Effects of Amino Acids, Ammonia and Leupeptin on Protein Synthesis and Degradation in Isolated Rat Hepatocytes

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Protein synthesis in isolated rat hepatocytes, as measured by the incorporation of [14C]-valine at constant specific radioactivity, proceeded at a rate of 0.3–0.5%/h in an unsupplemented medium, i.e. only about one-tenth the rate of protein degradation (4%/h). Leupeptin, which inhibits lysosomal protein degradation (previously found to be 75% of the total degradation in hepatocytes), had no effect on protein synthesis, showing that endogenous protein degradation supplied amino acids in excess of the substrate requirements for protein synthesis. The inhibition of protein synthesis by NH4Cl (another inhibitor of lysosomal protein degradation) as well as the stimulation by a physiological amino acid mixture must therefore represent indirect effects, either on general energy metabolism, or on unknown regulatory processes.

Amino acids stimulate hepatic protein synthesis in vivo (Munro, 1968; Sidransky et al., 1968; Pronczuk et al., 1970), in the isolated perfused rat liver (Jefferson & Korner, 1969; Ekren et al., 1971; Van Den Borre & Webb, 1972; Fausto, 1972; McGown et al., 1973; Woodside et al., 1974), in isolated rat hepatocytes (Grant & Black, 1974; Seglen, 1976a; Weigand et al., 1977; Seglen & Solheim, 1978a) and in a variety of other experimental systems in vitro (Eliasson et al., 1967; Smulson & Rideau, 1970; van Venrooij et al., 1970, 1972; Lee et al., 1971; Christian, 1973). The amino acids may exert their effect by being substrates for protein synthesis, by providing energy, or by unknown regulatory mechanisms (Seglen & Solheim, 1978b). To some extent the substrate requirement for protein synthesis can be satisfied by intracellular reutilization of amino acids from protein degradation (Gan & Jeffay, 1971; Righetti et al., 1971; Poole, 1971; Mortimore et al., 1972; Khairellah & Mortimore, 1976; Woodside, 1976; Hod & Hershko, 1976).

Isolated hepatocytes continuously release amino acids from protein degradation into the medium (Seglen, 1977a), and the initial lag in protein synthesis frequently observed (Craig & Porter, 1973; Seglen & Solheim, 1978a,b) and largely prevented by preincubation with amino acids (Seglen & Solheim, 1978a) could conceivably be due to a gradual increase in amino acid concentrations. In order to investigate further to what extent protein synthesis is rate-limited by the supply of amino acids from endogenous proteolysis, two inhibitors of lysosomal protein degradation in hepatocytes, NH4Cl (Seglen, 1975, 1976b, 1977b; Seglen & Reith, 1976) and leupeptin (Hopgood et al., 1977; P. O. Seglen & B. Grinde, unpublished work) were used in the present study. Previous observations suggested that protein synthesis was relatively unaffected by NH4Cl (Seglen, 1975; Seglen & Reith, 1977), but those experiments were done with radioactive amino acid mixtures of uncontrolled specific radioactivities (mostly tracer concentrations), and an inhibitory effect of the degradation blockade on protein synthesis could have been masked by a simultaneous increase in precursor specific radioactivity due to decreased radioactive isotope dilution. In the present paper, we have used [14C] valine as radioactive precursor, and established conditions under which its incorporation into protein can be used as a valid measure of protein synthesis, undisturbed by isotope dilution. NH4Cl and other inhibitors of lysosomal protein degradation have then been used as tools in an attempt to explore the interrelationship between protein synthesis and protein degradation.

Experimental

Isolated hepatocytes were prepared from the liver of 16h-starved male Wistar rats (250–300g) by the method of collagenase perfusion (Seglen, 1973, 1976c). The cells were suspended in a buffered balanced-salt solution (suspension buffer, Seglen, 1973) and incubated as 0.4 ml samples in rapidly shaking 15 ml centrifuge tubes at 37°C as previously described (Seglen, 1974). The standard cell concentration in the incubation was 70–90 mg wet wt./ml, unless otherwise specified. The cells were incubated for 1h, precipitated with 0.1 ml of 10% (w/v) HClO4
and left at 0°C for at least 15 min. The precipitate was then washed three times at 0°C with 4 ml of 2% (w/v) HClO₄ in the centrifuge tube (collected by centrifugation at 5000 rev./min for 5 min each time), dissolved in 0.5 ml of NaOH (0.3 mol/l), and mixed with 8 ml of scintillator (Scint. Hei 3, a pseudo-cumene-based scintillator from ing. F. Heidenreich, Oslo, Norway) and 0.1 ml of 1 M-HCl for liquid-scintillation counting of incorporated radioactivity.

Separate measurement of intracellular and extracellular radioactivity was performed as previously described (Seglen & Solheim, 1978a).

[14C]Valine was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. (CFB.75; 260 Ci/mol; 63 mCi/l; 0.24 nM) and used either at tracer concentrations (below 3 mmol/l) or mixed with unlabelled valine to the desired concentration and specific radioactivity.

[14C]Leucine from The Radiochemical Centre (CFB.67; 324 Ci/mol; 51 mCi/l; 0.16 nM) was added to an amino acid mixture (Seglen, 1976a) and incorporated at a concentration of 0.92 mmol/l and a specific radioactivity of 554 mCi/mol.

The non-labelled physiological amino acid mixture (with the omission of valine) was added in multiples of the normal concentration given (Seglen, 1976a).

Leupeptin was kindly donated by Dr. H. Umezawa, Institute of Microbial Chemistry, Shinagawa-ku, Tokyo 141, Japan; other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results and Discussion

Kinetics of [14C]valine incorporation

A prerequisite for quantitative estimation of protein synthesis by incorporation of radioactive amino acids is the maintenance of a constant specific radioactivity in the amino acid precursor pool. The precursor pool in isolated hepatocytes receives amino acids from the extracellular as well as from the general intracellular pool (Seglen & Solheim, 1978a), and in both of these pools radioactive isotope dilution may take place owing to the release of unlabelled amino acids from endogenous protein degradation (Seglen, 1976a).

This problem can be avoided by using a non-metabolizable precursor amino acid, [14C]valine, at such a high concentration that the dilution by proteolytically derived valine becomes insignificant. Valine equilibrates so rapidly across the plasma membrane (transport by facilitated diffusion; Seglen & Solheim, 1978a) that the precursor pool can be assumed to be uniform.

To determine the valine concentration needed for maintenance of reasonably constant precursor-pool conditions, hepatocytes were incubated with [14C]-valine (mixed with [12C]-valine) at different concentrations, but with the same initial specific radioactivity (i.e. the same 14C/12C ratio). The rate of incorporation of [14C]valine into protein increased with the valine concentration up to 5–10 mM (Fig. 1b;
cf. also Fig. 3). Incorporation (Fig. 1a) of \[^{14}\text{C}]\text{valine}\) showed an initial lag phase of variable duration (usually 10–20 min, but was sometimes absent; cf. Craig & Porter, 1973; Seglen & Solheim, 1978b), then proceeded linearly with time throughout the 60 min incubation period with 5 or 10 mM-valine. At 1 mM and below there was a pronounced decline in the incorporation rate in the later part of the incubation period (Fig. 1a), probably reflecting the combined effects of valine consumption and dilution of valine specific radioactivity.

High valine concentrations as such did not affect protein synthesis; the incorporation of \[^{14}\text{C}]\text{leucine}\) was found to be essentially unaffected by the addition of valine up to 10 mM.

Calculation of rates of protein synthesis

\[^{14}\text{C}]\text{Valine (5 mM; specific radioactivity 63 mCi/mol)}\) was chosen as the standard concentration for protein-incorporation experiments. With a uniform precursor pool of constant specific radioactivity thus established, the rate of protein synthesis could be quantitatively estimated from the incorporated radioactivity. Protein-incorporated \[^{14}\text{C}]\text{valine}\) was measured by liquid-scintillation counting with the same efficiency (85%) as free \[^{14}\text{C}]\text{valine}, and in an experiment using a tracer dose of \[^{14}\text{C}]\text{valine}\) it could be shown that the disappearance of acid-soluble radioactivity exactly equaled the accumulation of acid-insoluble (i.e. protein) radioactivity. By assuming a fractional valine content of 753 \(\mu\text{mol/g}\) of liver protein (Schreiber et al., 1971) and a hepatocytic protein content of 228 mg/g cellular wet wt., as measured by the micro-biuret method (Seglen, 1973), a protein-synthesis rate of 1%/h would result in the incorporation of 206 c.p.m./h per mg of cells with the chosen standard \[^{14}\text{C}]\text{valine}\) concentration of 5 mM and 63 mCi/mol. The incorporation rates of 60–110 c.p.m./h per mg of cells, as found in the present study for hepatocytes in unsupplemented medium, would thus correspond to protein-synthesis rates of 0.3–0.5%/h.

Effects of \(\text{NH}_4\text{Cl}\) and amino acids on \[^{14}\text{C}]\text{valine}\) incorporation

To investigate to what extent protein synthesis depended on the provision of amino acid precursors from protein degradation, \(\text{NH}_4\text{Cl (5 mM)}\) was added to the hepatocytes as an inhibitor of lysosomal protein degradation (Seglen & Reith, 1976). In Fig. 2, the incorporation of \[^{14}\text{C}]\text{valine}\) into protein has been measured as a function of cell concentration under various conditions in the presence and absence of \(\text{NH}_4\text{Cl}\). With a tracer dose of \[^{14}\text{C}]\text{valine (Fig. 2a)}, \(\text{NH}_4\text{Cl}\) was slightly stimulatory as previously observed (Seglen, 1975), but with 5 mM-[\(^{14}\text{C}]\text{valine (Fig. 2b)}, \(\text{NH}_4\text{Cl}\) markedly inhibited the incorporation of radioactivity into protein. Apparently \(\text{NH}_4\text{Cl}\) inhibits protein synthesis, but this effect is only seen when the specific radioactivity in the amino acid precursor pool is constant. With radioactive precursors at tracer concentrations, the continuous dilution of the specific radioactivity by unlabelled amino acids from protein degradation may be

![Fig. 2. Effect of \(\text{NH}_4\text{Cl}\) on \[^{14}\text{C}]\text{valine incorporation at various concentrations of amino acids}\) Isolated hepatocytes were incubated for 60 min at 37°C at the cell concentration indicated, in the presence (●) or absence (○) of \(\text{NH}_4\text{Cl (5 mM)}\). \[^{14}\text{C}]\text{Valine was included as a tracer dose (0.5 \(\mu\text{mol})\), or at a high concentration (5 mM; 63 mCi/mol) in the absence (b) or presence (c) of an amino acid mixture (20 times normal concentration). The net incorporation of radioactivity into protein during the incubation period was measured; each point is the mean of two cell samples. (a), (b) and (c) represent three different experiments; the absolute values are therefore not directly comparable.](image-url)
inhibited by NH₄Cl to approximately the same extent as protein synthesis, thus masking the latter inhibition.

There was no effect of NH₄Cl on [¹⁴C]valine uptake or distribution at any valine concentration, confirming previous observations (Seglen & Solheim, 1978a).

Incubation with [¹⁴C]valine at a high concentration (5 mM), but in the presence of an amino acid mixture, decreased the inhibition of protein synthesis by NH₄Cl (Fig. 2c). This antagonistic effect of amino acids is also indicated in Fig. 3 (compare a with b), which furthermore shows how the inhibition by NH₄Cl becomes increasingly more evident with increasing [¹⁴C]valine concentrations (i.e. with increasing stability of the specific precursor radioactivity).

The antagonism between amino acids and NH₄Cl might be taken as support for the idea that NH₄Cl inhibits protein synthesis by decreasing the endogenous provision of amino acids from protein degradation. However, as shown in Fig. 4, exogenous amino acids given to the cells at increasing concentrations stimulate protein synthesis to the same absolute extent in the presence and absence of NH₄Cl, resulting in a relative, rather than an absolute, decrease in the NH₄Cl effect. The data therefore suggest that NH₄Cl inhibits protein synthesis by a mechanism unrelated to amino acid provision.

The relationship between protein synthesis and degradation was further explored by using other inhibitors of lysosomal proteolysis. Both the NH₃ analogue methylamine and the peptide proteinase inhibitor leupeptin (Aoyaga & Umezawa, 1975; Kirschke et al., 1976; Hopgood et al., 1977) have been found to inhibit protein degradation in isolated hepatocytes to the same extent as NH₄Cl (P. O. Seglen et al., 1978).

Isolated hepatocytes were incubated for 60 min at 37°C with [¹⁴C]valine (5 mM; 63 mCi/mol) and various concentrations of an amino acid mixture. The net incorporation of radioactivity into protein in the presence (●) or absence (○) of NH₄Cl (5 mM) during the incubation period was measured. △, Relative inhibition by NH₄Cl (in %). Each point is the mean of two cell samples.

Graphs:
- Fig. 3. Dependence of NH₄Cl inhibition on [¹⁴C]valine concentration
  - Isolated hepatocytes were incubated for 60 min at 37°C, with no addition (a) or plus amino acids at 5 times normal concentration (b), with (●) or without (○) NH₄Cl (10 mM in a; 5 mM in b). [¹⁴C]Valine (constant specific radioactivity 63 mCi/mol) was included at various concentrations, and the net incorporation of radioactivity during the incubation period was measured. Each point is the mean of two cell samples. (a) and (b) represent different experiments; the absolute values are therefore not directly comparable.
Table 1. Effects of inhibitors of lysosomal protein degradation on protein synthesis

Isolated hepatocytes were incubated for 60 min at 37°C with [14C]valine (5mmol/l; 63mCi/mol) in the presence or absence of an amino acid mixture (12.5 times normal concentration), and the effects of leupeptin (125μmol/l = 60mg/l), NH4Cl (10mmol/l) or methylamine (10mmol/l) were tested on the incorporation of radioactivity into protein (acid-insoluble material) during the incubation period. Each value is the mean ± s.e. for four cell samples.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Control</th>
<th>+ Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
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</tr>
<tr>
<td>None</td>
<td>67.9 ± 1.0</td>
<td>77.6 ± 0.3</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>54.0 ± 0.6</td>
<td>76.4 ± 0.6</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>23.8 ± 0.5</td>
<td>59.7 ± 1.4</td>
</tr>
<tr>
<td>Expt. 2</td>
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<td></td>
</tr>
<tr>
<td>None</td>
<td>46.4 ± 1.8</td>
<td>—</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>46.8 ± 0.6</td>
<td>—</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>20.2 ± 1.1</td>
<td>—</td>
</tr>
<tr>
<td>Methylamine</td>
<td>35.7 ± 1.2</td>
<td>—</td>
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Seglen & B. Grinde, unpublished work). However, leupeptin failed to inhibit protein synthesis significantly (Table 1), in either the absence or presence of amino acids, and methylamine also inhibited much less than NH4Cl. Therefore it can be concluded that inhibition of lysosomal protein degradation as such does not affect protein synthesis.

The lysosomal pathway of protein degradation accounts for approx. 75% of the total protein degradation in isolated hepatocytes (Seglen, 1975; Seglen & Reith, 1976; P. O. Seglen & B. Grinde, unpublished work), but apparently the remaining 25% provides sufficient amounts of amino acids for protein synthesis. In an un-supplemented medium, the rate of protein synthesis (0.3-0.5%/h) is only about one-tenth the rate of protein degradation (4%/h; Seglen, 1977a); hence amino acid production by the non-lysosomal pathway(s) of degradation (Knowles & Ballard, 1976) alone should theoretically satisfy the amino acid requirements for protein synthesis.

Since protein synthesis in freshly isolated rat hepatocytes is apparently not rate-limited by substrate supply, both NH3 and exogenous amino acids must exert their effects indirectly, e.g. by alterations in energy metabolism or intermediary nitrogen metabolism. The stimulation by amino acids is prevented by the aminotransferase inhibitor amino-oxyacetate and mimicked by energy substrates, such as lactate and pyruvate (Seglen & Solheim, 1978b), suggesting that at least part of the amino acid effect may be nutritional. NH3 is a metabolically active amino-group donor that may interfere with hepatocyte metabolism in a variety of ways (Zahlten et al., 1974; Stubbs & Krebs, 1975; Meijer et al., 1975; Zaleski & Bryla, 1977). Other nitrogenous compounds such as adenosine and amino-oxyacetate also interfere with hepatocyte protein synthesis (Seglen & Solheim, 1978b), indicating that this process may be subject to regulation by a complex effector network (Seglen, 1976d) within the domain of intermediary nitrogen metabolism.

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References


