Intracellular protein degradation *in vitro*

The protein turnover observed in adult animals at a steady state suggests that in the intact cell at least some proteins are constantly synthesized and degraded and at comparable rates[1]. The synthetic mechanism is being actively investigated but the nature of the equally relevant and possibly related degradative mechanism has received little attention. The only demonstrated mechanism for protein degradation is that of hydrolysis of peptide bonds by digestive enzymes and by tissue cathepsins. However, the relationship of the cathepsins to the "physiological" protein degradation referred to above has not been established. The observations reported here on breakdown of cell proteins in surviving tissue slices suggest that this process may be more complex than direct catheptic hydrolysis.

Sprague-Dawley rats weighing 200–250 g and maintained on Purina chow *ad lib.* up to the time of sacrifice were used. Rats used in tracer experiments were injected intraperitoneally with 100–150 microcuries of labeled amino acid in 0.1 *M* phosphate buffer at pH 7.4, 3 days before the day of the experiment. The animals were decapitated and the liver and kidneys were rapidly removed and chilled. 400 mg aliquots of 0.5 mm tissue slices were incubated under O_2 at 37° C with 5 ml of a Ca^{++}- and Mg^{++}-free Krebs-Ringer phosphate medium at pH 7.4. Incubations were stopped by addition of 5 ml of 20% trichloroacetic acid (TCA). The entire flask contents —tissue plus medium— were thoroughly homogenized. After centrifugation the precipitate was washed twice with 10% TCA. One aliquot from the pooled supernatant and washings was taken for Kjeldahl nitrogen (NPN). Another aliquot was neutralized and passed over an 0.7 × 12 cm column of Dowex 50 (X8) in the H^+ form. The column was washed with H_2O and the fraction eluted by 10 ml. 2 *N* NH_4OH was collected, concentrated and plated directly for counting of radioactive "amino acids". This "amino acid" fraction would include small peptides, other ampholytes and possibly other cationic materials derivable from the injected labeled amino acid.

The protein precipitate was treated with hot TCA, washed twice with warm alcohol-ether (1:1) and once with ether. The residue was dissolved in 1 *N* NaOH and an aliquot was taken for Kjeldahl nitrogen determination. Another aliquot was reprecipitated with TCA, washed with alcohol and ether and plated for counting.

As shown in Table I, the release of labeled amino acid continued, although at a declining rate, for 4 hours. The release was inhibited by anaerobiosis and by dinitrophenol (DNP) in confirmation of SIMPSON's earlier observations[2]. In searching for more specific inhibitors it was found that *o*-fluorophenylalanine and *p*-fluorophenylalanine also inhibited the release. At $2 \cdot 10^{-2} M$ the inhibition varied from 32% to 61% in 4 experiments using 3-^{14}C-phenylalanine as the tracer. Results with liver and kidney slices were comparable. The inhibition of amino acid release appears to be general since similar results were obtained when the protein was labeled with alanine or with lysine.

TABLE I

INHIBITION OF PROTEIN BREAKDOWN IN PHENYLALANINE-LABELED LIVER SLICES

	Time of incubation (hrs)	*"Amino acid" radioactivity (c.p.m.)*	*"Amino acid" radioactivity released (c.p.m.)*	*Inhibition of release %*
Zero time control	0	130	—	—
Incubated control	1	440	310	—
	4	860	730	—
$2 \cdot 10^{-3} M$ DNP	1	270	140	55
	4	530	400	45
$2 \cdot 10^{-2} M$ *o*-fluoro-	1	360	230	26
phenylalanine	4	630	500	31
N_2 atmosphere	4	630	500	31

In these experiments, as in SIMPSON's experiments, a high concentration of unlabeled amino acid ($1.2 \cdot 10^{-2} M$) in the medium served as a "bank" of carrier to trap the labeled amino acids released. Hence the observed inhibitory effects might be due to prevention of the penetration of this carrier amino acid into the cell, decreasing the efficiency of the trapping. Furthermore, the isotope results might reflect an effect on an "exchange" process or on the dynamics of a reversible process without effect on net protein metabolism. For these reasons net protein degradation of liver slices was investigated.

The net increment in NPN during 4 hours incubation, expressed as per cent of total slice protein, averaged 2.7 ± 1 % in 11 experiments. With slices from a single rat liver the variability was less marked, averaging 2.9 ± 0.4 % in a typical experiment.

Amino acid nitrogen, determined on a Dowex 50-H^+ eluate as described above, accounted for about one-half the total NPN increment. Similar values were derived from 280/260 ratios measured in the unfractionated TCA supernatants[3]. There was no increase in free ammonia. The remainder of the NPN increment may be attributed to urea formation but the contribution from breakdown of nucleic acid or other TCA-insoluble nitrogenous constituents is not known.

As shown in Table II, net protein breakdown in kidney slices was strongly inhibited by DNP. Similar results were obtained with liver slices. On the other hand, the intrinsic catheptic activity of whole liver homogenates measured at pH 5.0 was inhibited only very slightly or not at all by similar concentrations of DNP. *o*-Fluorophenylalanine also inhibited net protein breakdown in slices and again was without effect on breakdown in homogenates at pH 5.0. Finally, it was observed that incubation under nitrogen inhibited net breakdown in slices by as much as 70 %.

The inhibitory effects of anaerobiosis and of DNP suggest a paradoxical energy requirement for the nominally exergonic process of protein degradation. However, as pointed out by SIMPSON[2], these effects may be indirect, secondary results of disruption of cellular organization and function. The inhibition by an amino acid analogue is more likely related specifically to protein metabolism *per se* but this remains to be established.

TABLE II

INHIBITION OF NET PROTEIN BREAKDOWN AND RELEASE OF RADIOACTIVE AMINO ACIDS FROM ALANINE-LABELED KIDNEY SLICES*

	$\frac{\text{mg NPN}}{\text{mg protein-N}}$	Protein-N released %	$\frac{\text{"Amino acid" radioactivity}}{\text{Protein radioactivity}}$	Protein radioactivity released %
Zero time control	0.244	—	0.012	—
Incubated control	0.292	4.8	0.084	7.2
$2 \cdot 10^{-2} M$ *o*-fluorophenylalanine	0.267	2.3	0.039	2.7
$2 \cdot 10^{-3} M$ DNP	0.257	1.3	0.023	1.1

* 4 h incubation.

It is noteworthy that the several inhibitors of protein degradation described here are likewise inhibitors of protein synthesis. Reversibility at one or more stages in protein synthesis would be compatible with these results and with previous results on non-uniform labeling of proteins[4]. The apparent energy requirements and the inhibition by the amino acid analogue would be compatible with a breakdown process involving a complex acceptor rather than water. For this there is an analogy in the mechanisms for degradation of other macromolecules such as glycogen[5] and ribonucleic acid[6].

Taken together the present results suggest that there is a continuing, organized process of intracellular protein degradation, a concept recently questioned by MONOD and co-workers[7]. While a considerable part of the "turnover" of proteins is undoubtedly a reflection of cell renewal and replacement of secreted proteins, the present studies indicate that there probably is in addition a true dynamic degradation and resynthesis of protein in mammalian cells.

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