RAPID PROTEIN CATABOLISM IN MAMMALIAN CELLS IS OBSCURED BY REUTILIZATION OF AMINO ACIDS[†]

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ABSTRACT: Protein bound radioactivity from maximally labeled cell cultures decreases abruptly following addition of medium containing unlabeled amino acids. Isotopically labeled amino acids appear briefly at increased concentrations in the acid soluble fraction and are then reutilized in protein. The release of amino acids from cells occurs at a rate of only one percent per hour and must be limiting in measurements of protein catabolism, especially in estimates made by pulse-chase techniques.

Enzyme levels in mammalian cells are determined by the equilibrium which exists between the synthetic and degradative processes (Schimke, 1969). Evidence from synchronized cell systems has indicated that protein catabolism is very rapid and that oscillations in enzyme protein are a rather general feature of the cell cycle (Klevecz, 1969; Vendrely, *et al.*, 1970).

Estimates of average protein half-life are often made by labeling cells with amino acids and then following the diminution of precipitable radioactivity in some manner (Jordan and Schmidt, 1961; Kolodny and Gross, 1969), or alternately, by adjusting culture conditions so that there is no net increase in cell protein

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and determining "excess" amino acid incorporation (Eagle, *et al.*, 1959). Although the latter method is preferred for measuring total protein catabolism, there is reason to question whether the values obtained are a true measure of the rate of degradation since the same objection which is applied to chase experiments, the recycling of amino acids (Loftfield and Harris, 1956; Gan and Jeffay, 1967), can be applied to continuous labeling experiments (Koch, 1962). Both methods tend to bias results in the direction of increased half-lives.

Following addition of medium containing normal or elevated levels of unlabeled amino acids to previously labeled cell cultures I noted sizable fluctuations in protein bound counts. The fluctuations diminished with time and if the experiment was carried out for several days, the estimated average protein half-life appeared to be in excess of 24 hours (Figure 1). To explain this effect it was proposed that immediately following the chase of highly labeled cultures the pool might fill with unlabeled amino acids. The synthetic machinery of the cell would then draw on a pool of lower specific activity while the degradative processes acted on maximally labeled proteins, releasing labeled amino acids back to the pool. Thus, while total protein was constant or increasing, the proportion of labeled protein would decrease. This would be a transient condition and one which should equilibrate rapidly.

The idea was tested first by labeling cells in F-10 medium depleted of glutamine and containing only a trace of radioactive isoleucine, so that no net protein synthesis could occur. Cultures were allowed to incorporate labeled isoleucine for 2 and 24 hours and were then chased in the same medium containing 2mM isoleucine (Figure 1). Several points are evident: first, total

protein bound radioactivity per culture does show a sharp but transient decrease. The concentration of acid soluble amino acids increases and in consequence specific activity decreases. Thus, the protein synthetic machinery of the cell is drawing initially upon an expanded and relatively unlabeled pool. As pool specific activity falls there is a concomitant decrease in radioactivity bound in protein. Curves of decreasing protein

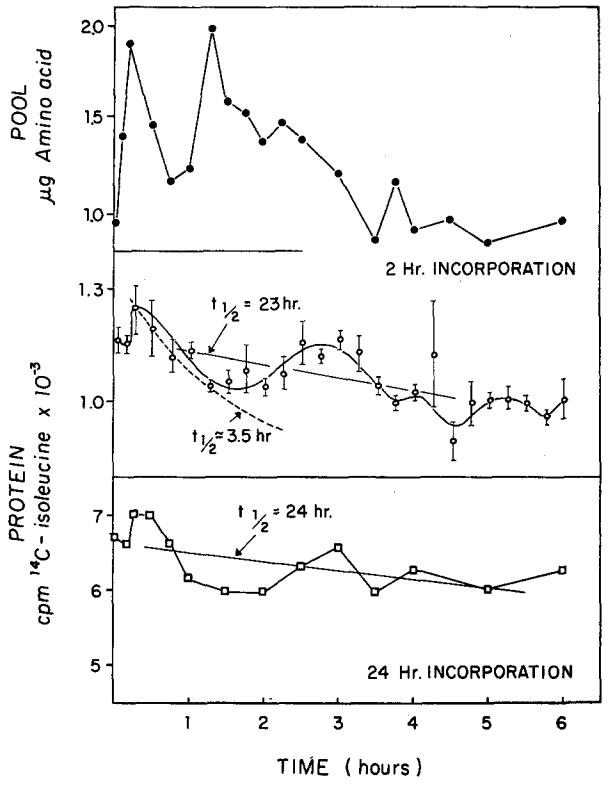

Figure 1. 1 x 10^5 Don C cells (Klevecz, 1969) were inoculated into sterile scintillation vials in F-10 medium lacking glutamine and isoleucine (Tobey and Ley, 1970) and allowed to grow for 24 hours. Cultures were then exposed to the same medium containing 0.5 μc/ml ^{14}C-isoleucine (uniformly labeled, specific activity, 180 mCi per mmole) for 2 hours or 24 hours and then washed twice and reincubated in F-10 medium containing 1mM glutamine and 2mM isoleucine. Protein bound radioactivity was determined following hot acid hydrolysis and lipid extraction. After extracting the 6 percent hot TCA hydrolysate twice with 7 volumes of ether and neutralizing the aqueous phase, amino acid concentration and specific activity were determined by the ninhydrin method (Rosen, 1957) using leucine and isoleucine as standards. Protein was determined by the Folin phenol method using bovine serum albumin as a standard.

radioactivity are retarded in phase with respect to free amino acid curves by about 15 minutes. As protein radioactivity decreases, counts begin to reappear in the pool and the two processes undergo decreasing fluctuations as pool size approaches its initial value. Although the kinetics of the process are similar whether the period of labeling is 2 or 24 hours, the relative decrease in labeled protein is greater for shorter labeling times.

Later experiments revealed that this phenomenon was not dependent on chasing with an excess of the unlabeled form of the precursor. In Figure 2 there was no increase in total free amino acids, but pool specific activity underwent a sharp decrease immediately following addition of unlabeled medium. If the release of labeled amino acids to the medium is measured during this period it is apparent that release of bound radioactivity goes on at the relatively constant rate of one to two percent per hour. This is an important finding because of the similarity of this value to that reported by Eagle, *et al.* (1959) for protein turnover. If the chase experiments described in Figures 1 and 2 are carried out for several days the average half-life for protein is 24 hours in Don cells and somewhat greater in other cell lines. This is in contrast with the kinetics of turnover observed in the first few hours of the chase and suggests that overall, the release of amino acids to the medium is a limiting step in measuring the rate of protein turnover.

Precipitation and counting of the chasing medium gave the same values as the blank, and labeled protein did not increase in the chasing media as labeled cell protein decreased. Thus, the loss of counts can only be attributed to degradation of protein.

This pattern was observed whether the cells were labeled with a single essential amino acid or a mixture of amino acids. The effect was not enhanced by chasing the labeled amino acid with an excess of the unlabeled form (compare Figures 1 and 2). In several instances, labeled cells were chased with medium which had been used to grow cells for a period of time equivalent to the labeling period (conditioned medium). Again there was no difference in the loss of counts from protein.

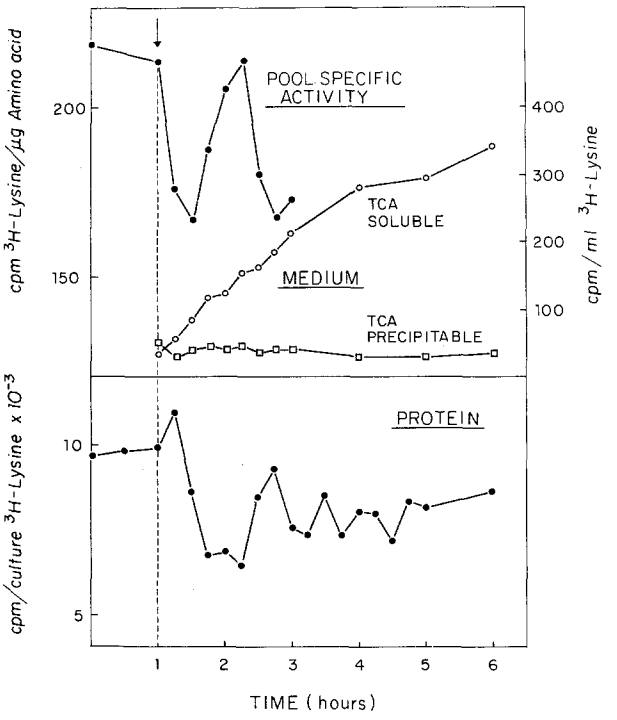

Figure 2. 1 x 10^5 Don (CCL 16) cells labeled for 24 hours in McCoy's 5a medium with 1 µc/ml ^{3}H-Reconstituted Protein Hydrolysate (^{3}H-RPH: Schwarz mixture, 3130-08) and then chased in the same McCoy's medium. Protein and pool specific activity were assayed as described in Figure 1. The medium was made 5 percent with respect to trichloroacetic acid and the soluble fraction analysed in the same manner as the soluble extracts of the cells. Protein precipitated from the medium was washed twice with 5 percent trichloroacetic acid, dissolved in 1N NaOH and an aliquot was counted. Arrow indicates the time of addition of chase medium.

Although it may be argued that this chase procedure tends to examine those proteins which are the most rapidly synthesized

and which, depending on their concentration in the cell, may be more rapidly catabolized, it nevertheless reveals a rather general process because losses equal to 30 percent of protein label are routinely observed.

In all studies which endeavor to determine rate constants or some other measure of protein degradation by following the loss of labeled precursor from protein, reutilization of isotopically labeled amino acids is a major problem. Normally, 50 percent of the free amino acids in liver may be derived from protein. Under starvation conditions this value may be as much as 90 percent (Gan and Jeffay, 1967). It seems reasonable to think that this situation would be aggravated in exponentially growing cells where there is an extensive increase in protein. In several Chinese hamster cell lines total protein per culture doubles every 9-10 hours. With the consequent flow of amino acids from the medium to cell protein, it is optimistic to expect that extensive reutilization of amino acids can be avoided by any methodology which involves chasing of label. This is an obvious consideration, but one which is commonly overlooked.

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