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 [^{14}C]-valine at constant specific radioactivity, proceeded at a rate of 0.3–0.5%/h in an un-supplemented 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 require-ments for protein synthesis. The inhibition of protein synthesis by NH_4Cl (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 (Jeffer-son & Korner, 1969; Ekren *et al.*, 1971; Van Den Borre & Webb, 1972; Fausto, 1972; McGown *et al.*, 1973; Woodside *et al.*, 1974), in isolated rat hepato-cytes (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; Christman, 1973). The amino acids may exert their effect by being sub-strates 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; Khairallah & 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 pre-incubation 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, NH_4Cl (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 syn-thesis was relatively unaffected by NH_4Cl (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 [^{14}C]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. NH_4Cl and other inhibitors of lysosomal protein degrad-ation have then been used as tools in an attempt to explore the interrelationship between protein syn-thesis 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 concen-tration in the incubation was 70–90 mg wet wt./ml, unless otherwise specified. The cells were incubated for 1 h, precipitated with 0.1 ml of 10% (w/v) $HClO_4$

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) HClO$_4$ 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 extra-cellular radioactivity was performed as previously described (Seglen & Solheim, 1978a).

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

[^{14}C]Leucine from The Radiochemical Centre (CFB.67; 324 Ci/mol; 51 mCi/l; 0.16 mM) was added to an amino acid mixture (Seglen, 1976a) and in-corporated 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 [^{14}C]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, [^{14}C]valine, at such a high concentration that the dilution by pro-teolytically 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 [^{14}C]valine (mixed with [^{12}C]valine) at different concentrations, but with the same initial specific radio-activity (i.e. the same ^{14}C/^{12}C ratio). The rate of incorporation of [^{14}C]valine into protein increased with the valine concentration up to 5–10 mM (Fig. 1b;
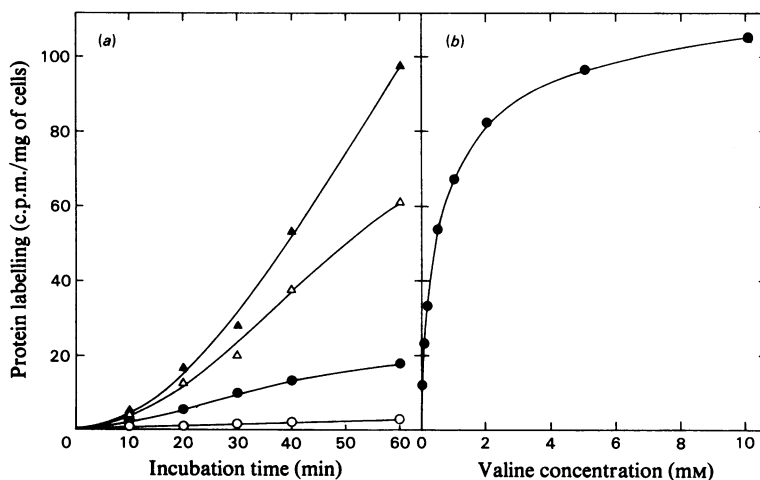

Fig. 1. *Effect of valine concentration on the time course and rate of [^{14}C]valine incorporation*
Isolated hepatocytes were incubated at 37°C with different concentrations of [^{14}C]valine of constant specific radio-activity (50 mCi/mol), and the incorporation of radioactivity into protein was measured. (*a*) Time course of incorpor-ation at [^{14}C]valine concentrations of 0.01 (○), 0.1 (●), 1 (△) and 10 (▲) mM. (*b*) Net incorporation during 60 min incubation with various concentrations of [^{14}C]valine. Each point is the mean of two cell samples.

cf. also Fig. 3). Incorporation (Fig. 1a) of [^{14}C]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}C]leucine was found to be essentially unaffected by the addition of valine up to 10 mM.

Calculation of rates of protein synthesis

[^{14}C]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}C]valine was measured by liquid-scintillation counting with the same efficiency (85%) as free [^{14}C]valine, and in an experiment using a tracer dose of [^{14}C]valine it could be shown that the disappearance of acid-soluble radioactivity exactly equalled the accumulation of acid-insoluble (i.e. protein) radioactivity. By assuming a fractional valine content of 753 μmol/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}C]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 NH_4Cl and amino acids on [^{14}C]valine incorporation

To investigate to what extent protein synthesis depended on the provision of amino acid precursors from protein degradation, NH_4Cl (5 mM) was added to the hepatocytes as an inhibitor of lysosomal protein degradation (Seglen & Reith, 1976). In Fig. 2, the incorporation of [^{14}C]valine into protein has been measured as a function of cell concentration under various conditions in the presence and absence of NH_4Cl. With a tracer dose of [^{14}C]valine (Fig. 2a), NH_4Cl was slightly stimulatory as previously observed (Seglen, 1975), but with 5 mM-[^{14}C]valine (Fig. 2b), NH_4Cl markedly inhibited the incorporation of radioactivity into protein. Apparently NH_4Cl 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
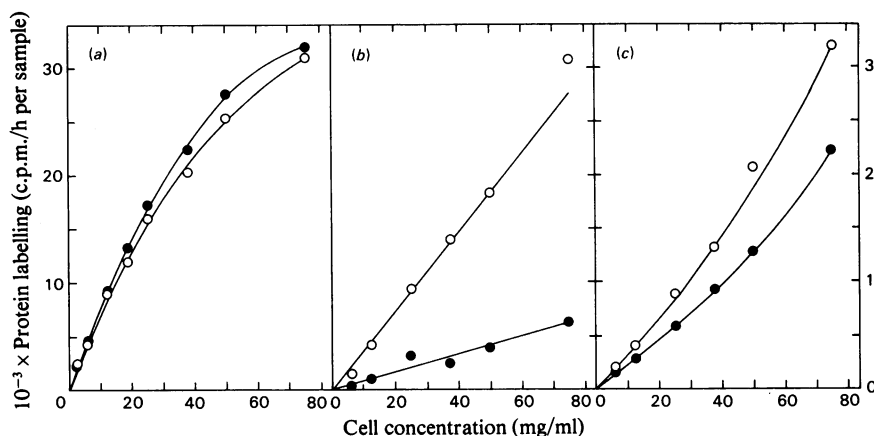

Fig. 2. *Effect of NH_4Cl on [^{14}C]valine incorporation at various concentrations ~' ~ nino acids*
Isolated hepatocytes were incubated for 60 min at 37°C at the cell concentration indicated, in the presence (●) or absence (○) of NH_4Cl (5 mM). [^{14}C]Valine was included as a tracer dose (0.5 μM; 250 Ci/moi, a), 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.

inhibited by NH$_4$Cl to approximately the same extent as protein synthesis, thus masking the latter inhibition.

There was no effect of NH$_4$Cl on [^{14}C]valine uptake or distribution at any valine concentration, confirming previous observations (Seglen & Solheim, 1978a).

Incubation with [^{14}C]valine at a high concentration (5mM), but in the presence of an amino acid mixture, decreased the inhibition of protein synthesis by NH$_4$Cl (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 NH$_4$Cl becomes increasingly more evident with increasing [^{14}C]valine concentrations (i.e. with increasing stability of the specific precursor radioactivity).

The antagonism between amino acids and NH$_4$Cl might be taken as support for the idea that NH$_4$Cl 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 NH$_4$Cl, resulting in a relative, rather than an absolute, decrease in the NH$_4$Cl effect. The data therefore suggest that NH$_4$Cl 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 NH$_3$

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 NH$_4$Cl (P. O.
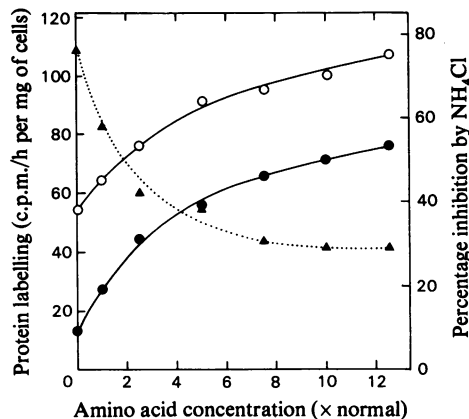

Fig. 4. *Dependence of NH$_4$Cl inhibition on amino acid concentration*

Isolated hepatocytes were incubated for 60 min at 37°C with [^{14}C]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 NH$_4$Cl (5 mM) during the incubation period was measured. ▲, Relative inhibition by NH$_4$Cl (in %). Each point is the mean of two cell samples.
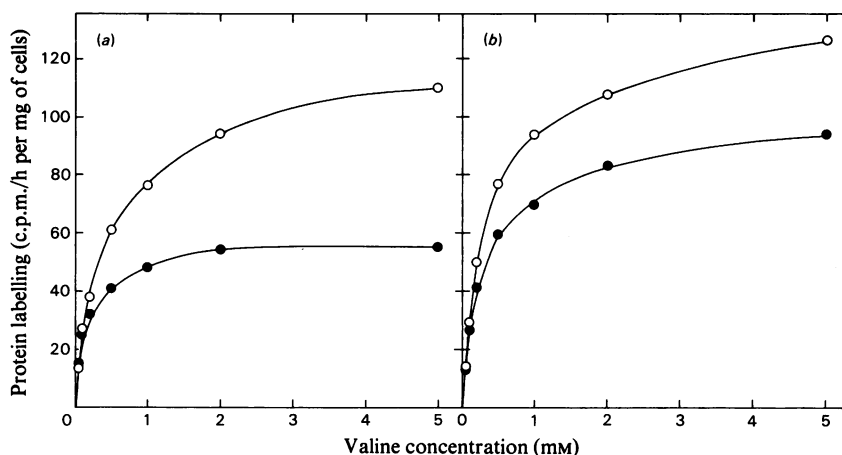

Fig. 3. *Dependence of NH$_4$Cl inhibition on [^{14}C]valine concentration*

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 (○) NH$_4$Cl (10 mM in a; 5 mM in b). [^{14}C]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 [^{14}C]valine (5 mmol/l; 63 mCi/mol) in the presence or absence of an amino acid mixture (12.5 times normal concentration), and the effects of leupeptin (125 μmol/l = 60 mg/l), NH$_4$Cl (10 mmol/l) or methylamine (10 mmol/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.

Additions	[^{14}C]Valine incorporation (c.p.m./h per mg of cells)	
	Control	+ Amino acids
Expt. 1		
None	67.9±1.0	77.6±0.3
Leupeptin	54.0±0.6	76.4±0.6
NH$_4$Cl	23.8±0.5	59.7±1.4
Expt. 2		
None	46.4±1.8	—
Leupeptin	46.8±0.6	—
NH$_4$Cl	20.2±1.1	—
Methylamine	35.7±1.2	—

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 NH$_4$Cl. 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 unsupplemented 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 NH$_3$ 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. NH$_3$ is a metabolically active amino-group donor that may interfere with hepato-

cyte 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.

References

Aoyagi, T. & Umezawa, H. (1975) in *Proteases and Biological Control* (Reich, E., Rifkin, D. B. & Shaw, E., eds.), pp. 429–454, Cold Spring Harbor Laboratory, Cold Spring Harbor

Christman, J. (1973) *Biochim. Biophys. Acta* **294**, 138–152

Craig, M. C. & Porter, J. W. (1973) *Arch. Biochem. Biophys.* **159**, 606–614

Ekren, T., Jervell, K. F. & Seglen, P. O. (1971) *Nature (London) New Biol.* **229**, 244–245

Eliasson, E., Bauer, G. E. & Hultin, T. (1967) *J. Cell Biol.* **32**, 287–297

Fausto, N. (1972) *Biochim. Biophys. Acta* **281**, 543–553

Gan, J. C. & Jeffay, H. (1971) *Biochim. Biophys. Acta* **252**, 125–135

Grant, A. G. & Black, E. G. (1974) *Eur. J. Biochem.* **47**, 397–401

Hod, Y. & Hershko, A. (1976) *J. Biol. Chem.* **251**, 4458–4467

Hopgood, M. F., Clark, M. G. & Ballard, F. J. (1977) *Biochem. J.* **164**, 399–407

Jefferson, L. S. & Korner, A. (1969) *Biochem. J.* **111**, 703–712

Khairallah, E. A. & Mortimore, G. E. (1976) *J. Biol. Chem.* **251**, 1375–1384

Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S., Bohley, P. & Broghammer, U. (1976) *Acta Biol. Med. Germ.* **35**, 285–299

Knowles, S. E. & Ballard, F. J. (1976) *Biochem. J.* **156**, 609–617

Lee, S. Y., Krsmanovic, V. & Brawerman, G. (1971) *J. Cell Biol.* **49**, 683–691

McGown, E., Richardson, A. G., Henderson, L. M. & Swan, P. B. (1973) *J. Nutr.* **103**, 109–116

Meijer, A. J., Gimpel, J. A., Deleeuw, G. A., Tager, J. M. & Williamson, J. R. (1975) *J. Biol. Chem.* **250**, 7728–7738

Mortimore, G. E., Woodside, K. H. & Henry, J. E. (1972) *J. Biol. Chem.* **247**, 2776–2784

Munro, H. N. (1968) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **27**, 1231–1237

Poole, B. (1971) *J. Biol. Chem.* **246**, 6587–6591

Pronczuk, A. W., Rogers, Q. R. & Munro, H. N. (1970) *J. Nutr.* **100**, 1249–1258

Righetti, P., Little, E. P. & Wolf, G. (1971) *J. Biol. Chem.* **246**, 5724–5732

Schreiber, G., Urban, J., Zähringer, J., Reutter, W. & Frosch, U. (1971) *J. Biol. Chem.* **246**, 4531–4538

Seglen, P. O. (1973) *Exp. Cell Res* **82**, 391–398

Seglen, P. O. (1974) *Biochim. Biophys. Acta* **338**, 317–336

Seglen, P. O. (1975) *Biochem. Biophys. Res. Commun.* **66**, 44–52

Seglen, P. O. (1976a) *Biochim. Biophys. Acta* **442**, 391–404

Seglen, P. O. (1976b) in *Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies* (Tager, J. M., Söling, H. D. & Williamson, J. R., eds.), pp. 245–256, North-Holland, Amsterdam

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

Seglen, P. O. (1976d) in *Progress in Differentiation Research* (Müller-Bérat, N., ed.), pp. 205–210, North-Holland, Amsterdam

Seglen, P. O. (1977a) *Biochim. Biophys. Acta* **496**, 182–191

Seglen, P. O. (1977b) *Exp. Cell Res.* **107**, 207–217

Seglen, P. O. & Reith, A. (1976) *Exp. Cell Res.* **100**, 276–280

Seglen, P. O. & Reith, A. (1977) *Biochim. Biophys. Acta* **496**, 29–35

Seglen, P. O. & Solheim, A. E. (1978a) *Eur. J. Biochem.* **85**, 15–25

Seglen, P. O. & Solheim, A. E. (1978b) *Biochim. Biophys. Acta* in the press

Sidransky, H., Sarma, D. S. R., Bongiorno, M. & Verney, E. (1968) *J. Biol. Chem.* **243**, 1123–1132

Smulson, M. E. & Rideau, C. (1970) *J. Biol. Chem.* **245**, 5350–5353

Stubbs, M. & Krebs, H. A. (1975) *Biochem. J.* **150**, 41–45

Van Den Borre, M. & Webb, T. E. (1972) *Life Sci.* **11**, 347–354

van Venrooij, W. J. W., Henshaw, E. C. & Hirsch, C. A. (1970) *J. Biol. Chem.* **245**, 5947–5953

van Venrooij, W. J. W., Henshaw, E. C. & Hirsch, C. A. (1972) *Biochim. Biophys. Acta* **259**, 127–137

Weigand, K., Wernze, H. & Falge, C. (1977) *Biochem. Biophys. Res. Commun.* **75**, 102–110

Woodside, K. H. (1976) *Biochim. Biophys. Acta* **421**, 70–79

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

Zahlten, R. N., Kneer, N. M., Stratman, F. W. & Lardy, H. A. (1974) *Arch. Biochem. Biophys.* **161**, 528–535

Zaleski, J. & Bryla, J. (1977) *Arch. Biochem. Biophys.* **183**, 553–562