Biochem. J. (1988) 251, 483–490 (Printed in Great Britain)



# Regulation of protein turnover versus growth state

## Studies on the mechanism(s) of initiation of acidic vacuolar proteolysis in cells of stationary ascites hepatoma

Luciana TESSITORE,* Gabriella BONELLI,* Giovanni CECCHINI,* Riccardo AUTELLI,* Joseph S. AMENTA† and Francesco M. BACCINO*‡

*Department of Experimental Medicine and Oncology, General Pathology Section, Torino University, Corso Raffaello 30, 10125 Torino, Italy, and †Department of Pathology, Pittsburgh University Medical School, Pittsburgh, PA 15261, U.S.A.

1. After transplantation, the rat AH-130 Yoshida ascites hepatoma enters a phase of exponential (log) growth, followed by a quasi-stationary (sta) state. Combining measurements made *in vivo* and *in vitro*, cessation of protein accumulation (growth) in sta phase has previously been shown to result from convergent reduction of protein synthesis and enhancement of protein breakdown [Tessitore, Bonelli, Cecchini, Amenta & Baccino (1987) Arch. Biochem. Biophys. **255**, 372–384]. 2. One day after labelling in the animal with [$^3$H]leucine, AH-130 cells were processed for short-term assays *in vitro* to measure rates of endogenous protein breakdown. 3. Exposure of AH-130 cells to inhibitors interfering with different steps of the acidic vacuolar pathway (AVP) showed that: (i) in log tumour cells the AVP was extensively suppressed; (ii) in sta tumour cells virtually all of the proteolytic acceleration was accounted for by activation of the AVP. 4. Treating log tumour cells with glucagon, cyclic AMP, or nutritional deprivation failed to elevate substantially the proteolytic rates. Nor could the elevation in proteolysis be explained by changes in free amino acids, which were more concentrated in the ascitic fluid of sta tumours. 5. The enhanced proteolysis in sta tumour cells was not associated with any increase in the intracellular activity levels of lysosomal cathepsins B, D, H, and L. 6. The above growth-related modulation of protein breakdown in AH-130 cells was probably a reflection of the tumour growth state rather than the direct effect of environmental stimuli.

## INTRODUCTION

Previous studies have shown that a rat ascites hepatoma (Yoshida AH-130) is a suitable model to investigate, both in the animal and *in vitro*, the alterations in protein turnover associated with growth-state transitions (Tessitore *et al.*, 1987*b*). After transplantation in the peritoneal cavity, this tumour enters a period of exponential (log) growth, followed by a quasi-stationary (sta) phase. Measuring protein radioactivity decay in the animal after labelling with [$^{14}$C]bicarbonate or [$^3$H]-leucine showed that the growth-state transition was associated with a change in the turnover of cell protein (slow-turnover pool), resulting from a combined reduction of synthesis rates and acceleration of breakdown rates. Associated with the accelerated protein degradation (PD) in the quasi-steady state neoplastic population was an appreciable rate of cell turnover suggesting a unique, but as yet undefined, relationship between cellular proteolysis and cell death.

By using our experimental model (*in vivo–in vitro*) (Tessitore *et al.*, 1987*b*), we observed that the elevation in protein degradation rates in sta tumours is entirely due to an activation of the acidic vacuolar proteolysis (AVP), which, by contrast, is totally suppressed in cells from log tumours. Briefly, cell proteins were labelled in the animal with [$^3$H]leucine during the log or sta growth phase and 24 h later cells were harvested and analysed *in vitro* in short-term assays. This combination of labelling *in vivo* and assaying *in vitro* offered two important advantages: (*a*) the rapid assays *in vitro* on the just-harvested cells provided quasi-instant estimates of protein degradation rates occurring in the animal; (*b*) the combined procedures offered a tool to extrapolate data directly from an analysis *in vitro* for the evaluation of mechanisms and factors involved in the regulation of protein turnover in the animal.

In the present paper this tool has been used for a detailed study of the proteolytic mechanisms operating in AH-130 cells and the factors responsible for the degradative alterations associated with growth-state transitions. The results support the hypothesis that the activation of AVP observed in sta ascites tumours resulted from intrinsic alterations (reflecting the growth state of the neoplastic population) and not from changes in the activity of lysosomal enzymes, alterations of amino acid concentration in the ascites fluid, or shifts in a number of other recognizable environmental signals. The possible relation of this high rate of AVP and associated cell death is discussed.

## MATERIALS AND METHODS

### Chemicals

L-[4,5-$^3$H(N)]leucine (59 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.), Picofluor 30 from Packard Instruments International

Abbreviations used: log, exponential (logarithmic) growth; sta, stationary (plateau) growth; AVP, acidic vacuolar pathway (for protein degradation); PD, protein degradation.

‡ To whom correspondence should be addressed.

(Zurich, Switzerland), proteinase inhibitors and peptidic substrates from Protein Research Foundation (Osaka, Japan), Dulbecco's minimal essential medium from Flow Laboratories (Irvine, U.K.) and other chemicals from Sigma (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Federal Republic of Germany). Carboxybenzoyl-Phe-Phe-$CHN_2$ was a generous gift from Dr. Elliot Shaw (Friedrich Miescher-Institut, Basel, Switzerland).

### Tumour growth

Yoshida ascites tumours were grown in male Wistar rats as previously described (Tessitore *et al.*, 1987*b*). In brief, this tumour was maintained by weekly intraperitoneal transplantation of approx. $3 \times 10^7$ cells, under aseptic conditions, discarding any occasional haemorrhagic tumour. The transplantation schedule was as follows: $10^8$ cells from log tumours were transplanted into rats, and cells were harvested 4 days later, before the end of the log growth phase. These cells were then transplanted into the experimental animals.

Transplanted animals, assigned to the experimental groups on a randomized basis, were killed by decapitation at different time intervals. The ascitic tumours were harvested with a syringe, adding 1/9 vol. of 0.135 M-sodium citrate as anticoagulant. The peritoneal cavity was then carefully washed with 5 ml of phosphate-buffered saline (0.14 M-NaCl/2.7 mM-KCl/10 mM-$Na_2HPO_4$/10 mM-$KH_2PO_4$, pH 7.2) to recover as many tumour cells as possible. Cells were counted with a Coulter counter Model ZM, equipped with a 100 $\mu$m diameter aperture tube, and cell viability was determined by Trypan Blue exclusion (Seglen, 1976).

Macrophages were separated from 3-day tumours by adhesion to a glass surface (Pretlow *et al.*, 1975). For this purpose, AH-130 cell suspensions were incubated in rotating glass bottles for 2 h at room temperature in a suspension buffer (Seglen, 1976) containing 70 $\mu$M-$CaCl_2$; the attached cells were then released with a further 1 h incubation in calcium-free medium.

### Endogenous proteolysis

Using the combined model (*in vivo–in vitro*) developed in our laboratory (Tessitore *et al.*, 1987*b*), cells from log or sta tumours were labelled with [$^3$H]leucine in the animal. These cells were harvested 24 h later and incubated for 2 h at 37 °C in ascites fluid from non-labelled paired tumours with added 5 mM cold L-leucine. Inhibitors were added directly to the ascitic fluid, as described in the Results section, and the rate of proteolysis was evaluated over the 2 h incubation period. Radioactivity measurements were made on trichloroacetic acid precipitates and soluble phases, as previously described (Tessitore *et al.*, 1987*b*).

### Enzyme assays

Enzyme activities were all assayed in the presence of 0.1% (w/v) Triton X-100. $\beta$-*N*-Acetylhexosaminidase (EC 3.2.1.30), acid DNAase (EC 3.1.4.6), acid phosphatase (EC 3.1.3.2), $\beta$-galactosidase (EC 3.2.1.23), cathepsin D (EC 3.4.23.5), cathepsin B (EC 3.4.22.1) (with benzoyl-D,L-arginine-2-naphthylamide as substrate) and cathepsin L (EC 3.4.22.15) (with azocasein as substrate) were determined as previously reported (Baccino *et al.*, 1982*a*,*b*). Cathepsin H (EC 3.4.22.16) was measured by the method of Barrett & Kirschke (1981) and peroxidase (EC 1.11.1.7) by the method of Herzog & Fahimi (1973). Cathepsins B and L activities were also assayed by fluorometric procedures with carboxybenzoyl-L-Arg-L-Arg-4-methylcoumaryl-7-amide and carboxybenzoyl-L-Phe-L-Arg-4-methylcoumaryl-7-amide as substrates respectively (Barrett & Kirschke, 1981). 5 $\mu$M-Carboxybenzoyl-L-Phe-L-Phe-$CHN_2$ was used as selective inhibitor to discriminate between the two activities (Kirschke & Shaw, 1981). Further details of enzymic assays have been previously reported (Baccino *et al.*, 1982*a*,*b*).

Tumour cell homogenates prepared by sonication in water were used for most assays. For cysteine proteinases, however, whole cell suspensions were added directly into the incubation mixture, which contained the substrate, 5 mM-dithiothreitol as activating agent, and the proper buffer. This procedure has been developed to prevent any loss of activity on homogenization (Bonelli *et al.*, 1986).

All activities are expressed in kat, except for cathepsins D and L (the latter when assayed with azocasein), for which 1 unit of activity corresponds to an increase of one absorbance unit at 280 or 366 nm, respectively, in the acid-soluble phase from a reaction volume of 6 or 2 ml.

### Chemical assays

Protein was determined by the method of Hartree (1972) and DNA by that of Fiszer-Szafarz *et al.* (1981), bovine serum albumin and calf thymus DNA being the working standards, respectively. Free amino acids in blood plasma and ascitic fluid were analysed by a chromatographic procedure described by Mondino (1969).

## RESULTS

### Effect of lysosomal and energy metabolism inhibitors on proteolysis

As previously reported (Tessitore *et al.*, 1987*b*), during the log phase, the transplanted tumour cells grew at a rate of approx. 3%/h for 4–5 days and reached a sta state by days 6–7. The proteolytic rate of cells from log tumours averaged 0.68%/h and increased to 1.36%/h in cells from sta tumours (Table 1); these rates, obtained during incubations carried out *in vitro*, were quite close to the degradative rates measured in the animal (Tessitore *et al.*, 1987*b*).

The ascites tumour cells were treated *in vitro* with agents known to interfere with the AVP at all of the known steps in this mechanism. For the sake of simplicity, we can distinguish 'late' from 'early' inhibitors, the former being those which interfere with the final proteolytic events in the lysosome. In view of its effectiveness in suppressing the AVP by increasing the intralysosomal pH (Seglen, 1983), ammonia was used as the reference 'late' inhibitor. At a concentration of 40 mM, this agent inhibited the proteolysis rate in cells from sta tumours only, reducing it virtually to the level observed in log growth cells (Fig. 1*a* and Table 1). Leupeptin also inhibited the increased proteolysis of cells from sta tumours, though less effectively than ammonia (Fig. 1*a* and Table 1). Neither of these agents appreciably affected the proteolytic rate observed in cells from log tumours.

These observations suggested that the AVP for PD was inactive in cells from log tumours and that the

**Table 1. Effects of lysosomal inhibitors on endogenous proteolysis in AH-130 cells**

Cells from log or sta tumours, harvested 24 h after labelling in the animal with [$^3$H]leucine and incubated for 2 h at 37 °C in ascitic fluid from non-labelled paired tumours in the absence (controls) and in the presence of the inhibitors listed (all assays in duplicate). Proteolytic rates were measured as generation of acid-soluble $^3$H radioactivity, expressed as fraction (%/h) of the initial total protein radioactivity. Data are presented as percentages ± S.D. of internal controls, all standardized on the basis of the general mean for log controls (0.68 ± 0.05%/h). The numbers of tumours are given in parentheses. By the end of the experimental period cell viability was not less than 99% (Trypan Blue exclusion test). Fractional rates were estimated by linear regression analysis. Differences in fractional rates were evaluated by calculating the correlation coefficients, which were then tested using the Student's *t*-test (Lee & Lee, 1982). Significance of the differences versus internal controls: $*P < 0.05$, $**P < 0.01$. The amino acid mixture was L-leucine/L-phenylalanine/L-tyrosine/L-tryptophan/L-histidine/L-asparagine/L-glutamine, each at 5 mM.

| Inhibitor | $^3$H radioactivity (% of initial radioactivity/h) | |
|---|---|---|
| | Log tumours | Sta tumours |
| None | 100 ± 7 (16) | 200 ± 28 (18) |
| $NH_4Cl$, 40 mM | 98 ± 4 (6) | 114 ± 14 (4)** |
| Leupeptin, 0.5 mM | 98 ± 8 (4) | 146 ± 15 (6)** |
| Pepstatin, 0.5 mM | 94 (2) | 188 (2) |
| Pepstatin, 0.5 mM + leupeptin, 0.5 mM | 103 (2) | 142 (2) |
| Amino acid mixture | 94 ± 7 (4) | 156 ± 12 (4)* |
| Leucine, 10 mM | 106 ± 6 (4) | 152 ± 22 (6)** |
| Asparagine, 10 mM | 102 ± 9 (4) | 158 ± 24 (7)** |
| Cycloheximide, 1 mM | 101 ± 13 (4) | 142 ± 32 (6)** |
| 6-Dimethylaminopurine riboside, 1 mM | 94 ± 4 (4) | 150 ± 24 (6)** |
| Puromycin aminonucleoside, 1 mM | 106 (2) | 148 ± 38 (6)** |
| 3-Methyladenine, 10 mM | 100 (2) | 140 ± 26 (4)** |
| Insulin, 100 munits/ml | 101 ± 6 (4) | 178 (2) |

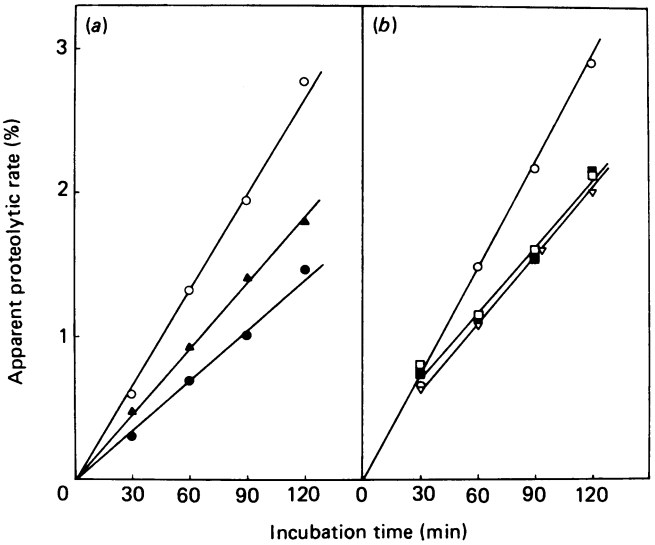


**Fig. 1. Kinetics of the effects of lysosomal inhibitors on endogenous proteolysis in AH-130 cells**

Prelabelled cells from sta tumours were incubated with the following additions. (*a*) Controls (○), 40 mM-$NH_4Cl$ (●), 1 mM-leupeptin (▲). (*b*) Controls (○), 10 mM-leucine (■), 10 mM-asparagine (□), 1 mM-6-dimethylaminopurine riboside (▽). Each point is the mean of two tumours (assayed in duplicate).

increase in proteolytic rates observed in cells from sta tumours was due to activation of the AVP. Both conclusions were substantiated by additional experiments in which tumour cells were exposed to a variety of other inhibitors. These are currently assumed (Seglen, 1983) to interfere in various ways with different steps in the same PD pathway; namely intravacuolar sequestration of substrates, fusion of autophagosomes with lysosomes and intravacuolar protein breakdown. All of these inhibitors, while suppressing proteolytic rates in sta tumour cells to different degrees, did not affect proteolysis in log tumour cells (Table 1). At variance, no effect on proteolytic rates in either log or sta tumour cells was exhibited by pepstatin, a group-specific aspartic proteinase inhibitor, used alone or in combination with leupeptin (Table 1). Presumably, the experimental period was too short to allow any significant amount of this drug to enter cells by pinocytosis (Shaw & Dean, 1980).

Different inhibitors of the autophagic process were used in relation to their specific effects (Seglen, 1983), such as inhibition of sequestration (amino acid mixtures, leucine, 3-methyladenine) or fusion (asparagine) or suppression of protein synthesis (cycloheximide). In sta tumour cells (Table 1) all of these agents afforded a similar degree of inhibition (25–30%), which was not additive with that effected by ammonia (results not shown). The inhibition was not as prompt as that afforded by ammonia or leupeptin, however, as judged by extrapolation; a lag period of approx. 30 min was always necessary to attain the maximal effect (Fig. 1*b*). This lag period probably reflected the time required for cells to degrade the protein substrates already sequestered intravacuolarly when exposed to inhibitors. In general, these inhibitors of early steps in the AVP for PD, such as sequestration or fusion, affected proteolytic rates less than ammonia. Either these 'early' inhibitors lack the ability to block the AVP as effectively as 'late' inhibitors do, or proteins are supplied to this pathway by more

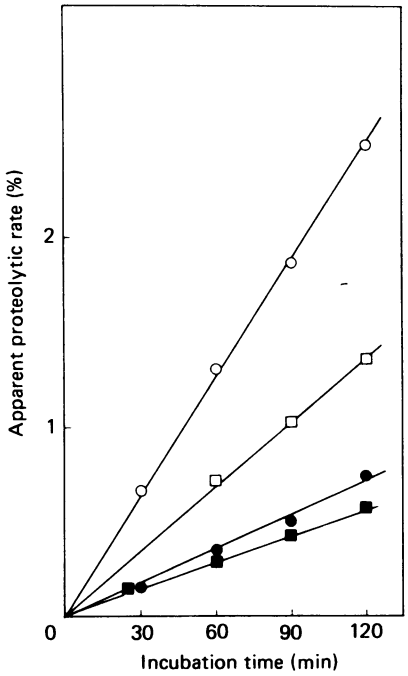


**Fig. 2. Effect of rotenone on endogenous proteolysis in AH-130 cells**

Prelabelled cells from log (□, ■) or sta (○,●) tumours. Controls (open symbols), 0.25 mM-rotenone (closed symbols). Each point is the mean of two tumours (assayed in duplicate). Similar results were obtained with other energy metabolism inhibitors such as deoxyglucose, iodoacetate, and NaF–KCN (not shown). By the end of the experimental period, the viability of cells incubated with rotenone was 89% (Trypan Blue exclusion test).

**Table 2. Free amino acid concentrations (μmol/l) in the AH-130 ascitic fluid**

Six animals were tested per group.

| Amino acid | Concentration (μmol/l) Day 4 | Day 10 |
|---|---|---|
| α-Amino-n-butyric acid | 10±2 | 10±4 |
| Alanine | 520±99 | 722±200 |
| Arginine | 67±15 | 104±30 |
| Asparagine | 8±3 | 46±18 |
| Aspartic acid | 13±5 | 8±3 |
| Citrulline | 39±6 | 96±21 |
| Cyst(e)ine | 18±4 | 36±11 |
| Glutamine | 1±1 | 92±23 |
| Glutamic acid | 57±15 | 71±24 |
| Glycine | 572±168 | 543±122 |
| Histidine | 66±10 | 88±5 |
| Isoleucine | 11±7 | 94±36 |
| Leucine | 23±10 | 210±86 |
| Lysine | 462±100 | 419±69 |
| Methionine | 16±3 | 29±10 |
| Ornithine | 19±5 | 45±7 |
| Phenylalanine | 33±7 | 61±8 |
| Proline | 172±27 | 301±113 |
| Serine | 53±14 | 48±15 |
| Taurine | 102±23 | 121±40 |
| Threonine | 137±35 | 176±38 |
| Tryptophan | 34±13 | 37±19 |
| Tyrosine | 33±6 | 67±20 |
| Valine | 25±11 | 249±102 |
| Total amino acids | 2559±371 | 3667±187 |

than one mechanism and some of the 'early' inhibitors do not affect all of them (cf. Seglen & Gordon, 1984).

Energy metabolism inhibitors extensively suppressed PD in cells from both log and sta tumours, without any appreciable lag period (Fig. 2), down to residual rates which did not markedly differ in the two growth states. Combining these with the above observations, it appears as if the overall PD in these tumour cells resulted from three major components: (1) one energy-dependent and operated by the AVP; (2) another energy-dependent, but not taking place in the AVP; (3) a third, neither energy-dependent nor operated by the AVP. Of these, only the first one appears to be subjected to extensive modulations associated with the growth state.

### Study of medium factors as possible signals for proteolytic regulations

To account for the differences in proteolytic rates between cells in different growth phases, we first considered the composition of the ascitic fluid. Since amino acids have been shown to affect AVP (Seglen & Gordon, 1984; Mortimore & Pösö, 1986), we first compared the free amino acid composition of the ascites fluid. The overall amino acid concentration was markedly lower in the fluid from log tumours (Table 2), while amino acid concentrations in sta tumour fluid generally approached those in blood plasma (not shown). Such observed values likely reflect both the elevated amino acid requirement of log growing tumours, on the one hand, and the higher generation of amino acids from protein breakdown in sta tumours, on the other. Therefore, AVP activation in sta tumour cells was apparently not elicited by shortage of amino acids.

In sta tumours, the ascites fluid is known to contain considerable amounts of lactic acid and to be depleted of glucose and oxygen (Del Monte & Rossi, 1963; Olivotto & Paoletti, 1981). Incubating log tumour cells in ascitic fluid derived from sta tumours and vice versa resulted in small effects on proteolytic rates in the direction expected. However, the extent of the changes observed was far from that which would fully account for the differences in proteolytic rates between log and sta tumour cells (Table 3).

Incubation of sta tumour cells at 37 °C in air, in a medium composed of homologous ascitic fluid diluted 1:5 with a saline solution enriched with glucose, is adequate for cells to resume the progression into cycle up to the S phase (Olivotto & Paoletti, 1980). However, a 2 h incubation in such a medium did not reduce proteolytic rates in sta tumour cells.

The possibility that nutritional deprivation itself could cause the acceleration of PD in sta tumour cells was ruled out by experiments in which log tumour cells were incubated in the above medium, containing ascitic fluid diluted 1:5 with a saline solution (Olivotto & Paoletti, 1980), or even in saline alone. An acceleration of proteolytic rates was barely detectable. However, under these circumstances, an appreciable fraction of proteolysis was then suppressed by ammonia, which had no effect on control cells (Fig. 3). The results might suggest

**Table 3. Effect of different incubation conditions on proteolytic rates of AH-130 cells**

Proteolytic rates expressed as percentages of the values measured in complete, homologous ascitic fluid (see Table 2). The numbers of experiments are given in parentheses. *$P < 0.05$.

| Conditions used | Proteolytic rate (%) | |
|---|---|---|
| | Log tumours | Sta tumours |
| Complementary ascitic fluid† | 119±14 (4)* | 180±12 (4) |
| Saline medium‡ | 115±17 (8)* | 208±34 (4) |
| Glucagon,§ 0.01 μM | 100 (2) | 196 (2) |
| 0.1 μM | 109 (2) | – |
| 1 μM | 115±4 (3)* | – |
| Dibutyryl cyclic AMP, § 0.1 mM | 14±6 (4)* | 194±16 (4) |

† Cells from log tumours incubated in ascitic fluid from sta tumours and vice versa.

‡ See the Materials and methods section for composition of this solution (Seglen & Gordon, 1984).

§ Glucagon and dibutyryl cyclic AMP added to homologous ascitic fluid.

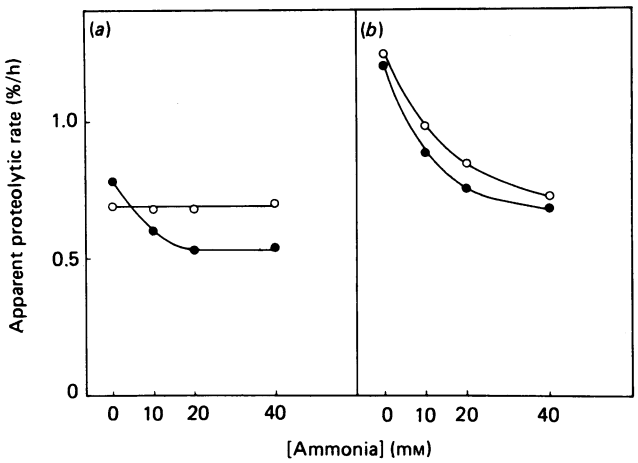


**Fig. 3. Effects of the incubation in saline medium on endogenous proteolytic rates in log (*a*) and sta (*b*) tumour cells**

Prelabelled cells were incubated for 2 h in ascitic fluid (○) or saline (●) in the presence of $NH_4Cl$ at the concentrations indicated. Each point is the mean of two tumours (assayed in duplicate).

that, when log tumour cells were placed in saline medium, some of the PD normally occurring in a non-lysosomal basal mechanism was shifted over to the AVP. We are not aware that such a shift of PD from basal to AVP has been previously reported.

We also attempted to modify proteolytic rates of log AH-130 cells through the action of 'environmental' agents, such as glucagon or dibutyryl cyclic AMP (Table 3), which induce autophagy in different cell types (e.g. Woodside *et al.*, 1974; Mortimore & Pösö, 1986). These agents, however, had little or no effect on proteolysis in either log or sta tumour cells (for glucagon resistance of hepatoma cells, cf. Fehlmann *et al.*, 1983).

## Lysosomal hydrolase activities

The specific activities of a number of acid hydrolases in AH-130 cells are reported in Table 4, which also lists activities in adult rat liver tissue for purposes of comparison. On a total protein basis, activity levels in ascites hepatoma cells were much lower than in liver tissue. For some activities, including cathepsins B, D, and L, the differences approximated to an order of magnitude. Of interest is the fact that such differences by far exceed those observed between normal adult and regenerating or developing rat liver (Baccino *et al.*, 1982*a*).

Table 4 also compares specific activity data for log and sta tumour cells, while Fig. 4 details the specific activities in the course of tumour growth. The emerging pattern is

**Table 4. Lysosomal hydrolase and peroxidase activities in AH-130 ascites hepatoma cells and adult rat liver**

| Enzyme | Activity (pkat/mg of protein)† | | |
|---|---|---|---|
| | Log AH-130 (Day 3) | Sta AH-130 (Day 9) | Rat liver |
| Cathepsin B | 1.33±0.15 (9) | 0.61±0.11** (7) | 19.3±4** (14) |
| | 0.29±0.07 (6)‡ | 0.14±0.02** (6)‡ | |
| Cathepsin D | 45.0±8.8 (8) | 44.4±3.8 (8) | 360±59** (16) |
| Cathepsin H | 1.17±0.13 (6) | 0.83±0.06** (6) | – |
| Cathepsin L | 6.69±1.3 (8) | 5.40±2.7 (6) | 80.0±9** (8) |
| | 0.48±0.09 (6)‡ | 0.39±0.03** (6)‡ | |
| β-Galactosidase | 8.43±1.1 (8) | 11.8±1.4* (7) | 30.5±4** (15) |
| Acid phosphatase | 54.4±6.4 (8) | 67.8±8.0 (8) | 581±74** (16) |
| Acid DNAase | 52.0±7.5 (9) | 61.6±4.5 (8) | 97.2±23** (14) |
| β-*N*-Acetylhexosaminidase | 59.3±5.4 (8) | 70.7±8.1 (8) | 193±54** (12) |
| Peroxidase | 1049±159 (9) | 304±94** (9) | – |
| Protein/DNA (mg/mg) | 37±6 (4) | 41±8 (4) | 100±7 (10) |
| Dry weight (mg/$10^6$ cells) | 0.36±0.07 (5) | 0.40±0.10 (6) | – |

† Activities/mg of protein are expressed in pkat, except for cathepsins D and L (units × $10^{-7}$). Student's *t*-test was calculated between log and sta AH-130 cells (asterisks in the second column) or between sta AH-130 cells and adult rat liver (asterisks in the third column). *$P < 0.05$, **$P < 0.01$. The numbers of animals are given in parentheses.

‡ Fluorometric assay (see the Materials and methods section).

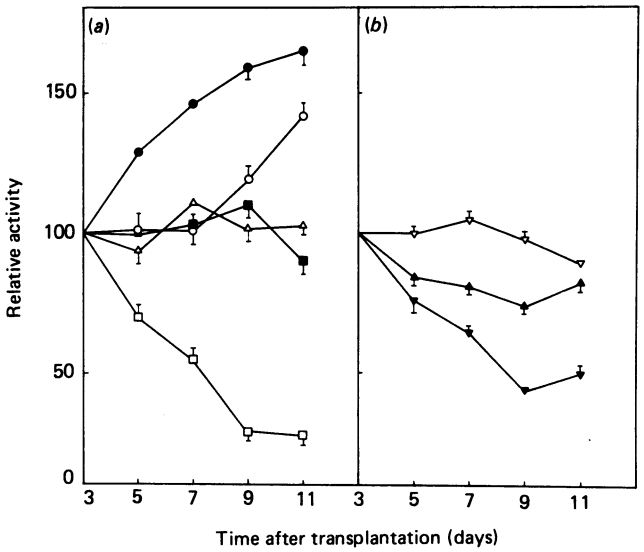


**Fig. 4. Cellular levels of peroxidase and hydrolase activities during the growth of the AH-130 tumour**

Activities per mg of protein are expressed as percentages of the values for 3-day tumours. (*a*) β-Galactosidase (●), β-*N*-acetylhexosaminidase (○), acid DNAase (△), acid phosphatase (■), and peroxidase (□) activities. (*b*) Cathepsin B (▼), cathepsin D (▽), and cathepsin L (▲) activities. Each point is the mean of 9–25 tumours. Vertical bars denote S.E.M. (some of them are concealed by the symbols).

quite complex and suggestive for marked individual heterogeneity in the regulation of lysosomal hydrolases (cf. Baccino *et al.*, 1982*a*,*b*). The activity of β-galactosidase markedly and steadily increased during tumour growth, while that of β-*N*-acetylhexosaminidase increased only in correspondence with the sta phase. Cathepsin D, acid DNAase, and acid phosphatase activities remained essentially constant, except for a slight decrease of acid phosphatase in late stages. By contrast, cathepsin L and, particularly, cathepsin B activities, progressively decreased during tumour growth and levelled on attainment of the sta phase. Also cathepsin H activity was reduced in sta compared with that in log tumour cells (Table 4). The data on lysosomal proteinases should be considered with caution, since measurements could have been affected by cytoplasmic inhibitors (e.g. Katunuma & Kominami, 1983). To minimize such an interference, however, in the present assays whole cells were disrupted directly in the incubation mixture (Bonelli *et al.*, 1986).

A possible explanation for the reduction of cell proteinase activities with the progress of tumour growth might have been the declining contamination of the neoplastic population with reactive cells (Tessitore *et al.*, 1987*b*). Peroxidase activity, which is often taken as a marker for inflammatory cells, did indeed strongly decline with time (Fig. 4). In an additional study, macrophages were purified from the ascitic populations and compared with tumour cells for enzyme activities. As shown in Table 5, differences in activities were relatively low for cathepsins B and D, as well as for peroxidase, while macrophages exhibited higher levels of cathepsin L activity. On the whole, values of enzyme activities in the macrophages (perhaps not yet fully matured; cf. Mørland & Pedersen, 1979) were close to the enzyme activities observed in the AH-130 tumour cells. Therefore, the decrease in some enzyme activities occurring in the total cell population over the course of the experiment cannot be accounted for by the shift in the relative proportion of tumour and reactive cells.

## DISCUSSION

Growth-associated regulations of cell PD have consistently been found to result from changes in the activity of AVP (e.g. Amenta *et al.*, 1976, 1977*a*; Warburton & Poole, 1977; Sparkuhl & Sheinin, 1980; Hendil, 1981; Baccino *et al.*, 1982*c*, 1984; Polet, 1983; Tushinski &

**Table 5. Enzyme activities in macrophages isolated from AH-130 ascites tumours 3 days after transplantation**

Macrophages separated from tumour cell suspensions, identified by morphological and cytochemical criteria, and counted as specified in the Materials and methods section. $n = 6$. Enzyme activities are expressed as pkat (cathepsin B and peroxidase) or units $\times 10^{-7}$ (cathepsins D and L) per mg of protein. The data in the 2nd and 3rd columns are expressed as percentages of those in the 1st column.

| | Total cell suspension | Cell suspension after removal of macrophages | Macrophages purified from tumours |
|---|---|---|---|
| Cell composition (%) | | | |
| Tumour cells | 92.5±0.5 | 97.9±0.4 | 0 |
| Macrophages | 5.0±0.4 | 0.4±0.1 | 99.0±0.4 |
| Granulocytes | 2.2±0.8 | 1.2±0.2 | 1.0±0.3 |
| Lymphocytes | 0.3±0.2 | 0.5±0.2 | 0 |
| Enzyme activity (pkat/mg of protein) | | | |
| | 1.36±0.4 | 94±20**† | 135±8**‡ |
| Peroxidase | 1654±468 | 97±15 | 126±45 |
| $10^7$ × Enzyme activity (units/mg of protein) | | | |
| Cathepsin D | 42.1±8.2 | 99±24 | 104±18 |
| Cathepsin L | 6.33±0.5 | 102±25** | 547±41** |

† Significance of the difference versus macrophages (**$P < 0.01$).
‡ Significance of the difference versus total cell suspensions (**$P < 0.01$).

Stanley, 1983; Gronostajski *et al.*, 1984; Libby & O'Brien, 1984; Spanier *et al.*, 1984). The increased proteolysis we observed in Yoshida ascites tumours in sta phase is another example of this growth-associated regulation of PD by the AVP.

The AVP can be discriminated from other pathways for cell PD by the use of selective inhibitors, as shown by a large body of evidence accumulated in the past decade (Amenta *et al.*, 1977*a*, 1978; Seglen, 1983). The AVP itself has been further dissected with agents believed to act at different steps in this process (Seglen, 1983). The present results, obtained with a series of such inhibitors, are in general agreement with the existing published results, suggesting that in highly deviated tumour cells, such as the AH-130, the AVP does not mechanistically differ from that in normal cells, particularly in hepatocytes (Seglen *et al.*, 1979; Seglen & Gordon, 1984). Consistently, the fractional volume of autophagic vacuoles has been found to be increased 4-fold in sta compared with log tumour cells (U. Pfeifer, F. M. Baccino, L. Tessitore, G. Bonelli & F. M. Baccino, unpublished work).

The final effectors of PD in the AVP are a battery of endo- and exo-peptidases (Tanaka *et al.*, 1984) with an optimum pH ranging from neutral to very acidic (pH 3.0–3.5). Admittedly, the rate-limiting step in AVP is the sequestration of substrates into the vacuolar system, by autophagy or related processes, rather than the availability of enzymes (Baccino *et al.*, 1982*c*; Spanier *et al.*, 1984). In many cell types the proteolytic potential of the lysosomal endo- and exo-peptidases apparently far exceeds the needs for PD. In spite of evidence consistent with the opposite view (e.g. Amenta *et al.*, 1977*b*, 1978; Baccino *et al.*, 1981, 1982*a*,*b*; Kirschke & Shaw, 1981), rates of PD often have little or no relation to levels of enzyme activities (e.g. Baccino *et al.*, 1982*b*; Spanier *et al.*, 1984). This conclusion is further strengthened if one considers that, while the PD rate in sta AH-130 cells was quite close to the average daily rate in adult liver tissue (Baccino *et al.*, 1982*b*), lysosomal proteinase and other hydrolase activities were much lower in AH-130 tumour cells; in most cases about an order of magnitude. On the other hand, hepatocytes as well as other cells have the ability to extensively modulate the AVP, even achieving a several-fold increase of PD over basal rates (Vidrich *et al.*, 1977; Baccino *et al.*, 1982*b*,*c*, 1984; Seglen & Gordon, 1984; Khairallah *et al.*, 1985; Mortimore & Pösö, 1986). The range allowed to tumour cells for such regulation seems much more restricted (cf. Feo & Bonelli, 1971), suggesting that the AVP in sta AH-130 cells may be operating 'at saturation'.

On the whole, these AH-130 tumour cells seem deregulated with respect to host cells, not only in terms of their ability to vary PD in response to environmental stimuli (see below), but possibly also in their lack of the enzymic tools required to operate any larger regulation than that corresponding to the observed shift between log and sta growth phases.

## Signals for the AVP modulations

The present observations leave open the question of which signals activated the AVP and accelerated PD in AH-130 cells when attaining the sta phase. Growth arrest of AH-130 tumours has been ascribed, mainly, if not entirely, to shortage of oxygen and glucose in the overcrowded peritoneal cavity (Del Monte & Rossi, 1963; Olivotto & Paoletti, 1981). The supply of nutrients by diffusion becomes progressively inadequate and the host organism quite exhausted. When AH-130 growth ceases, cachexia is already overtly established in the host (Tessitore *et al.*, 1987*a*). Oxygen and glucose are essential for energy metabolism. Yet cell PD is mostly an energy-requiring, active process, only operated by fully viable cells. Indeed, PD was largely suppressed by energy metabolism inhibitors in both log and sta AH-130 cells, being most pronounced in the latter (Fig. 2). Therefore, it is unlikely that enhanced PD in sta tumour cells may reflect impaired cell metabolism due to the lack of oxygen or glucose.

It has been suggested that amino acids, some more so than others, exert a regulatory role on PD (Seglen & Gordon, 1984; Mortimore & Pösö, 1986). Our results clearly show that amino acids were available to sta tumour cells at concentrations not lower than or, as in most instances, higher than those for log tumours. Therefore, it is unlikely that lack of nutrients or accumulation of putative toxic metabolites accounts for the difference in PD rates between log and sta AH-130 cells.

Having eliminated alternative explanations, one is left with the tentative conclusion that the differences in PD between log and sta AH-130 cells are a reflection of the tumour growth state, rather than being caused directly by environmental factors. Growth is barely controlled in this ascitic neoplasm; yet growth ceased when the cell population attained a very large size. DNA flow cytometric patterns suggest (cf. Holley *et al.*, 1976) that cessation of growth was not an expression of real growth control, since cells were distributed over the different cell-cycle phases (G1, S, G2/M) (Tessitore *et al.*, 1987*b*). Moreover, some proliferation still occurred in sta tumours, even if balanced by, and often even if exceeded by, cell loss, as suggested by DNA radioactivity decay and results from mitotic and [$^3$H]thymidine-labelling indices (Tessitore *et al.*, 1987*b*). Growth of AH-130 tumours ceases only under rather extreme conditions, when the host organism is already severely affected. Whether this depends on oxygen or glucose deprivation (Del Monte & Rossi, 1963; Olivotto & Paoletti, 1981), insufficient autocrine stimulation of growth (growth and transforming factors have been repeatedly reported to be released by ascites hepatomas), or even some residual susceptibility of AH-130 cells to hypothetical inhibitory factors, is impossible to decide. The important conclusion remains, however, that changes in PD rates in these Yoshida ascites hepatoma cells seem linked directly to their growth state rather than to any environmental stimulus.

In honour of Professor Valdo Mazzi (Dipartimento di Biologia Animale, Università di Torino), for his 70th birthday. This work was supported by grants from the Consiglio Nazionale delle Ricerche, Rome (Special Project 'Oncology', grant No. 86.00297.44; bilateral Italy–U.S.A. program, CT 86.00012.04), Ministero della Pubblica Istruzione, Rome, and Associazione Italiana per la Ricerca sul Cancro, Milan. Parts of this work have been presented in a preliminary form at the 5th International Symposium on Intracellular Protein Catabolism (Baccino *et al.*, 1985).

## REFERENCES


Amenta, J. S., Baccino, F. M. & Sargus, M. J. (1976) Biochim. Biophys. Acta **451**, 511–516

Amenta, J. S., Sargus, M. J. & Baccino, F. M. (1977*a*) Biochem. J. **168**, 223–227

Amenta, J. S., Sargus, M. J. & Baccino, F. M. (1977*b*) Biochim. Biophys. Acta **476**, 253–261

Amenta, J. S., Sargus, M. J. & Baccino, F. M. (1978) J. Cell. Physiol. **97**, 267–284

Baccino, F. M., Messina, M., Musi, M. & Tessitore, L. (1981) Acta Biol. Med. Ger. **40**, 1249–1258

Baccino, F. M., Fiszer-Szafarz, B., Messina, M., Nadal, C., Barrera, G., Guevara de Murillo, A. & Tessitore, L. (1982*a*) Biol. Cell **46**, 21–28

Baccino, F. M., Tessitore, L., Cecchini, G., Messina, M., Zuretti, M. F., Bonelli, G., Gabriel, L. & Amenta, J. S. (1982*b*) Biochem. J. **206**, 395–405

Baccino, F. M., Messina, M., Musi, M. & Tessitore, L. (1982*c*) in Recent Trends in Chemical Carcinogenesis (Pani, P., Feo, F. & Columbano, A., eds.), pp. 83–100, ESA, Cagliari

Baccino, F. M., Tessitore, L. & Bonelli, G. (1984) Toxicol. Pathol. **12**, 281–287

Baccino, F. M., Tessitore, L., Bonelli, G., Rubano, R. & Terzuolo, S. (1985) in Intracellular Protein Catabolism (Khairallah, E. A., Bond, J. S. & Bird, J. W. C., eds.), pp. 595–597, Alan R. Liss, New York

Barrett, A. J. & Kirschke, H. (1981) Methods Enzymol. **80**, 535–561

Bonelli, G., Tessitore, L., Isidoro, C., Musi, M. & Baccino, F. M. (1986) Cell Biol. Int. Rep. **10**, 209

Del Monte, U. & Rossi, C. B. (1963) Cancer Res. **23**, 363–367

Fehlmann, M., Crettaz, M. & Kahn, C. R. (1983) Biochem. J. **214**, 845–850

Feo, F. & Bonelli, G. (1971) Experientia **27**, 317–318

Fiszer-Szafarz, B., Szafarz, D. & Guevara de Murillo, A. (1981) Anal. Biochem. **110**, 165–170

Gronostajski, R. M., Goldberg, A. L. & Pardee, A. B. (1984) J. Cell. Physiol. **121**, 189–198

Hartree, E. F. (1972) Anal. Biochem. **48**, 422–427

Hendil, K. B. (1981) Exp. Cell Res. **135**, 157–166

Herzog, V. & Fahimi, H. D. (1973) Anal. Biochem. **55**, 554–562

Holley, R. W., Baldwin, J. H., Kiernan, J. A. & Messmer, T. O. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 3229–3232

Katunuma, N. & Kominami, E. (1983) Curr. Top. Cell. Regul. **22**, 71–101

Khairallah, E. A., Bartolone, J., Brown, P., Bruno, M. K., Makowski, G. & Wood, S. (1985) in Intracellular Protein Catabolism (Khairallah, E. A., Bond, J. S. & Bird, J. W. C., eds.), pp. 373–383, Alan R. Liss, New York

Kirschke, H. & Shaw, E. (1981) Biochem. Biophys. Res. Commun. **101**, 454–458

Lee, J. D. & Lee, T. D. (1982) Statistics and Numerical Methods in BASIC for Biologists, vol. 1, pp. 185–205, Van Nostrand Reinhold, New York

Libby, P. & O'Brien, K. V. (1984) J. Cell. Physiol. **118**, 317–323

Mondino, A. (1969) J. Chromatogr. **39**, 262–272

Mørland, B. & Pedersen, A. (1979) Lab. Invest. **41**, 379–384

Mortimore, G. E. & Pösö, A. R. (1986) Adv. Enzymol. **25**, 257–276

Olivotto, M. & Paoletti, F. (1980) Cell Tissue Kinet. **13**, 605–612

Olivotto, M. & Paoletti, F. (1981) J. Cell. Physiol. **107**, 243–249

Polet, H. (1983) Exp. Cell Res. **148**, 345–362

Pretlow, T. G. II, Weir, E. E. & Zettergren, J. G. (1975) Int. Rev. Exp. Pathol. **14**, 99–102

Seglen, P. O. (1976) Methods Cell Biol. **13**, 29–83

Seglen, P. O. (1983) Methods Enzymol. **96**, 737–764

Seglen, P. O. & Gordon, P. B. (1984) J. Cell Biol. **99**, 435–444

Seglen, P. O., Grinde, B. & Solheim, A. E. (1979) Eur. J. Biochem. **95**, 215–225

Shaw, E. & Dean, R. T. (1980) Biochem. J. **186**, 385–390

Spanier, A. M., Clark, W. A., Jr. & Zak, R. (1984) J. Cell. Biochem. **26**, 47–64

Sparkuhl, J. & Sheinin, R. (1980) J. Cell. Physiol. **105**, 247–258

Tanaka, K., Ikegaki, N. & Ichihara, A. (1984) J. Biol. Chem. **259**, 5937–5944

Tessitore, L., Bonelli, G. & Baccino, F. M. (1987*a*) Biochem. J. **241**, 253–261

Tessitore, L., Bonelli, G., Cecchini, G., Amenta, J. S. & Baccino, F. M. (1987*b*) Arch. Biochem. Biophys. **255**, 372–384

Tushinski, R. S. & Stanley, E. R., (1983) J. Cell. Physiol. **116**, 67–75

Vidrich, A., Airhart, J., Bruno, M. K. & Khairallah, E. A. (1977) Biochem. J. **162**, 257–266

Warburton, M. J. & Poole, B. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2427–2431

Woodside, K. H., Ward, W. F. & Mortimore, G. E. (1974) J. Biol. Chem. **249**, 5458–5463


Received 10 August 1987/4 November 1987; accepted 10 December 1987