h after cold treatment.

During constant conditions, the ratio of free to conjugated ubiquitin (Figure 8) persisted at about 11. Cold treatment caused it to decrease temporarily to approx. 8 immediately after cold treatment. The ratio returned to its original value at 2 h after cold treatment. At the end of observation period the ratio decreased to 8, but was within the range of standard error of the ratio at constant conditions.

Rate constants of ubiquitin conjugation (Figure 9), deconjugation (Figure 10), synthesis (Figure 11) and degradation (Figure 12) did not change in the course of observation at constant

conditions and remained at values of 0.035 h^{-1}, 0.005 h^{-1}, 750 $ng.h^{-1}$ and 0.11 h^{-1} respectively. During first 4 h after cold treatment k_c increased from 0.040 h^{-1} to 0.075 h^{-1} and returned back to its original value 16 after cold treatment (Figure 9). A similar timecourse was observed for k_s, which increased from 700 $ng.h^{-1}$ to 1250 $ng.h^{-1}$ during first 4 h after cold treatment (Figure 11). Rate constant k_D peaked 8 h after cold treatment at 0.20 h^{-1} and then decreased attaining its final value of 0.09 h^{-1} which was slightly lower than its initial value 0.10 h^{-1} (Figure 12). No changes in k_{DC} were observed (Figure 10). Half life of ubiquitin $t_{\frac{1}{2}}$ calculated from the constant of degradation remained unchanged at a value of about 6.5 hours during cultivation in constant conditions, but decreased from approx. 6 hours to 3.5 hours during first four hours after the cold treatment (Figure 13).

4. Discussion

Chlamydomonas cultures were synchronised (Figure 5) in order to obtain a population of uniform cells. Periodic changes in content of individual proteins during synchronised growth (Howel *et al.* 1977) could be easily explained as a part of a cell cycle program (Halvorson *et al.* 1971). While this is probably true for proteins directly involved in various phases of cell cycle, changes in protein synthesis in general appear to be a reaction of cells to changing environmental (light) conditions (Rollins *et al.* 1983). Most of the periodic fluctuation in protein synthesis in synchronised *Chlamydomonas* cultures can be suppressed by subjecting them to constant conditions (for example changing 12/12h dark/light regime to continuous light); fluctuations in protein synthesis diminish during first continuous light cycle, while synchronicity of cell cultures persists for 3-4 cell cycles (Rollins *et al.* 1983). As far as ubiquitin is concemed, no changes in ubiquitin conjugation, ubiquitin-dependent proteolysis and isopeptidase activities were observed in cycling *Xenopus* egg extracts (Mahaffey *et al.* 1993). Therefore it can be assumed that ubiquitin metabolism in *Chlamydomonas* cultures cultivated as described was influenced by environmental changes but not by various phases of the cell cycle. Furthermore, cells were treated while at the same stage of the cell cycle as the controls. The temperature changes the cells were subjected to were not lethal, since only 0.3% of cells were observed dead after one hour of incubation at 4°C (Figure 6).

The ratios of free, as well as conjugated, ubiquitin to total protein increased more than two-fold four hours after the exposure of cells to 4°C, but remained unchanged during cultivation at constant conditions (Figure 7). Also, ratio of free to conjugated ubiquitin decreased from 11 to 8 immediately after the cold treatment and returned back to its original value of 11 within 2 hours (Figure 8). These observations imply that both free ubiquitin and ubiquitin-conjugate levels

increased as a result of an exposure to low temperature. An increase in accumulation of ubiquitin conjugates after a temperature treatment has been reported in *Chlamydomonas* previously: Shimogawara & Muto (1989) reported that heat stress caused a burst of high MW conjugates during a treatment and a reciprocal decrease of 31 and 28 kDa ubiquitinated polypeptides and free ubiquitin. The 28 kDa polypeptide was later identified as ubiquitinated histone H2B (Shimogawara & Muto 1992). In another study (Wettern *et al.* 1990) the disappearance of 29 kDa protein (perhaps representing the same species as 28 kDa histone reported by Shimogawara & Muto) was confirmed, as well as increase of accumulation of high MW ubiquitin-protein conjugates during a treatment. Conjugates disappeared slowly during recovery. No changes were observed in the free ubiquitin pool. (Treatments described in the mentioned paper were performed in high light intensity or dark environment to elucidate the effect of photoinhibition on ubiquitination, and can not be therefore directly compared with results presented in this work.) Similar results were obtained in wheat roots (Ferguson *et al.* 1990) and in *Drosophila* (Niedzwiecki & Fleming 1993). In this work changes are observed after the cold treatment, presumably only uppon return to physiological temperature allowing metabolic activity (or perhaps because of the shortness of the cold treatment).

The changes in plant metabolism upon exposure to low temperatures have been studied extensively (for review see for example Sakai & Larcher 1987, Guy 1990, or Huner *et al.* 1993), and similarities between changes induced by heat and cold stress have been reported (Collins *et al.* 1993). The only report dealing specifically with reaction of ubiquitin metabolism to low temperature is that of Gindin & Borochov (1992). After 48h exposure of the Mediterranean plant *Clerodendrum speciosum* to 4°C, they observed 90% decline in free ubiquitin levels. Discrepancy between these results and findings presented in this work may be due to the sensitivity of *Clerodendrum* to chilling; chilling injury in this plant is manifested by leakage of cellular

electrolytes, indicating damage of cellular membranes (Gindin & Borochov 1992). The loss of free ubiquitin, as well as reported loss of total protein, would be then consistent with electrolyte leakage as a sign of cellular death. Increased conjugation of ubiquitin to cellular proteins is difficult to reconcile with this (Gindin & Borochov 1992). The mentioned discrepancy can be also attributed to the difference in the length of the cold treatment:- 48 h in Gindin & Borchov (1992) compared to 1 h in this experiment.

There appear to be two discrepancies between changes of ubiquitin and ubiquitin conjugates following cold shock described in this thesis and the findings pertaining to heat shock discussed above. First, ubiquitin-protein conjugates appear to accumulate during heat shock but after cold shock. Slowing down of the rate of enzymatic reaction at temperature much lower than physiological temperature may explain this. Events serving as signals for ubiquitin conjugation (see below) occur at low temperature, but the cell presumably does not have a chance to respond to them accordingly, until the ambient temperature is high enough for enzymatic reactions to proceed at sufficient rate. The second discrepancy, the decrease (or no change) of ratio of free ubiquitin to total protein during heat shock as opposed to increase in this ratio during cold shock might be explained, at least in part, by the same mechanism. It is possible that during cold shock induction of ubiquitin conjugation system lags behind activation of ubiquitin genes, allowing growth of the pool of free ubiquitin. This might be caused, for example, by differential sensitivities of enzymes engaged in protein synthesis and ubiquitin conjugation towards cold and heat stress/denaturation.

Figure 9 shows that rate constant of ubiquitin conjugation k_c increased about two-fold during first 3 hours after transfer of culture from cold conditions and after reaching its maximum it decreased slowly until reaching its original value. This means activity of ubiquitin-conjugating

system was induced by cultivation at low temperature and that ubiquitin moved faster to the conjugate pool. As expected, rate constant remained unchanged during incubation at 25°C.

No changes were observed in rate constant of ubiquitin deconjugation k_{DC} (Figure 10). This suggests that neither the 26S proteasome, the enzymatic system responsible for degradation of ubiquitin-conjugated proteins, nor isopeptidase system was activated by the cold treatment, or that increase of activity of one was compensated by decrease of activity of the other. Although a heat shock protein has been identified as a regulatory component of the complex (Tsubuki *et al.* 1994), little is known about the exact mechanism of proteasome regulation. It seems that under conditions employed in this experiment no regulatory mechanisms were induced.

These two findings indicate that ubiquitin conjugates accumulated after a cold treatment as a result of increased ubiquitin conjugation, which is most likely caused by increased activity of enzymes of ubiquitin conjugating system or by increase of their content in cell.

Four hours after the exposure of cells to 4°C an almost two-fold increase in rate constant of ubiquitin synthesis k_S (Figure 11) was observed as compared to constant conditions, indicating that synthesis of ubiquitin increased as a result of incubation at 4°C. It is reasonable to suspect that some of the ubiquitin genes of *Chlamydomonas* were induced by the cold treatment. Changes in ubiquitin expression as a reaction to other forms of stress have been reported at the transcriptional level. Run-on transcription in maize seedlings following heat shock revealed a 4-5 fold increase in polyubiquitin gene expression (Christensen & Quail 1989). Maize polyubiquitin genes *Ubi1* and *Ubi2* has been identified as at least partially responsible for this increase (Christensen *et al.* 1992). In potato tubers, several ubiquitin genes (represented by their cDNAs) have been reported to be induced by various kinds of stress (Garbarino *et al.* 1992). These authors demonstrated the independent regulation of the different members of the ubiquitin gene family in response to various kinds of stress. Genschik *et al.* (1992) arrived to the same

conclusion: In tobacco four different size classes of ubiquitin mRNA responded differently to heat shock, $HgCl_2$ treatment, viral infection giving rise to a hypersensitive reaction, and an *Agrobacterium tumefaciens* infection which resulted in tumour formation. Ubiquitin mRNA content in antarctic alga *Plocamium cartilagineum* exhibited after heat shock at 5°C (normal growth temperature for this organism is around 0°C) the same pattern of increase as heat shock protein HSP70 (Vayda & Yuan 1994).

Two heat shock elements were found in promoter regions of polyubiquitin gene of *Volvox carteri* (Schiedlmeier & Schmitt 1994). In contrast, no heat shock element sequences have been identified in the promoter region of heat non-inducible polyubiquitin gene *ubi4-2* of *Petroselinum crispum* (Kawalleck *et al.* 1993). According to the mechanism of induction of the heat shock proteins proposed by Morimoto *et al.* (1992), heat shock gene expression is regulated by negative feedback regulation by heat shock proteins capable of sequestering proteins with exposed hydrophobic surfaces. Appearance of such proteins seems to be a common consequence of a wide array of cellular stress (Kabakov & Gabai 1993). It is known that low temperature can cause protein denaturation (Azuaga *et al.* 1992, Damaschun *et al.* 1993), which means that mechanism proposed by Morimoto *et al.* (1992) for heat shock response could be applied as a starting hypothesis for study of induction of cold shock response.

Cold shock treatment resulted in two fold increase of the rate constant of ubiquitin degradation k_D during 9 hours after cold shock (Figure 12), lagging behind the induction of k_S (Figure 11) and k_C (Figure 9). The corresponding decrease of $t_{½}$ which is calculated directly from k_D according to the Equation (12), was from approximately 6 h to 3.5 hours (Figure 13). During cultivation in constant conditions there was no change in k_D and $t_{½}$; half-life of ubiquitin remained constant at approximately 6 hours. These findings suggest that degradation of ubiquitin increased after transfer of culture from cold conditions, but later than conjugation and synthesis of ubiquitin.

Increased degradation is likely to be a way by which the cell ensures return of the ubiquitin levels to the normal values after the perturbation disappeared and high levels of Ub are no longer needed.

Haas & Bright (1987) conducted pulse-chase experiments using ^{3}H-leucine with cultured human lung fibroblast cells and determined ubiquitin having half-life of 28-31 hours in conditioned and freshly fed cultures. Withdrawal of serum from cultures led to a rapid decline in total ubiquitin during which the ratio of conjugated to free ubiquitin remained constant. Haas & Bright showed that increased turnover after the removal of the serum is likely to involve lysosomal autophagy. In this context it is worth noting that a ubiquitin-conjugating enzyme is involved in biogenesis of peroxisomes (Wiebel & Kunau 1992), organelles related to lysosomes, and that ubiquitin was reported necessary for stress-induced lysosomal degradation of cellular proteins (Gropper *et al.* 1991). After feeding with fresh serum content of free ubiquitin in cells rose by about 50% within 4 days, while ratio of free to conjugated ubiquitin remained constant at about 50%. Stimulation of ubiquitin synthesis was explained by the presence of a specific serum factor rather than by stress caused by the change of cell environment. According to Gropper *et al.* (1991) the increase of ubiquitin content was due to approximately 1.8-fold increase in its synthetic rate. Ubiquitin synthesis was assessed by a pulse experiment using ^{3}H-leucine.

Chin *et al.* (1982) introduced ^{125}I-labelled ubiquitin into HeLa cells by erythrocyte-mediated microinjection. Their data show that ubiquitin was degraded with half-life of approximately 10 hours. Similar technique was used to inject ^{125}I-labelled ubiquitin into human diploid fibroblast culture (Neff *et al.* 1981). Observed half-life of ubiquitin was in this case 320 hours. Variability between these results and between the results presented in this work ($t_{½}$ = 6 h) can be certainly attributed to a large extent to differences in organisms (or cell type) and growth conditions. It is worth noting that in all mentioned experiments a recyclable label (^{125}I and ^{3}H-Leu) was used.

Using such a label requires special accommodations of rate constant calculation (Reiner 1953, Zak *et al.* 1979) none of which were used in these studies. They calculated the degradation rate constants from the equation for the first-order decline of the label in the protein, not compensating for recycling of the label through amino acid pool back to the protein and thus resulting half-lifes are overestimated. Instead of avoiding these errors by solving complicated differential equations, tritiated water was used to label ubiquitin in pulse parts of experiments presented in this work. The use of ^3H_2O to measure turnover of proteins was developed by Humphrey & Davies (1975). The method is based on the assumption that when cells are incubated with ^3H_2O, ^3H rapidly equilibrates with hydrogen on the α-carbon atoms of most amino acids, owing to an exchange reaction catalyzed by transaminases. When cells are transferred back to non-radioactive medium, the transaminases ensure that the reverse sequence occurs and hydrogen replaces ^3H on the α-carbon. However, if an amino acid labelled with ^3H on the α-carbon atom is incorporated into protein, the ^3H is no longer exchangeable and remains in the protein when the cells are transferred back to non-radioactive medium.

During radioactive pulse not only ubiquitin is labelled with ^3H, but also the rest of the protein population of the cell. When radioactivity of ubiquitin-protein conjugates is counted an error of unknown size is introduced by labelled proteins that are linked to ubiquitin. Unfortunately it proved to be difficult to develop any assay that would separate covalently linked ubiquitin from its substrates. If similar experiments are to be conducted in the future, it might be necessary to use isopeptidases to perform this step. A potential source of these enzymes is wheat germ (Sullivan *et al.* 1990).

The ratio of free to conjugated ubiquitin previously observed was 0.5 in human lung fibroblasts (Haas & Bright 1987), 0.8 in rabbit reticulocytes and 0.30 in erythrocytes (Haas & Bright 1985), whereas results of this work suggest ratio 10:1 (Figure 8). This is no doubt caused

by differential sensitivity of conjugated and free ubiquitin to homogenous and solid phase immunoassays; homogenous phase assays (used with anti ubiquitin antibody raised in rabbit) are about 8 times more sensitive towards free than to conjugated ubiquitin, whereas sensitivity of solid phase assays is reported to be the same for both (Haas & Bright 1985). At the time when these studies were conducted no device was available to analyze solid phase blots.

In conclusion, after incubation of *C. reinhardtii* at 4°C the rate constants of synthesis and conjugation increased, the increase of the rate constant of ubiquitin degradation laged behind these two and the rate constant of deconjugation did not not change. Half life of ubiquitin decreases from 6 to 3.5 hours. This sugests that the accumulation of free ubiquitin and conjugated ubiquitin following the cold treatment is caused by increased ubiquitin synthesis and conjugation and delayed onset of ubiquitin degradation.

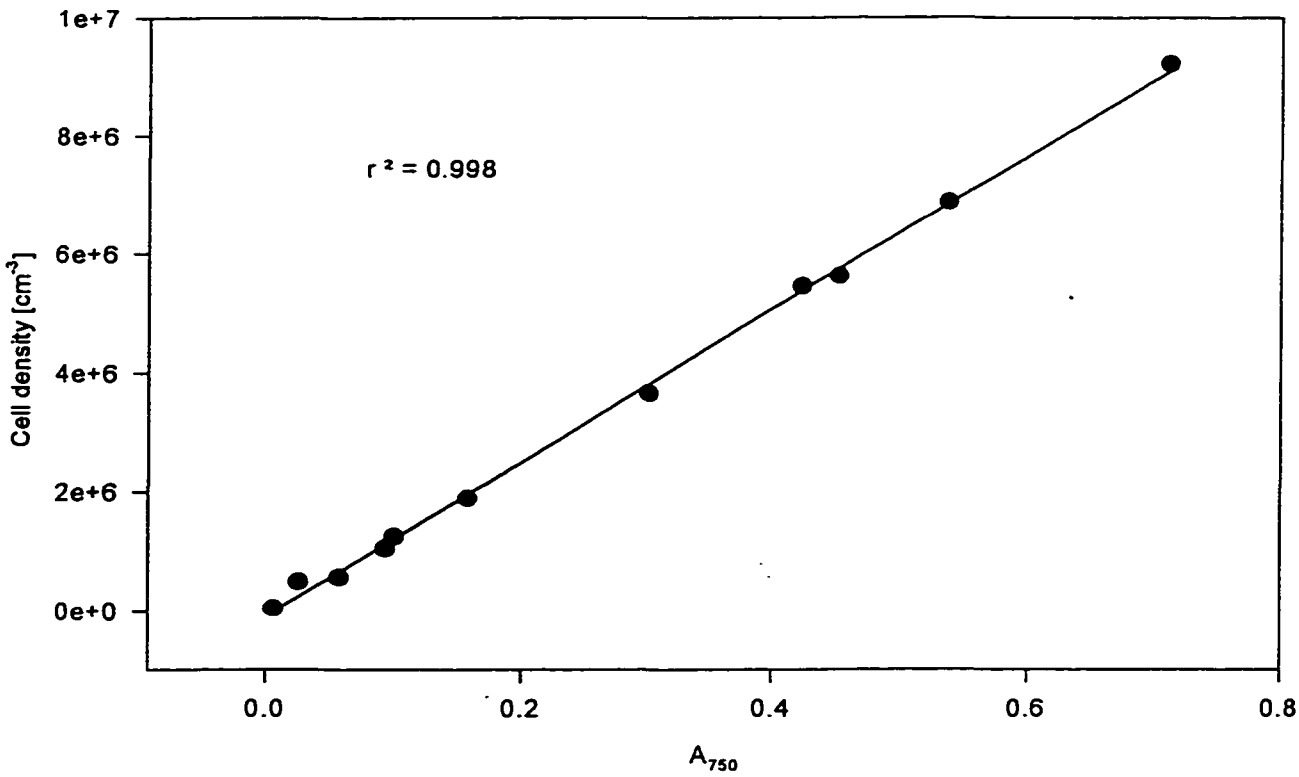

Figure 1. Standard curve used for spectrophotometric determination of cell densities of *Chlamydomonas* cultures.

Data points were acquired by direct counting of cell densities using a haemocytometer. Each data point represents an average of at least 5 independent experiments. A_{750} - absorbance at 750 nm.

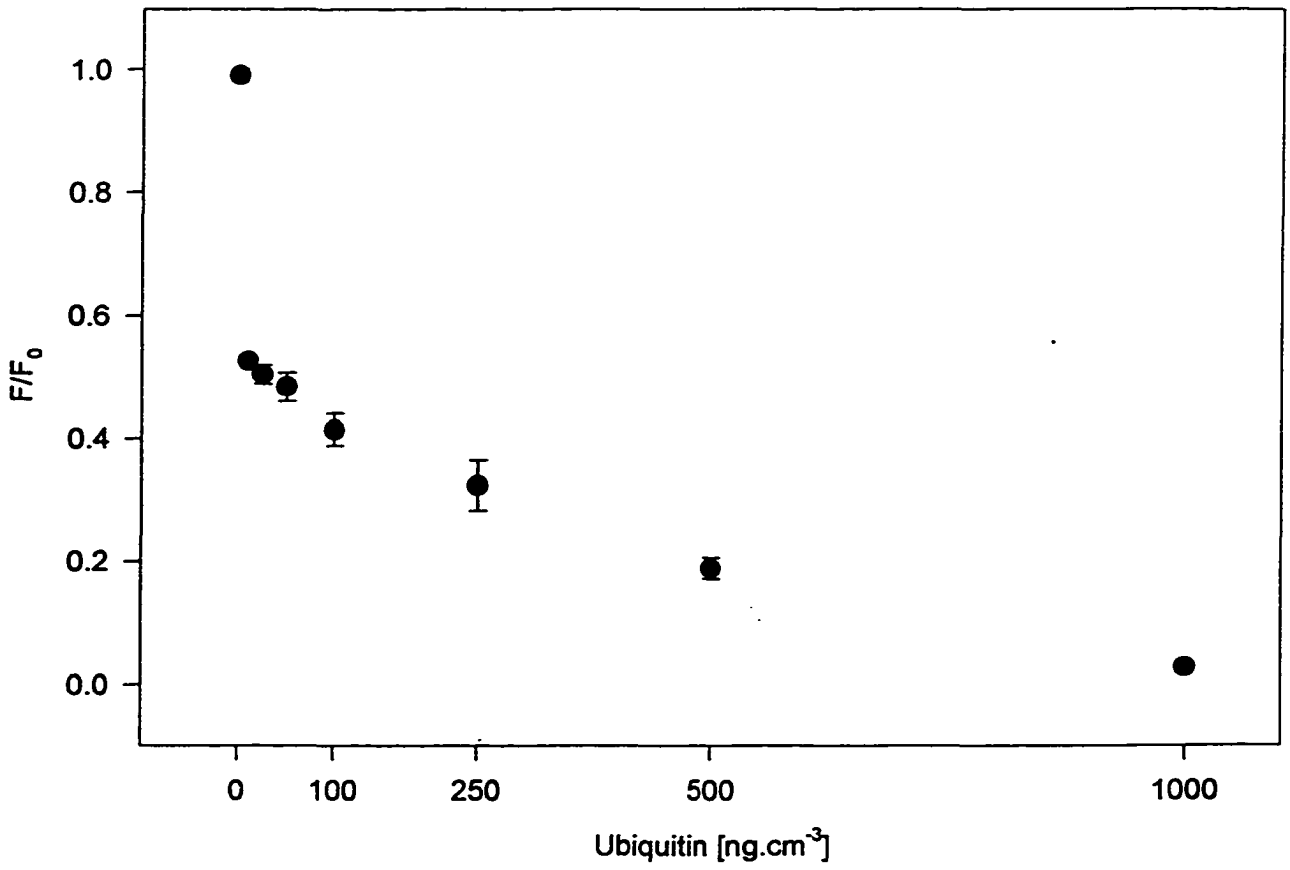

Figure 2. A typical standard curve for fluorescence immunoassay of ubiquitin, before probit reduction.

Error bars represent standard deviation of a mean calculated from 3 idependent measurements during single experiment. F/F_0 - ratio of sample fluorescence to fluorescence of standard 0.

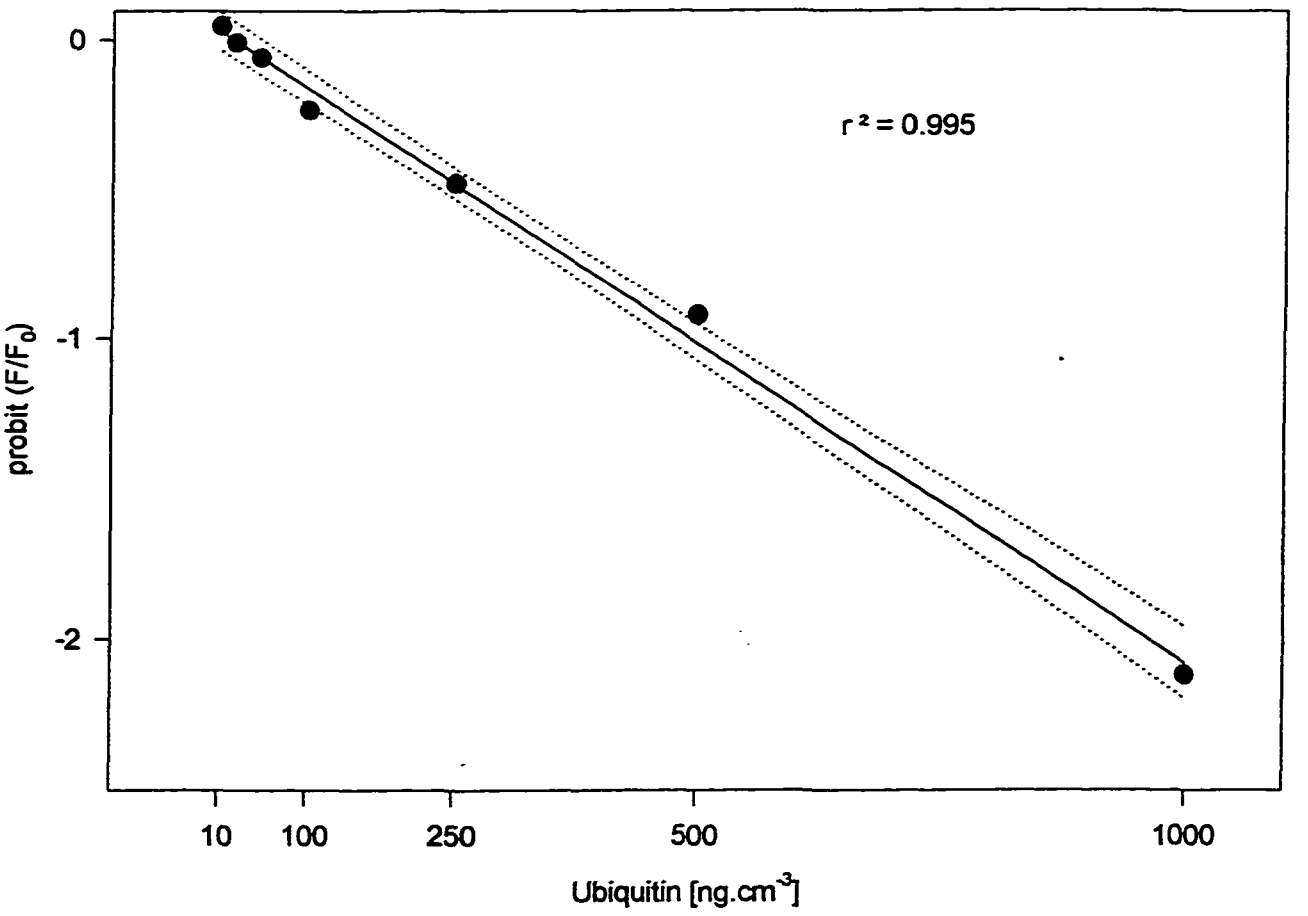

Figure 3. A typical standard curve for FIA of ubiquitin. Data were reduced using probit function. Each data point is a mean calculated from 3 idependent measurements during single experiment. F/F_0 - ratio of sample fluorescence to fluorescence of standard 0. (——) a least square linear regression line; (.....) 95% confidence interval for regression

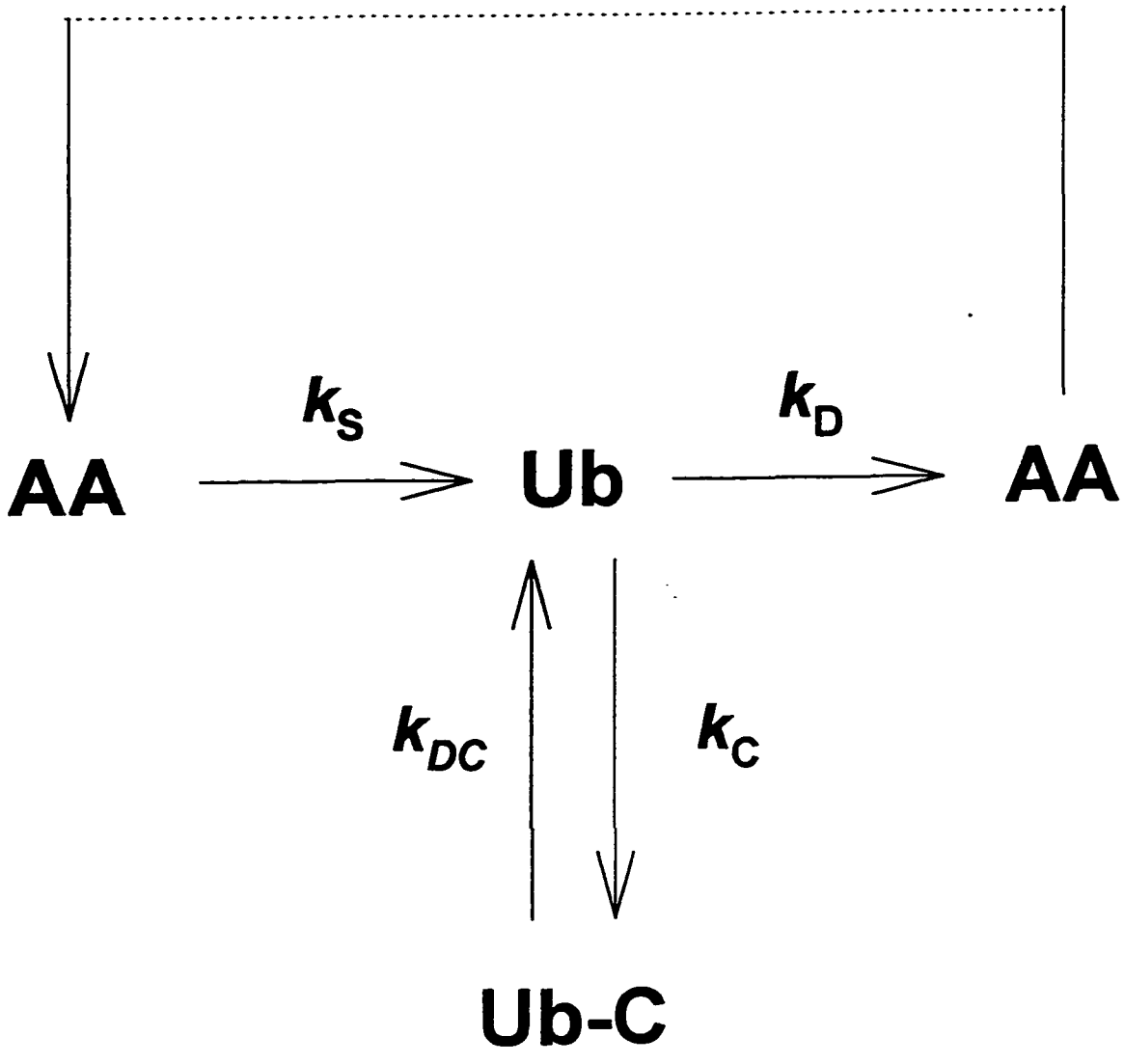

Figure 4. A model of ubiquitin metabolism in *Chlamydomonas reinhardtii*. Explanation in text.

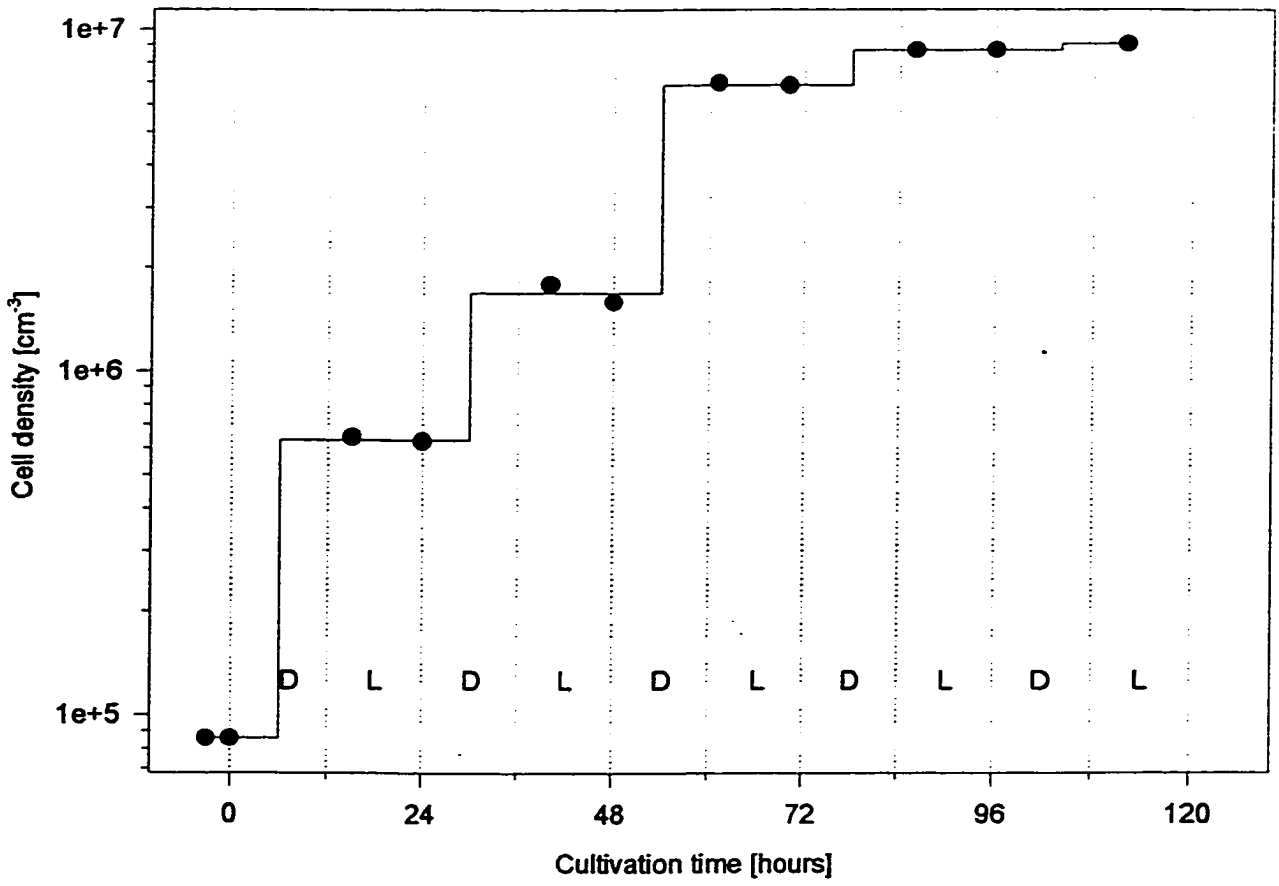

Figure 5. Growth of *Chlamydomonas reinhardtii* culture cultivated under 12h/12h light/dark regime at 25°C.

D - dark period, L - light period; The curve demonstrates the *Chlamydomonas* cell cycle (division in dark, growth in light). Each data point is an average of 3 independent measurements during single experiment.

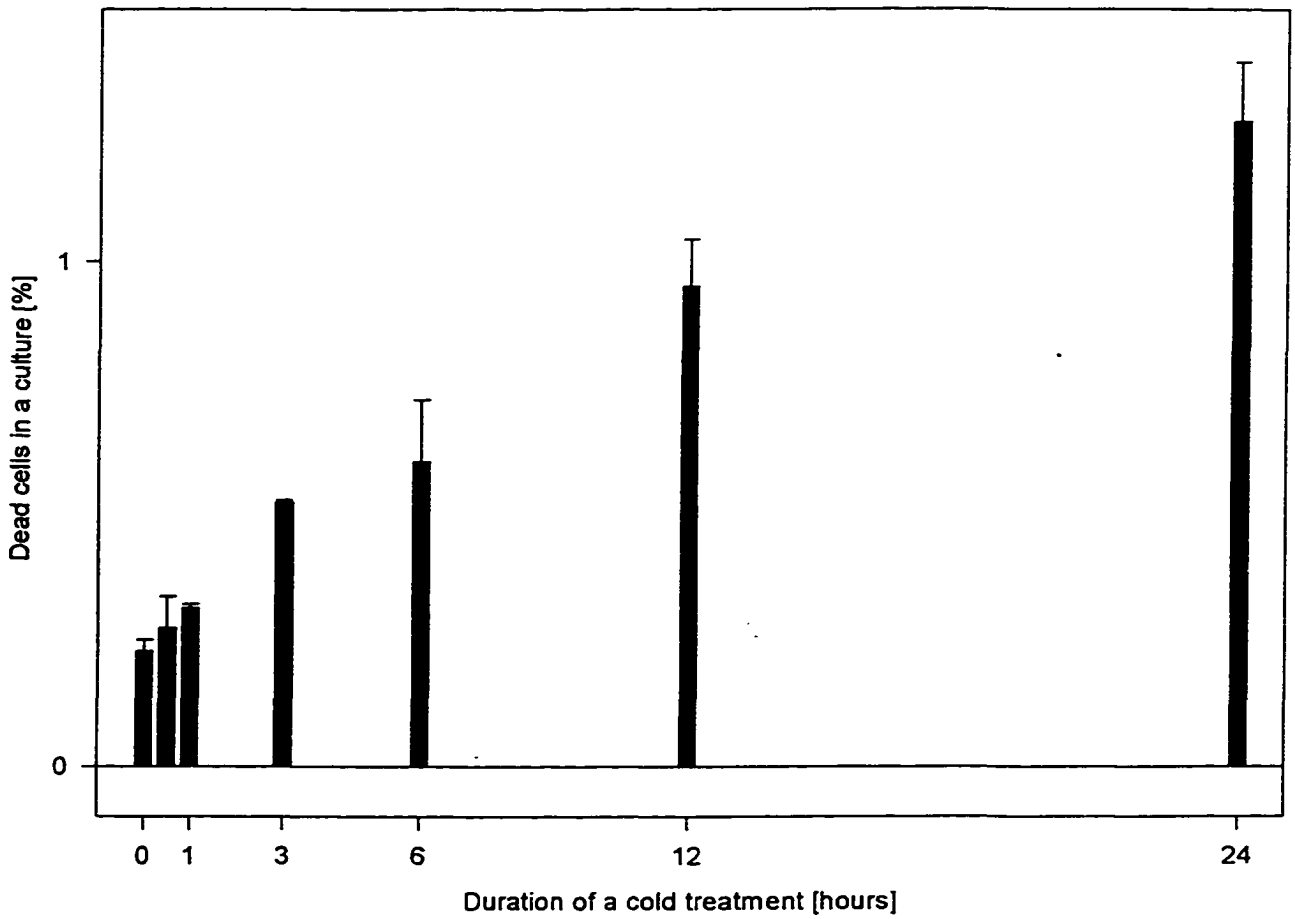

Figure 6. Percentage of dead cells in a *Chlamydomonas* culture after cold treatments of various duration

Error bars represent standard deviation of a mean calculated from 2 independent experiments.

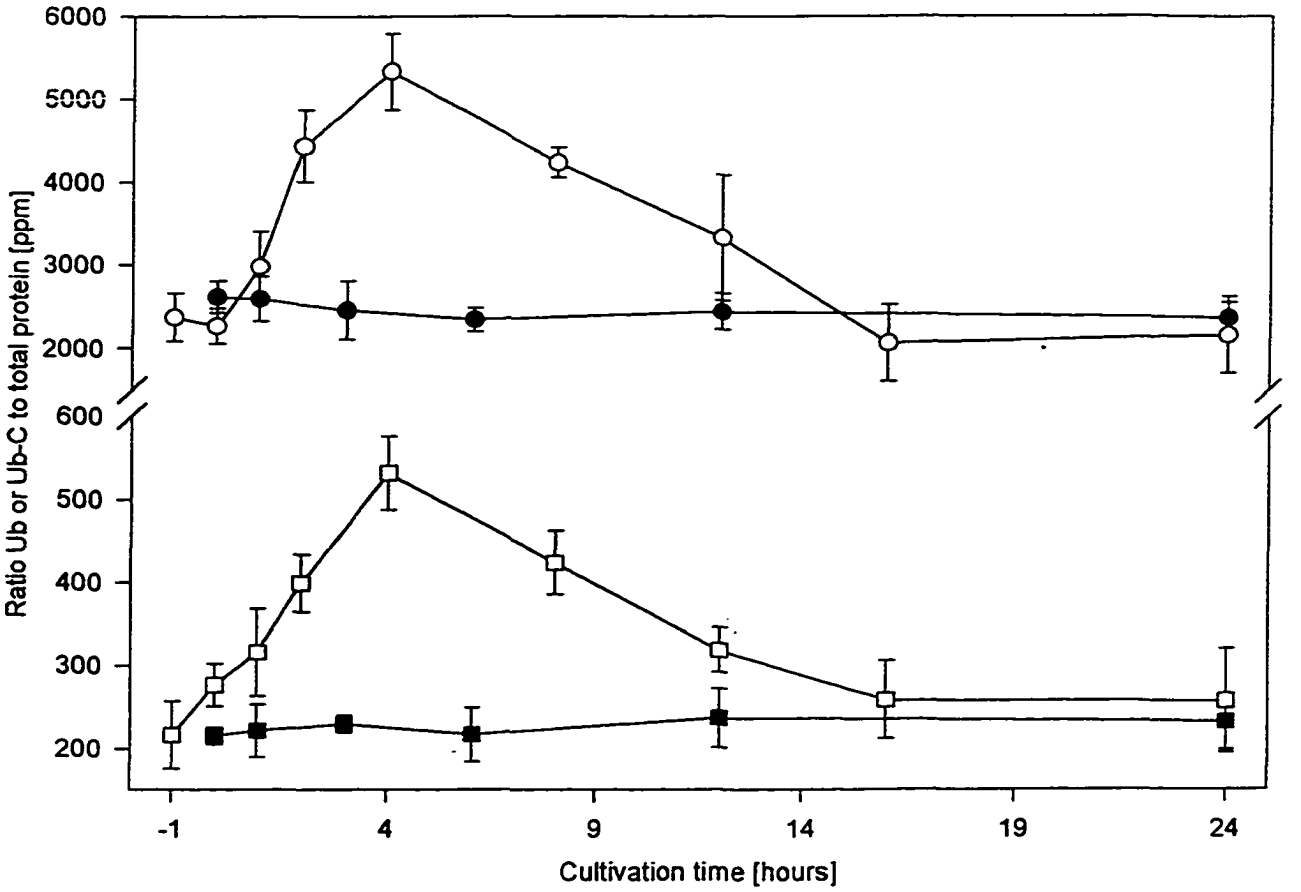

Figure 7. Ratio of free and conjugated ubiquitin to total protein at constant conditions and after a cold treatment.

Ratio of free (●○) and conjugated (■□) ubiquitin to total protein, at constant conditions (■●) and after a cold treatment (□○). Error bars represent standard deviation of a mean calculated from 3 (constant conditions) and 2 (cold treatment) independent experiments. Time -1 denotes the beginning of the 1 hr cold treatment.

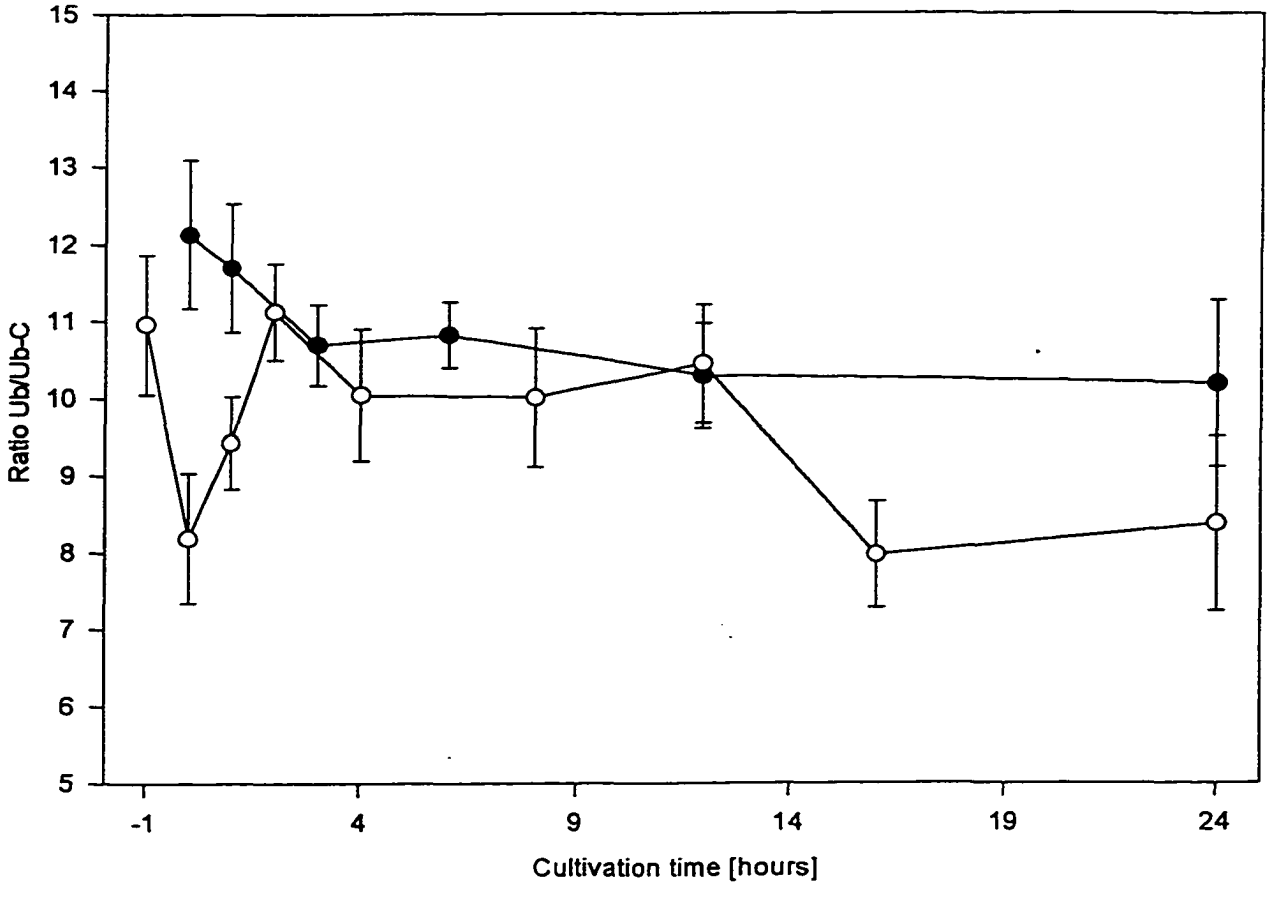

Figure 8. Ratio of free to conjugated ubiquitin during constant conditions and after a cold treatment.

Ratio of free to conjugated ubiquitin, during constant conditions (●) and after a cold treatment (○). Error bars represent standard deviation of a mean calculated from 3 (constant conditions) and 2 (cold treatment) independent experiments.

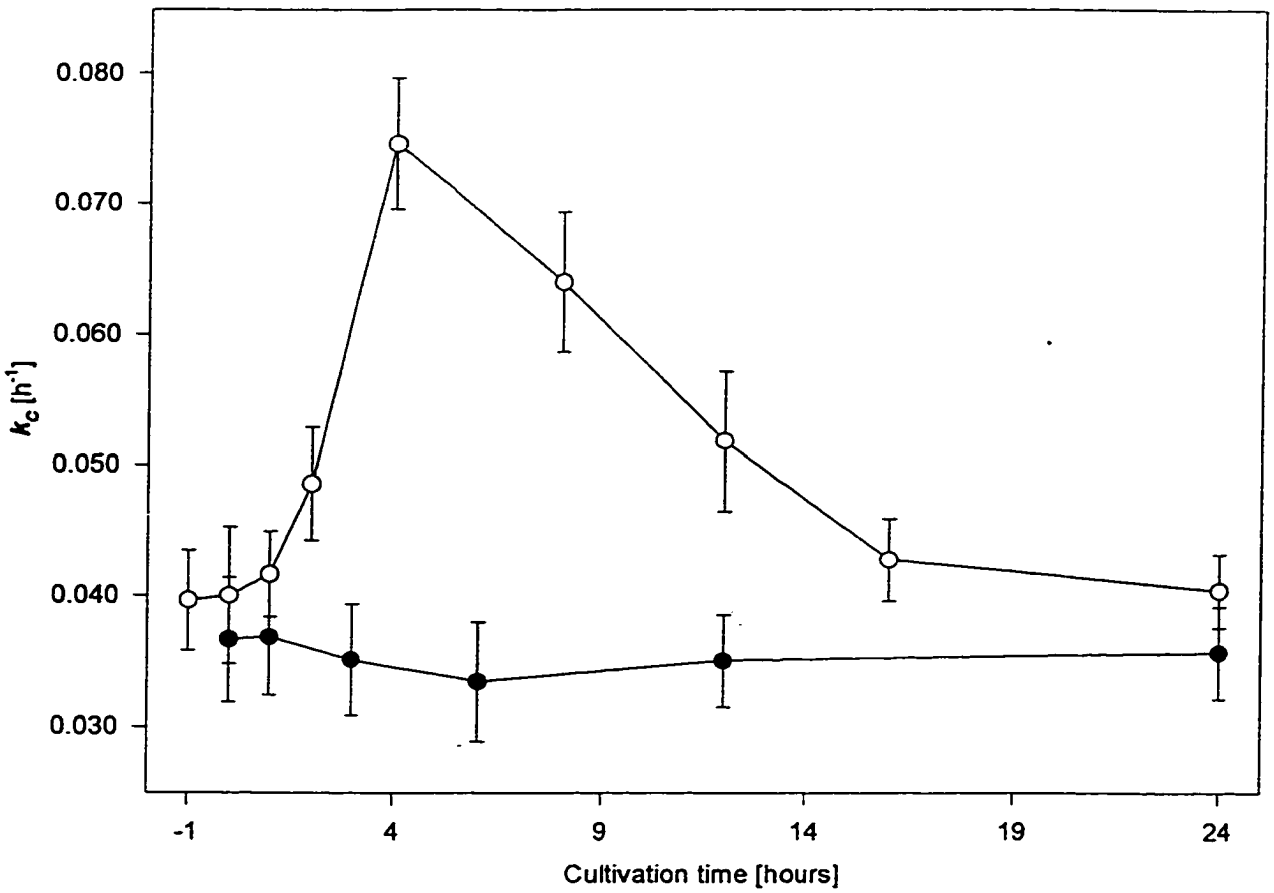

Figure 9. Rate constant of conjugation (k_c) during constant conditions and after a cold treatment. Rate constant of conjugation (k_c) during constant conditions (●) and after a cold treatment (○). Error bars represent standard deviation of a mean calculated from 3 (constant conditions) and 2 (cold treatment) independent experiments.

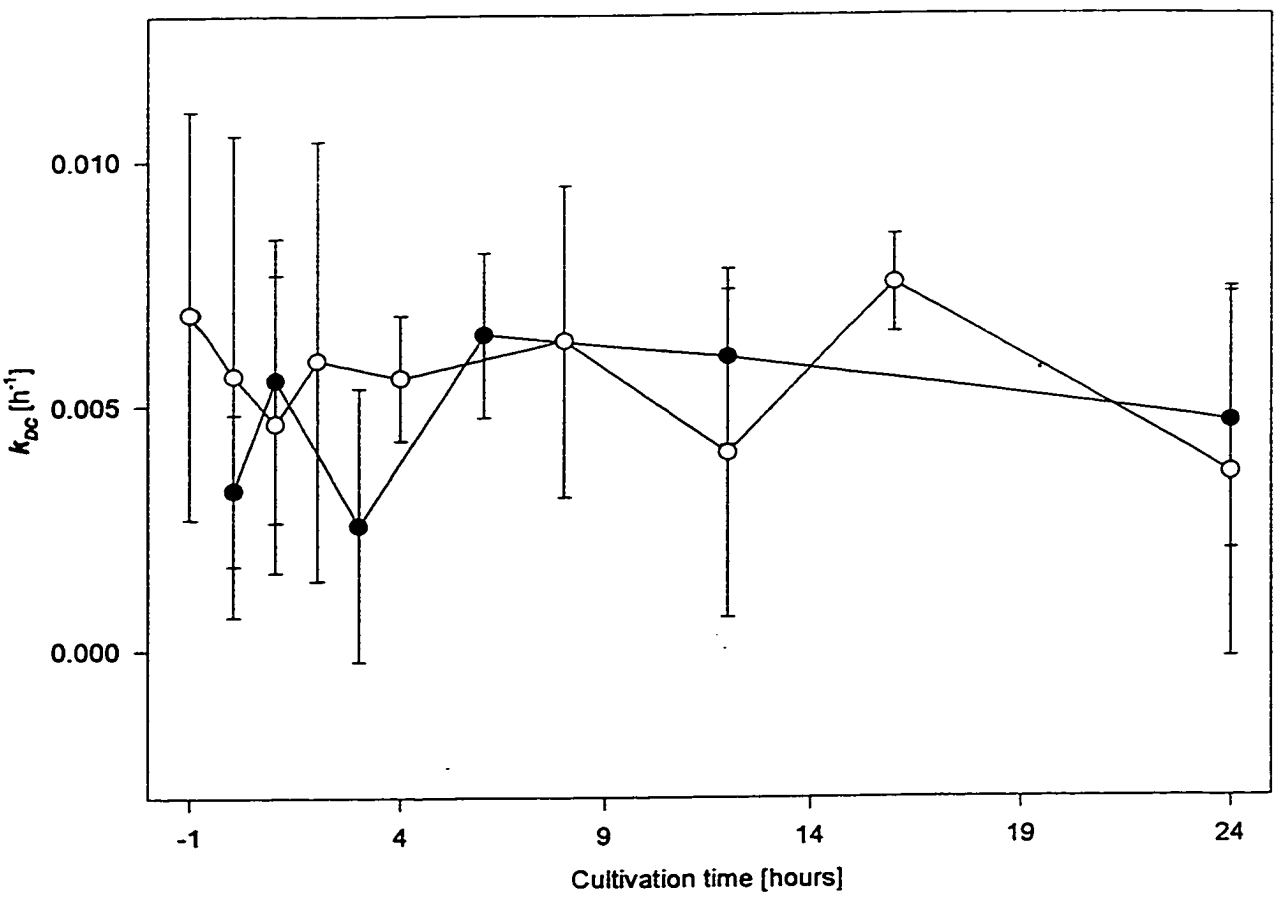

Figure 10. Rate constant of deconjugation (k_{DC}) during constant conditions and after a cold treatment.

Rate constant of deconjugation (k_{DC}) during constant conditions (●) and after a cold treatment (○). Error bars represent standard deviation of a mean calculated from 3 (constant conditions) and 2 (cold treatment) independent experiments.

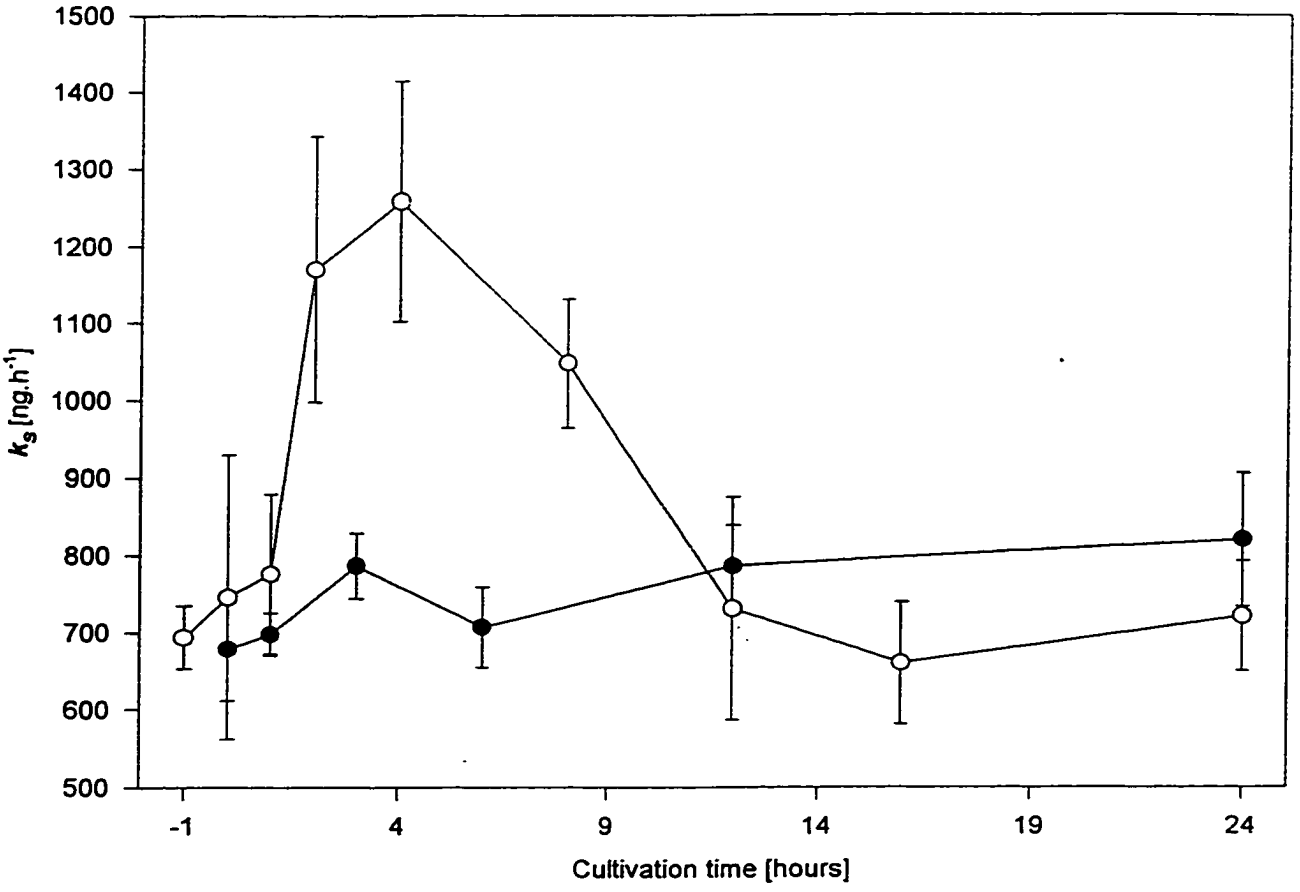

Figure 11. Rate constant of ubiquitin synthesis (k_s) during constant conditions and after a cold treatment.

Rate constant of ubiquitin synthesis (k_s) during constant conditions (●) and after a cold treatment (○). Error bars represent standard deviation of a mean calculated from 3 (constant conditions) and 2 (cold treatment) independent experiments.

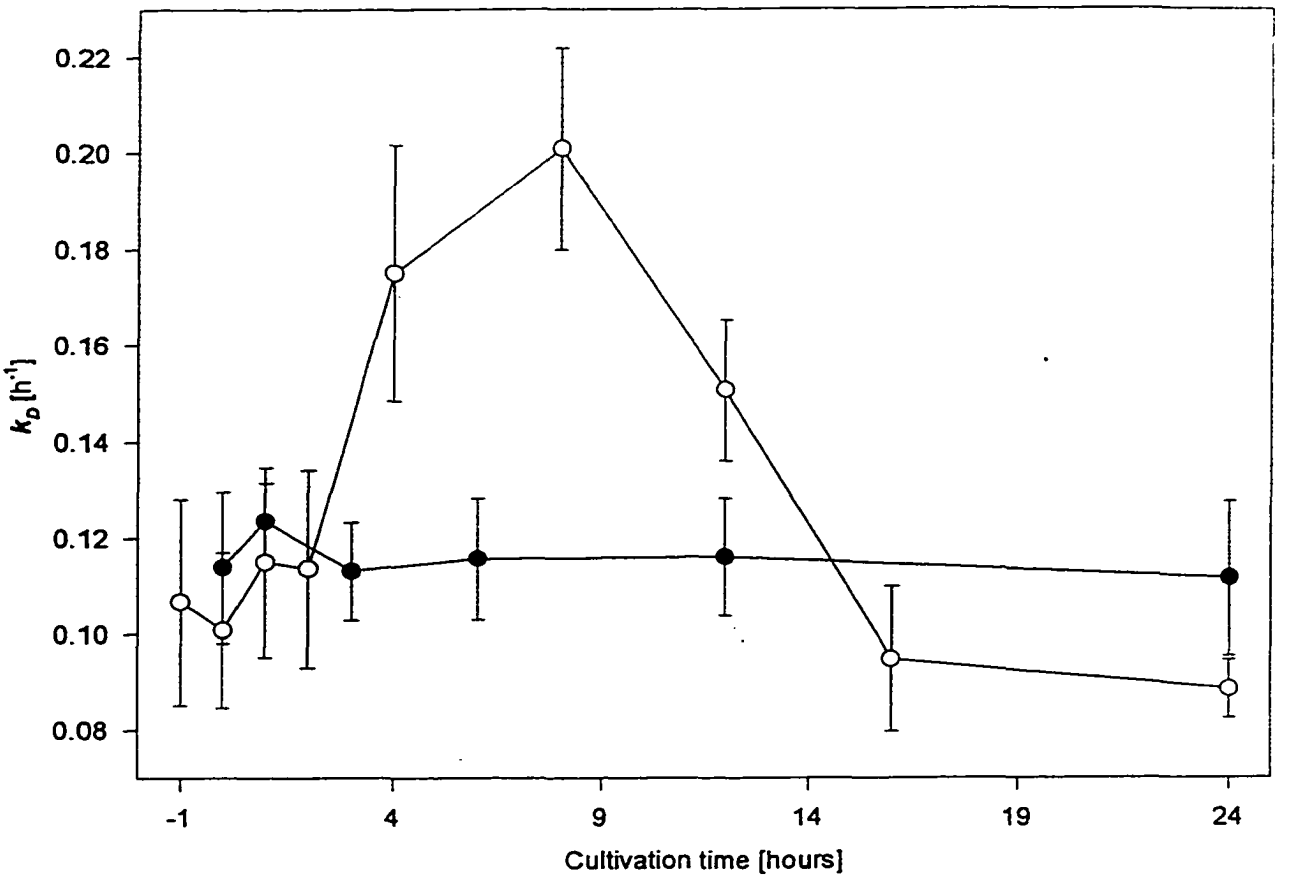

Figure 12. Rate constant of ubiquitin degradation (k_D) during constant conditions and after a cold treatment.

Rate constant of ubiquitin degradation (k_D) during constant conditions (●) and after a cold treatment (O). Error bars represent standard deviation of a mean calculated from 3 (constant conditions) and 2 (cold treatment) independent experiments.

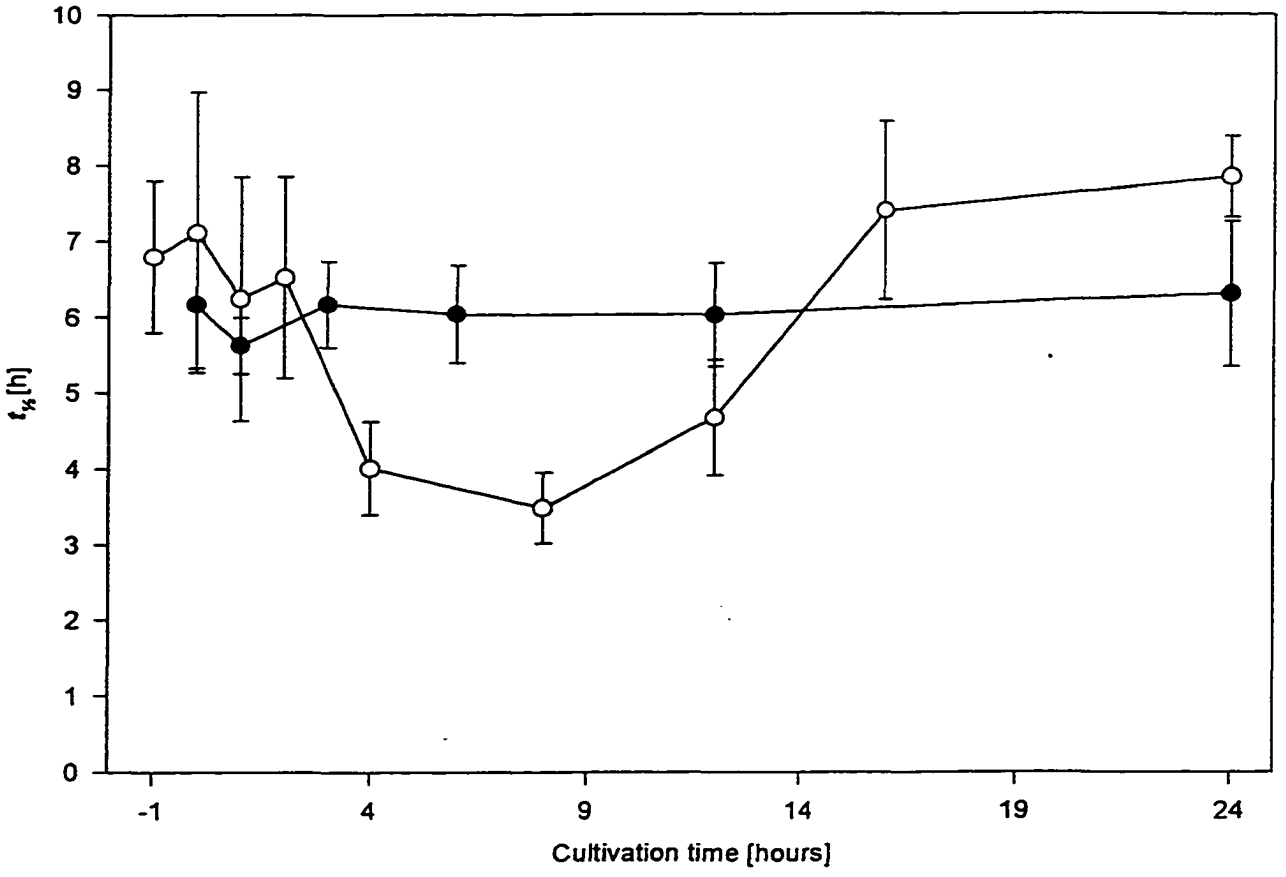

Figure 13. Half-life of ubiquitin ($t_{½}$) during constant conditions and after a cold treatment. Half-life of ubiquitin ($t_{½}$) during constant conditions (●) and after a cold treatment (○). Error bars represent standard deviation of a mean calculated from 3 (constant conditions) and 2 (cold treatment) independent experiments.

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IMAGE EVALUATION
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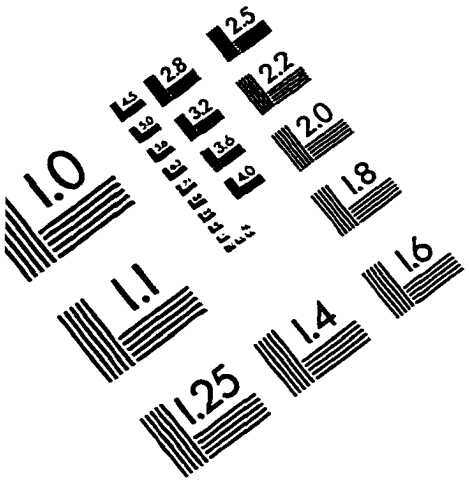

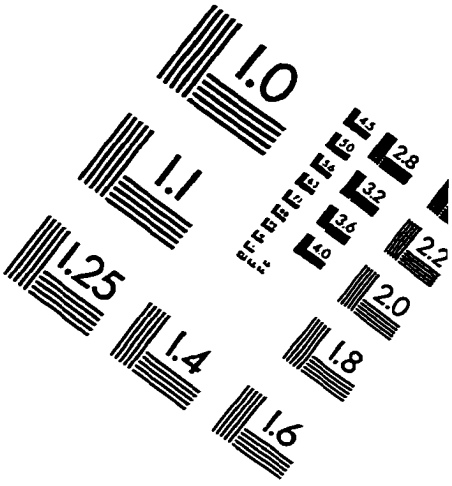

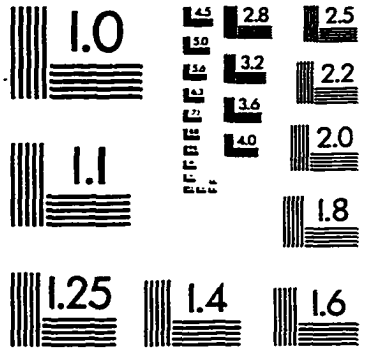

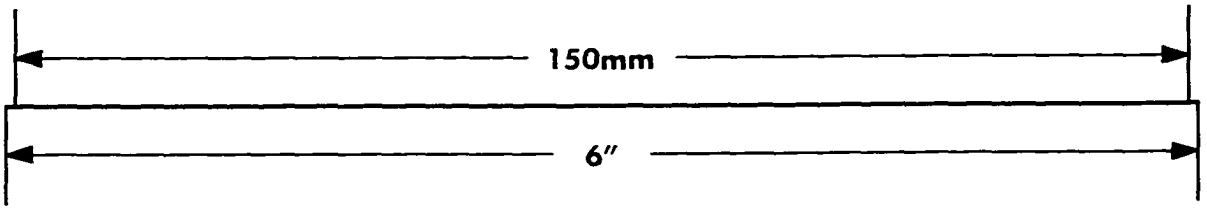

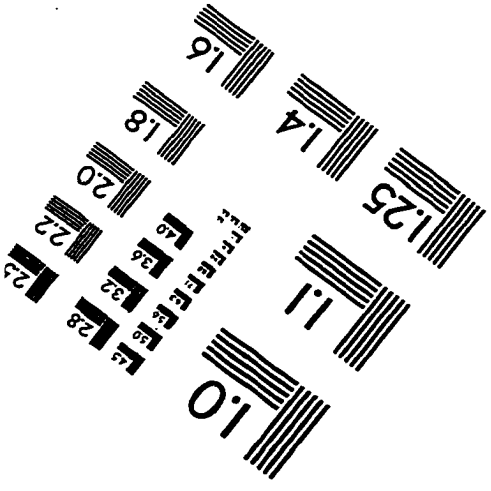

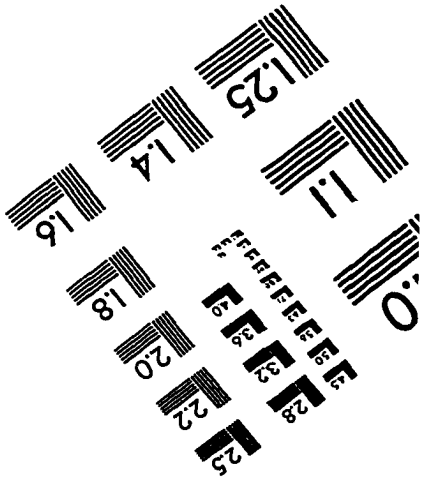

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