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Increased Degradation Rates of Protein Synthesized in Hepatoma Cells in the Presence of Amino Acid Analogues

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1. Reuber H35 hepatoma cells incorporate the arginine analogue canavanine into cell protein when arginine is omitted from the incubation medium. 2. By labelling arginine-containing proteins with [^{14}C]leucine and then canavanine-containing proteins with [^{3}H]leucine in the same cells, it is possible to measure the degradation of both types of protein during a subsequent 'chase' period. With this technique it has been shown that canavanine-containing proteins are degraded at a rate severalfold greater than normal proteins. Comparable results were found when 6-fluorotryptophan was used as an analogue to tryptophan. 3. Control experiments in which the labelling order was reversed or where the amino acid and its analogue were incubated in separate cell cultures support the conclusion that aberrant proteins are rapidly degraded *in vivo*.

The large differences between rates of degradation of different proteins in mammalian tissues can occur because either the range of protein-degradation rates may reflect a range of effectiveness of the proteins as substrates for a single degrading system, or there may be several degrading systems each of which acts on a particular class of proteins (Schimke & Doyle, 1970). A prediction common to both proposals is that a protein in which the primary sequence of amino acids has been altered may be recognized as different by a proteolytic degrading sequence within the cell. The altered protein may then be degraded at a different rate to the normal protein.

Such a prediction has been confirmed in *Escherichia coli*, where altered proteins are degraded rapidly *in vivo* (Goldschmidt, 1970; Platt *et al.*, 1970; Pine, 1967; Goldberg, 1972*a*) and *in vitro* (Goldberg, 1972*b*). We wished to extend these studies to mammalian tissues and have used Reuber H35 hepatoma cells to test whether amino acid analogues could be incorporated into cell protein. We have used the arginine analogue canavanine in most experiments and have shown rapid degradation of canavanine-containing proteins in the hepatoma cells.

Experimental

Materials

Disposable plastic Petri dishes (90cm^2 surface area) were purchased from Camelec Ltd., Camden Park, S. A., Australia. Eagle's Minimal Essential Medium and Earle's salts were from Grand Island Biological Co., Grand Island, N.Y., U.S.A. Trypsin and foetal calf serum were from Commonwealth Serum Laboratories, Melbourne, Australia. Dexamethasone phosphate was from Merck, Sharp and Dohme, Granville, N.S.W., Australia. Streptomycin sulphate and penicillin G were from Glaxo–Allenburys Ltd., Boronia, Vic., Australia. NCS solubilizer was from Amersham/Searle Corp., Arlington Heights, Ill., U.S.A. L-[4,5-^{3}H]Leucine (specific radioactivity 50–60Ci/mmol) and L-[U-^{14}C]leucine (specific radioactivity 300–350mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. The amino acids and vitamins required to prepare arginine-free and tryptophan-free media, L-canavanine, DL-6-fluorotryptophan, theophylline and dibutyryl cyclic AMP were from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Cell culture conditions

Reuber H35 hepatoma cells (Reuber, 1961) adapted to tissue culture (line H4IIE; Pitot *et al.*, 1964) were obtained from Dr. J. E. Becker and Dr. V. R. Potter, University of Wisconsin, and grown under sterile conditions as monolayers at 37°C in glass culture bottles. The growth medium used was Eagle's Minimal Essential Medium (Eagle, 1959) supplemented with vitamins and amino acids at twice the original formulation, 17mM-glucose, 9% (v/v) foetal calf serum, 100mg of streptomycin/litre and 60mg of penicillin G/litre. Initially, 1×10^6–2×10^6 cells were added to 10ml of medium in each culture bottle of 80cm^2 surface area, equilibrated with a mixture of CO_2+air (5:95), and stoppered. The medium was changed every 2 days, and by 1 week the cells were

confluent. At this stage the medium was poured off, the monolayer rinsed with 10ml of Dulbecco salts solution (Paul, 1970), washed for 15s with 10ml of Dulbecco salts solution containing 0.1% trypsin and the medium poured off. When the cells were partially dislodged 10ml of Dulbecco salts solution was added and the suspension sucked up into and blown out of a pipette repeatedly for 2–3min. Approx. 1×10^6–2×10^6 of these cells were either transferred to culture bottles as described above or added to 10ml of growth medium in plastic Petri dishes and incubated at 37°C in a moist atmosphere containing CO_2+air (5:95). The medium was changed in the Petri dishes every 2 days and after 1 week each dish contained approx. 4mg of cell protein.

Measurements of protein degradation

In the three different types of experiments the cells were maintained at 37°C, equilibrated with a mixture of CO_2+air (5:95), and kept under sterile conditions.

Type 1 *experiment.* This experiment is similar to that carried out by Johnson & Kenney (1973). The growth medium was removed from confluent cells that were growing in Petri dishes, and replaced with 10ml of a serum-free medium otherwise similar to the growth medium but with arginine absent and with 0.5mM-dibutyryl cyclic AMP, 1mM-theophylline and 5μM-dexamethasone added. These compounds were added as inducers of phosphoenolpyruvate carboxykinase (GTP) so that degradation of this enzyme could be measured in the same experiments (Knowles *et al.*, 1975). After 2h, 2.5μCi of [^{14}C]leucine was added and the cells were incubated for 15h to label the newly synthesized proteins with ^{14}C. The medium was then replaced with a similar medium but with [^{14}C]leucine omitted and 0.5mM-canavanine and 20μCi of [^{3}H]leucine added. After a further 3h, this medium was removed, the dishes were rinsed three times with Dulbecco salts solution, and 10ml of growth medium with 2mM-leucine present and serum absent was added. Dishes were taken at various times during the subsequent 'chase' period for the measurement of ^{3}H and ^{14}C in released amino acids, cell amino acids and cell protein. To make these measurements, the medium was removed, the cells were scraped off the dish surface, transferred in 5ml of 0.25M-sucrose to a 10ml polypropylene tube and centrifuged at 1000*g* for 5min. The supernatant was discarded. Water (1ml) was added to the cell pellet, the mixture was homogenized in a Dounce homogenizer, frozen and thawed three times and samples were taken for the measurement of radioactivity in protein and amino acids.

Type 2 *experiment.* The aim of this experiment was to reverse the sequence of the type 1 experiment, and label the canavanine-containing protein first and the arginine-containing proteins second. The details were the same as described for type 1 experiment except that 0.5mM-canavanine was added with the inducers and [^{14}C]leucine was added 2h later. After the 15h labelling period, the medium was replaced with similar medium but with [^{14}C]leucine omitted and 0.5mM-arginine and 20μCi of [^{3}H]leucine added. The second labelling period, the washing procedures and the degradation conditions were as described for type 1 experiment.

Type 3 *experiment.* In this experiment canavanine-containing proteins and arginine-containing proteins were labelled in different culture dishes. The cells were grown and inducers added with arginine absent as described for type 1 experiment. After induction overnight (17h) the medium was replaced with a similar solution but containing 0.5mM-canavanine in one group of dishes and 0.5mM-arginine in the other. Then 20μCi of [^{3}H]leucine was added and 3h later the radioisotope and inducers were removed by rinsing the dishes three times with Dulbecco salts solution. Degradation of radioactive protein and measurement of ^{3}H in amino acids was measured as described for type 1 experiment.

6-*Fluorotryptophan experiments.* These were carried out exactly as the type 1 experiment but with 1.2mM-arginine present in all media, L-tryptophan absent and with 1mM-DL-6-fluorotryptophan replacing canavanine.

Measurement of radioactivity in amino acids in the medium

Samples (200μl) of medium from Petri dishes at different times during the degradation period were added to 50μl of 30% (w/v) trichloroacetic acid, mixed and centrifuged at 3000*g* for 5min. Portions (50μl) of the supernatant were added to 250μl of NCS solubilizer in 5ml of toluene scintillation fluid (Hopgood *et al.*, 1973) and ^{3}H and ^{14}C radioactivity was measured by liquid-scintillation spectrometry. In some experiments the radioactivity in trichloroacetic acid-insoluble material was also determined, but this 'medium protein' radioactivity was always less than 1% of that found in cell protein and was ignored.

Measurement of radioactivity in cell proteins and cell amino acids

Samples (50μl) of frozen and thawed cell homogenate were added to 100μl of 10% (w/v) trichloroacetic acid, mixed and centrifuged at 3000*g* for 5min. Portions (50μl) of the supernatant were added to 250μl of NCS solubilizer in 5ml of toluene scintillation fluid. The remainder of the supernatant was discarded, the pellet was washed by suspension in 0.5ml of 10% (w/v) trichloroacetic acid, centrifuged at 3000*g* for 5min, and then dissolved in 250μl of NCS solubilizer. This solution was transferred to 5ml of toluene scintillation fluid and the radioactivity measured as described above.

Table 1. *Degradation of normal and canavanine-containing proteins*

Details for this example of a type 1 experiment are given in the Experimental section. The radioactivity in amino acids is the sum of radioactivity in the medium and in cellular trichloroacetic acid-soluble material. Values are the means±S.E.M. for determinations of three Petri dishes at each degradation period.

Degradation period (h) ...	0	1	2	3	4	6
Protein (mg)	3.42±0.84	2.81±0.85	3.62±1.07	4.13±0.24	4.91±0.53	4.94±0.73
$10^{-4}\times{}^{3}$H-labelled protein (d.p.m.)	55.1±12.8	46.2±9.2	48.7±8.8	45.6±2.6	50.4±5.4	48.4±5.2
$10^{-4}\times{}^{3}$H-labelled amino acids (d.p.m.)	7.3±1.0	24.4±4.0	37.2±4.9	47.9±2.1	56.0±0.9	63.0±3.3
$10^{-4}\times{}^{14}$C-labelled protein (d.p.m.)	40.3±6.8	36.9±5.7	40.3±6.3	40.8±1.9	44.6±4.9	48.0±5.0
$10^{-4}\times{}^{14}$C-labelled amino acids (d.p.m.)	1.1±0.2	2.6±0.4	4.9±0.8	6.8±0.3	9.2±0.1	11.1±0.5
% degradation of ^{3}H-labelled protein	12.2±1.2	34.9±1.1	46.8±1.9	51.2±0.9	53.0±2.7	57.6±1.5
% degradation of ^{14}C-labelled protein	2.7±0.4	6.6±0.2	10.8±0.2	14.3±0.5	17.4±1.4	20.8±1.9

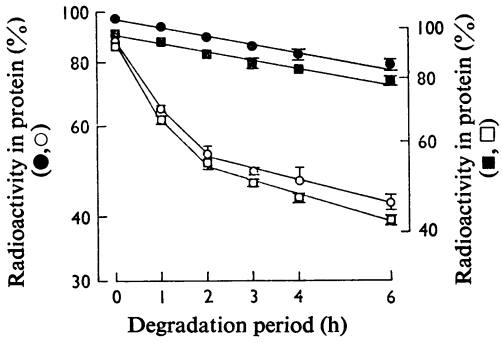

Fig. 1. *Degradation of ^{3}H-labelled canavanine-containing proteins and ^{14}C-labelled normal proteins*

Hepatoma cells were grown and labelled and degradation was measured as described for a type 1 experiment in the Experimental section. The derivation of percentage radioactivity in protein is outlined in the Results section. Two separate experiments are reported, with the vertical logarithmic scales off-set for clarity. Each point represents the mean for determinations on three culture dishes. Variability is indicated by the S.E.M. (bar) where it is sufficiently large. ○, □, ^{3}H-labelled canavanine-containing proteins; ●, ■, ^{14}C-labelled normal proteins.

Protein measurements

The protein contents of cell homogenates was determined as described by Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Amino acid analysis

To measure directly the incorporation of canavanine into cell proteins, the cells were grown under normal conditions until they were confluent, after which they were incubated for 15h with serum omitted and with 0.5mM-canavanine replacing arginine in the inducing medium. The medium was then removed, the monolayer rinsed with Dulbecco salts solution, and the cells were harvested, washed with 0.25M-sucrose and homogenized in 1ml of water. Samples of the homogenate were added to 2vol. of 10% (w/v) trichloroacetic acid, centrifuged and the precipitates washed twice with 10% trichloroacetic acid. The proteins were hydrolysed in 6M-HCl *in vacuo* at 100°C and the amino acids separated and detected with a Jeol model 6 AH Amino Acid Analyser. The methods are essentially those described by Benson & Patterson (1965) for amino acids in physiological fluids (Resin C).

Results

In each experiment where protein degradation was measured, three Petri dishes were taken at the beginning of the degradation period and usually at 1h intervals thereafter. Measurements were made on media and cells of each dish so that the values reported are the average of three determinations. To illustrate the extent and variability of the primary measurements made in each experiment we show the complete data for an experiment of type 1 (Table 1). The cell protein content in each dish is approx. 4mg, but since the cells are grown from a small inoculum in each dish, some variability is expected. This variability in protein content is also reflected in the measurements of protein and amino acid radioactivity, but when degradation is expressed as percentage degradation, e.g. with ^{3}H:

$$\%\ \text{degradation of } {}^{3}\text{H-labelled protein} = 100\times\frac{{}^{3}\text{H in amino acids}}{{}^{3}\text{H in protein}+\text{amino acids}}$$

the variability within a group is substantially decreased (Table 1, lines 6 and 7). In this experiment, canavanine was added with [^{3}H]leucine, so that degradation of ^{3}H-labelled protein represents degradation of canavanine-containing protein. Normal proteins were labelled with [^{14}C]leucine. A comparison of lines 6 and 7 in Table 1 shows that during the first 2h the degradation of ^{3}H-labelled protein is much more rapid than the degradation of ^{14}C-labelled protein.

The rapid degradation of canavanine-containing protein is illustrated more clearly in Fig. 1, where the data from the experiment reported in Table 1 and another similar experiment have been expressed as 100 minus the percentage degradation, i.e. the percentage of total radioactivity retained in protein. The results have been plotted on a logarithmic scale, so that straight lines indicate a single rate constant. For the arginine-containing proteins (Fig. 1, closed symbols), loss of radioactivity does approximate to a simple first-order process and has a half-time of 20h. However, the proteins labelled under conditions where canavanine replaces arginine in the medium are rapidly degraded, with only one-half of the radioactivity remaining in the protein pool after 2h of incubation. Between 2 and 6h the rate of radioactivity loss from cell proteins is diminished and approximates to that found with normal proteins. This slower rate may occur because either canavanine is not incorporated into all proteins or the amount of canavanine incorporated is insufficient for the protein to be recognized as aberrant.

The results in Table 1 and Fig. 1 can be interpreted in ways other than those discussed above. For example, it is possible that canavanine is not incorporated into general proteins, but perhaps influences the degradation system by some direct stimulation. This alternative is unlikely, because degradation of putative error proteins and normal proteins are measured in the same cells, at the same time. Nevertheless, we have obtained qualitative evidence that canavanine is incorporated into cell protein. Cell protein formed in the presence of canavanine was hydrolysed and amino acids were detected by using the amino acid analyser. A discrete peak found at the elution position expected for canavanine contained approx. 5% of the ninhydrin-reacting material that was present in the arginine peak. Larger amounts would probably not be expected if canavanine-containing proteins were rapidly degraded.

A second alternative explanation for the rapid degradation of ^{3}H-labelled proteins is that normal cell protein contains a substantial proportion of labile protein. If such protein was present the 3h of [^{3}H]leucine labelling in a type 1 experiment would be sufficient for any ^{14}C-containing labile protein to be degraded. It would not therefore be detected in a type 1 experiment. To circumvent this possibility, we reversed the sequence of labelling so that canavanine is added first (type 2 experiment). We would expect the labile proteins formed from canavanine and [^{14}C]leucine to be degraded during the ^{3}H-labelling period, but any initial rapid degradation of normal proteins would now be measured. This experiment (Fig. 2) shows a slightly greater rate of degradation of ^{3}H-labelled (normal) proteins. However, the rate is far less than that shown in Fig. 1 for the putative error proteins.

The double-labelling experiments reported in Figs. 1 and 2 have the advantage that both normal and canavanine-containing proteins are labelled in the same cells, but it is not possible to label both groups of proteins simultaneously. We have performed a third type of experiment in which [^{3}H]leucine is used to label normal and canavanine-containing proteins in different Petri dishes. The results of this type 3 experiment (Fig. 3) show a difference between the degradation rates of normal proteins and canavanine-containing proteins that is comparable with the data of Fig. 1.

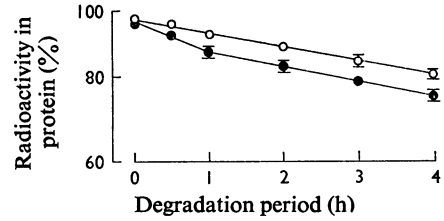

Fig. 2. *Degradation of ^{3}H-labelled normal proteins and ^{14}C-labelled canavanine-containing proteins*

Details are given in the Experimental section for this example of a type 2 experiment. Values are expressed as means±S.E.M. (bars) for measurements on three culture dishes at each time-point and are presented as a semi-logarithmic plot. ○, ^{14}C-labelled canavanine-containing proteins that were labelled first; ●, ^{3}H-labelled normal proteins labelled in the period from 3h before to the beginning of the degradation period.

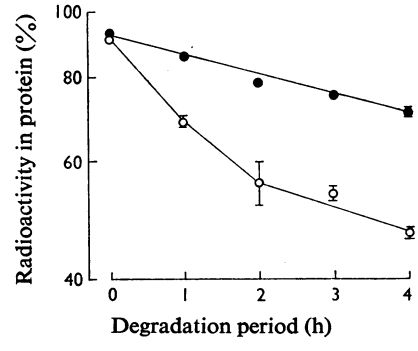

Fig. 3. *Degradation of normal and canavanine-containing proteins in different culture dishes*

Details are given in the Experimental section for this example of a type 3 experiment. ○, ^{3}H-labelled canavanine-containing proteins in one group of Petri dishes; ●, ^{3}H-labelled normal proteins in other dishes. Values are means±S.E.M. (bars) for three Petri dishes at each time-point and are presented as a semi-logarithmic plot.

Table 2. *Half-times of degradation*

Values for the half-times of degradation have been determined graphically from the experiments in Figs. 1–3 and additional experiments. In the presence of canavanine, component A represents the initial rate of degradation and component B the degradation from 2h onwards.

	Half-lives (h) in the presence of:		
		Canavanine components	
Expt.	Arginine	A	B
1A	21	2.1	11.5
1B	20	2.3	13.5
1C	18	1.9	5.7
2A	15	—	15
2B	14	—	15
3	11	2.5	8.4

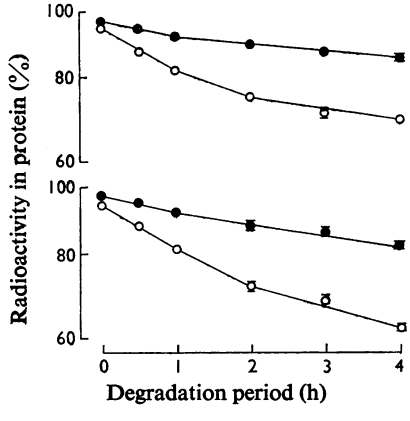

Fig. 4. *Degradation of ^{3}H-labelled 6-fluorotryptophan-containing proteins and ^{14}C-labelled normal proteins*

Two separate experiments were performed exactly as the examples of type 1 experiments that are given in Fig. 1. ○, Degradation of ^{3}H-labelled 6-fluorotryptophan-containing proteins; ●, ^{14}C-labelled normal proteins. Values are means±S.E.M. (bars) for data on three Petri dishes at each time-point in each experiment and are presented as semi-logarithmic plots.

Half-times of degradation for proteins labelled in the presence of arginine are listed in Table 2 for three experiments of type 1, two of type 2 and one of type 3. The values range from 11 to 21h, the longer periods probably being more reliable, since they were obtained from experiments covering a longer degradation period. For proteins formed from canavanine, two degradation components are reported, the first (Table 2, A) calculated from the rate of radioactivity loss during the initial 1h of degradation, and the second (Table 2, B) determined from degradation after 2h. The rapid component gives a half-time of approx. 2h, a value that is a maximum since values between 0 and 1h were not usually obtained. The second degradation rate was rather variable, but in some experiments it approached that found for arginine-containing proteins.

Another amino acid analogue, 6-fluorotryptophan, has been used to replace tryptophan in two type 1 experiments (Fig. 4). In these experiments, degradation of the ^{3}H-labelled 6-fluorotryptophan-containing protein was substantially greater than for normal protein.

Discussion

The concept that error proteins are recognized as such, and rapidly degraded in intact cells, was developed from work with strains of *Escherichia coli* that require certain amino acids for growth (Pine, 1967). Thus auxotrophs for arginine or tryptophan could be used to study the effect of arginine and tryptophan analogues on bacterial protein degradation. Goldberg (1972*a*,*b*) found a greater degradation rate of such proteins whether degradation was measured in the intact cell or by specific proteinases *in vitro*. Other experiments showed that puromycin-terminated peptides and incorrect proteins caused by a lack of fidelity in protein synthesis were also rapidly degraded (Goldberg, 1972*a*; Prouty & Goldberg, 1972*a*). In addition to these measurements of the turnover of total cell proteins, it has been shown that certain deletion or termination mutants result in the production of proteins that are recognized as mistakes and are rapidly degraded. For example, a mutant *lac* repressor (Platt *et al.*, 1970) and the X90 fragment of β-galactosidase (Goldschmidt, 1970) are degraded with half-times of 7 and 20min respectively, whereas the 'correct' enzymes are stable.

Although similar mutants would probably be lethal and thus unlikely to occur in higher organisms, Rabinovitz & Fisher (1964) found that protein synthesized in isolated reticulocytes in the presence of α-amino-β-chlorobutyric acid, a valine antagonist, was rapidly degraded by the cells.

The present data with canavanine and possibly with 6-fluorotryptophan show that hepatoma cells can incorporate the amino acids into cell protein, and the proteins once formed are much more labile than normal proteins. It is unresolved whether all cell proteins incorporate the analogues during protein synthesis but only some of the proteins are labile, or whether only some of the proteins formed have the analogues inserted.

We recognize that the situation tested in this paper

is artificial, since the incorporation of amino acid analogues was only measured under conditions when the correct amino acid was omitted from the incubation medium. It is important, therefore, to resolve whether cells normally make mistakes in protein synthesis and thus produce aberrant molecules. Certain experiments on aging (Holliday & Tarrant, 1972; Gershon & Gershon, 1973; Anderson, 1974) agree with that conclusion, and it has been shown that fibroblasts growing in normal media form a significant amount of protein that is rapidly degraded (Poole & Wibo, 1973).

It is tempting to speculate that the Reuber H35 cells and possibly all cells have evolved a degradation system that functions to clear the cells of aberrant protein, but this point cannot be established until the degrading system has been characterized. Research with *E. coli* has established that error proteins are removed by a degrading system different from that operating on normal proteins, for not only do the sensitivities of the two processes differ in their response to nutritional alterations and to proteolytic inhibitors (Prouty & Goldberg, 1972*b*; Goldberg, 1972*a*), but a mutant has been isolated which lacks the system for degrading error proteins (Bukhari & Zipser, 1973). Comparable experiments may clarify the situation in higher organisms.

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