An Energy Requirement for the Degradation of Intravenously Injected ¹²⁵I-Labelled Albumin in Mouse Liver and Kidney Slices

By JOHN L. MEGO and RODERICK M. FARB

Department of Biology, University of Alabama, University, AL 35486, U.S.A.

(Received 15 August 1977)

Liver and kidney slices prepared 30 min after intravenous injections of formaldehyde-treated ¹²⁵I-labelled bovine serum albumin into mice degrade approx. 25–40% of the protein to a trichloroacetic acid-soluble form during 60 min incubation at 37°C. The presence of bicarbonate in Krebs–Ringer phosphate medium inhibited intracellular proteolysis, and similar results were obtained at pH 5 or pH 7 in kidney or liver slices. Cellular integrity was required to obtain substantial rates of proteolysis. This intralysosomal intracellular degradation of an exogenous protein was partially inhibited by inhibitors of oxidative ATP formation, such as cyanide, azide, 2,4-dinitrophenol and absence of oxygen. Arsenite and iodoacetamide were also effective inhibitors, but the effects of fluoride were variable. These results suggest that an energy requirement exists for intralysosomal proteolysis in intact cells and are consistent with the hypothesis that energy may be required to maintain intralysosomal acidity.

Intracellular degradation of endogenous proteins is inhibited by inhibitors of ATP production. This observation, first reported by Simpson (1953), has been confirmed by Brostrom & Jeffay (1970) and Herskho & Tomkins (1971), and by Poole & Wibo (1973) and Hopgood *et al.* (1977), using cell suspensions. In recent years, evidence has accumulated suggesting that intracellular protein catabolism is accomplished in lysosomes (Hopgood *et al.*, 1977; Dean, 1975*a,b,c*; Neely & Mortimore, 1974; Mortimore *et al.*, 1973). An involvement of lysosomes in the degradative aspect of protein turnover might offer an explanation for the apparent energy requirement, since energy might be necessary for protein uptake into lysosomes, maintenance of lysosome integrity, or perhaps for generation of the intralysosomal acidity required for hydrolase functions. However, cells that do not contain lysosomes also require energy for intracellular proteolysis. In particular, bacteria (Goldberg, 1976; Goldberg *et al.*, 1975; Pine, 1975) and rabbit reticulocytes (Etlinger & Goldberg, 1977) demonstrate this requirement.

Thus the degradative aspect of protein turnover has not been clearly defined. The energy requirement for the process has also not been adequately explained and it is possible that more than one system exists. We have demonstrated at least 2-fold stimulation of proteolysis by ATP in mouse kidney phagolysosomes in cell-free preparations (Mego *et al.*, 1972; Mego, 1975). This stimulation is manifested only in alkaline or neutral media (pH 7–8) and it is abolished by the proton ionophores nigericin and 2,4-dinitrophenol (Mego, 1975), suggesting that ATP may function in this system to maintain or enhance intralysosomal

acidity in some manner, perhaps by energizing a proton pump. Substantial rates of proteolysis in mouse liver or kidney phagolysosomes occur in media buffered at pH 7–8 (Mego, 1971), suggesting that intralysosomal acidity is maintained by a proton Donnan equilibrium, as proposed by Reijngoud & Tager (1973, 1975), Reijngoud *et al.* (1976) and Henning (1975). Stimulatory effects of ATP therefore may reflect an auxiliary mechanism that functions to maintain acidic pH only in the presence of alkaline buffers or perhaps under the neutral conditions existing in the cell. In the present paper, we have attempted to determine if an energy requirement exists for intracellular proteolysis of intravenously injected ¹²⁵I-labelled albumin in intact cells.

Materials and Methods

Preparation of ¹²⁵I-labelled bovine serum albumin and treatment of this protein with formaldehyde to induce uptake into phagolysosomes after intravenous injections have been described elsewhere (Mego, 1971, 1973*b*). Adult Swiss albino mice were injected intravenously via the caudal vein with 1–2 mg of labelled protein (0.1–0.2 ml; about 4×10^6 c.p.m.). About 30 min after injections, the animals were anaesthetized with diethyl ether and livers and/or kidneys removed and immediately chilled on ice in modified (bicarbonate-free) Krebs–Ringer phosphate solution. The organs were sliced with a Stadie–Riggs tissue slicer in the cold-room. The slices were carefully washed with the cold Krebs–Ringer solution and placed in 25 ml Erlenmeyer flasks containing 5 ml of preincubated (37°C) media. Incubations were carried

out in a Dubnoff shaking water bath, generally for 1 h at 37°C. The suspensions were then transferred to chilled Teflon-glass homogenizers (A. H. Thomas Co., Philadelphia, PA, U.S.A.) and homogenized on ice for 30 s at about 300 rev./min. A portion (2 ml) of each homogenate was added to 2 ml of 10% (w/v) trichloroacetic acid in a 16 mm × 150 mm plastic counting tube. These were counted for radioactivity for at least 1 min in a gamma well counter with a 2 in (5 cm) thallium-activated NaI crystal and automatic sampler changer. The samples were then chilled in ice and centrifuged briefly (1600g for 5 min) at room temperature (20–22°C) to pack the precipitated protein. The supernatants were decanted into clean counting tubes and counted again for radioactivity to obtain the acid-soluble radioactivity, which is a measure of proteolytic activity.

Modified (bicarbonate-free) Krebs-Ringer phosphate solution was prepared by mixing 100 ml of 0.9% (w/v) NaCl with 4 ml of 1.5% (w/v) KCl, 3 ml of 1.22% (w/v) CaCl₂, 1 ml of 2.11% (w/v) KH₂PO₄ and 21 ml of water instead of the 1.3% NaHCO₃ normally required for this solution. In some preliminary experiments (described in the text), 0.155 M-potassium phosphate buffer, pH 7.5, was used in place of the KH₂PO₄.

Experiments on the effects of metabolic inhibitors and other substances on proteolysis in tissue slices were performed in the bicarbonate-free Krebs-Ringer phosphate, pH 5.2. Liver or kidney slices prepared from a mouse injected with formaldehyde-treated ¹²⁵I-labelled albumin were incubated in Erlenmeyer flasks with and without inhibitors. Samples of slices were homogenized at zero time and after 60 min incubation and these were analysed for trichloroacetic acid-soluble radioactivity. In experiments on the effects of anaerobiosis, the preincubated flasks were flushed with N₂, the slices were added and the flasks were flushed again, stopped and incubated. Control flasks for these experiments were incubated as usual in unflushed unstoppered flasks.

A measure of cell viability was determined by lactate dehydrogenase activities in the incubation media at zero time and after 1 h incubation after centrifugation for 5 min at 2000g in a refrigerated centrifuge at 4°C to remove intact tissues and other debris. Lactate dehydrogenase was also measured in the total tissue after the incubation period. For total activities, the tissues were homogenized on ice in the incubation media and centrifuged at 2000g for 5 min. The enzyme was measured in the resulting supernatant. Lactate dehydrogenase was determined by measuring the initial rate of decrease in A₃₄₀ of NADH in a 3 ml reaction mixture containing 0.33 mM-sodium pyruvate, pH 7.4, 66 μM-NADH and 30 mM-sodium phosphate buffer, pH 7.4. Enzyme activities were calculated as μmol of NADH oxidized/min per ml of medium. Cell viability was determined by sub-

tracting zero-time activity from the activity at 60 min. The resulting value was taken as a measure of cell disruption during the incubation period. This was subtracted from the total lactate dehydrogenase activity in the slices to determine the percentage of viable (intact) cells remaining.

NaN₃, 2,4-dinitrophenol, iodoacetamide, mercaptoethanol, NADH, sodium pyruvate and crystalline bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Other chemicals used were A.C.S.-certified reagent-grade or Baker analysed reagents.

Results

Initial studies were performed to determine a suitable medium for incubating tissue slices prepared from mice injected with formaldehyde-treated ¹²⁵I-labelled bovine albumin. Higher proteolytic rates of labelled albumin in the slices were obtained in 0.24 M-sucrose than in Krebs-Ringer phosphate containing bicarbonate or in water. If bicarbonate was left out of the Krebs-Ringer solution, proteolytic rates were approximately the same as in 0.25 M-sucrose. Replacing 1.3% NaHCO₃ with an equal volume of water results in a pH of about 5.2 rather than the normal 7.2 in Krebs-Ringer solution. An experiment was therefore carried out to determine if the inhibition of proteolysis by complete Krebs-Ringer phosphate was due to pH or to the bicarbonate present. In this experiment, proteolytic activity was compared in mouse kidney and liver slices incubated in Krebs-Ringer solution containing bicarbonate, in Krebs-Ringer solution containing an equal volume of water instead of the bicarbonate, and in Krebs-Ringer solution lacking bicarbonate but containing 0.155 M-potassium phosphate buffer, pH 7.2, in place of the KH₂PO₄ component. The results (Table 1) show that proteolysis in tissue slices incubated in bicarbonate-containing medium was considerably lower than in media lacking this component, and pH had little or no effect. The results shown in Table 1 also demonstrate typical values obtained in these experiments.

Intralysosomal proteolysis in cell-free suspensions of mouse liver or kidney phagolysosomes filled with formaldehyde-treated ¹²⁵I-labelled albumin incubated in media containing 25 mM-bicarbonate or pH 8 buffers is decreased by about 50% compared with suspensions incubated at pH 5 or in unbuffered media (Mego, 1971; Mego *et al.*, 1972). Proteolysis in these suspensions was stimulated about 2-fold by additions of ATP (Mego, 1975; Mego *et al.*, 1972). However, additions of ATP to mouse liver or kidney slices incubated in Krebs-Ringer phosphate solution containing bicarbonate in the present studies did not stimulate proteolysis. This suggests that these tissue suspensions did not contain significant quantities of

Table 1. *Effect of bicarbonate on proteolysis of intravenously injected ^{125}I -labelled bovine serum albumin in mouse liver and kidney slices*

Slices were washed in ice-cold 0.25M-sucrose, and a zero-time sample was homogenized in cold sucrose. The total and acid-soluble radioactivity (c.p.m.) respectively in these zero-time samples were: kidney, 8246 and 686 (8.3% acid-soluble); liver, 4393 and 371 (8.4% acid-soluble). Samples were then incubated in Krebs-Ringer phosphate media for 60min at 37°C. They were then homogenized and counted for total and trichloroacetic acid-soluble radioactivity. Values in parentheses are the acid-soluble radioactivities as percentages of the total in the samples. $\Delta/60$ refers to the total acid-soluble radioactivity at 60min minus the zero-time acid-soluble radioactivity as a percentage of the total.

Tissue	Krebs-Ringer phosphate medium	Radioactivity (c.p.m.) at 60min		$\Delta/60$
		Total	Acid-soluble	
Kidney	Complete (pH 7.2)	7753	2042 (26.3)	18.0
	-NaHCO ₃ (pH 7.2)	11338	4645 (41.0)	32.7
	-NaHCO ₃ (pH 5.2)	8975	3616 (40.3)	32.0
Liver	Complete (pH 7.2)	4428	1152 (26.0)	17.6
	-NaHCO ₃ (pH 7.2)	2619	883 (33.7)	25.3
	-NaHCO ₃ (pH 5.2)	2718	1044 (38.4)	30.0

free phagolysosomes in which proteolysis was inhibited by bicarbonate. To provide further evidence that proteolytic activity in the tissue-slice experiments was not occurring in phagolysosomes liberated from the cells during slice preparation and to demonstrate that cellular integrity was required for significant rates of intracellular proteolysis, kidney and liver slices from mice injected with labelled albumin were homogenized in cold bicarbonate-free (pH 5.2) Krebs-Ringer phosphate solution. Control slices from the same animal were not homogenized. Samples of the homogenized and intact tissues were taken at zero time and at intervals during incubation at 37°C, and these were analysed for trichloroacetic acid-soluble radioactivity. Results illustrated in Fig. 1 show that substantially lower rates of proteolysis occurred in the homogenized samples, indicating that cellular integrity was required for intralysosomal proteolysis in the medium used in these experiments.

An experiment was also performed to determine if rates of proteolysis were the same in all sections obtained from mouse kidneys. Slices were carefully prepared from two kidneys as shown in Fig. 2, and these were incubated separately for 1 h in modified Krebs-Ringer phosphate solution. The results indicate that proteolysis was about the same in each slice.

Effects of some inhibitors of oxidative metabolism and ATP formation and of glycolysis and substrate-level phosphorylation are shown in Table 2. Significant inhibitory effects were obtained with 2,4-

Fig. 1. *Effects of tissue integrity on proteolysis in mouse kidney (—) and liver (---)*

Liver and kidney slices were prepared from a mouse previously injected with ^{125}I -labelled albumin and each set of slices was divided into two approximately equal parts. One part was homogenized in 6ml of ice-cold modified (bicarbonate-free) Krebs-Ringer phosphate solution on ice. The remaining slices were divided into four parts. Each part was washed and transferred to 3ml of preincubated modified Krebs-Ringer solution. The 6ml tissue homogenates were added to 6ml of preincubated media. These and the intact slices were then incubated at 37°C. Samples (3ml) of the homogenates (○) and one flask of each tissue-slice preparation (●) were homogenized at the times indicated and trichloroacetic acid-soluble radioactivity was determined as shown in Table 1.

dinitrophenol, anaerobiosis (N₂ atmosphere), NaCN, NaN₃ and iodoacetamide. Inhibitory effects of fluoride, although consistent in the liver, were more variable than those of the other substances tested. Fluoride was less effective in kidney slices than in liver, and the inhibition shown in Table 2 is perhaps not significant. Proteolysis rates were also more variable in liver slices than in the kidney preparations. This was perhaps due to the greater difficulty in obtaining uniform slices of liver tissue, or to variable rates of liver-cell disintegration during incubation (Table 3). Therefore no attempts were made to calculate S.E.M. values in experiments with liver slices.

Table 3 shows the percentage of viable (intact) cells remaining after 1 h incubations at 37°C as determined by lactate dehydrogenase activities in the incubation media as described in the Materials and Methods section. Although a relatively large proportion of the liver cells disintegrated during incubation, kidney cells were more resistant and the results obtained with inhibitors present were not significantly different

Slice	$\Delta/60$
1	37.1
2	36.9
3	34.1
4	35.2
5	36.1
6	34.8
7	36.7
8	33.2

Fig. 2. Proteolysis in individual slices prepared from kidneys removed from a mouse injected with ^{125}I -labelled serum albumin

Each kidney was sliced into six slices and numbered as shown. Those marked 0 (two slices from each kidney) were homogenized before incubation and were taken as the zero-time acid-soluble radioactivity. The remaining slices were incubated individually in modified (bicarbonate-free) Krebs-Ringer phosphate solution at 37°C for 1 h. The percentage acid-soluble radioactivity in the zero-time samples was subtracted from the percentages after incubation and the resulting values ($\Delta/60$) are shown for each slice. Slices 1, 2, 3 and 4 were from one kidney and slices 5, 6, 7 and 8 were from the other kidney. Slices 0 (two from each kidney) were the zero-time samples.

from control preparations to which no inhibitors were added. The results obtained with liver slices were also considerably more variable, which probably accounted for the greater variations obtained with inhibitors noted previously. The data in Table 3 therefore suggest that the inhibitory effects of the substances added to the tissue suspensions were not due to cell disruptions.

Discussion

The energy requirement for the catabolic aspect of protein turnover has been demonstrated in a variety of cells and tissues (Goldberg, 1976). This requirement is based on studies similar to the present one in which inhibitors of ATP formation decrease intracellular protein catabolism in intact cells and tissues.

Table 2. Effects of some metabolic inhibitors on proteolysis of intravenously injected ^{125}I -labelled serum albumin in mouse liver and kidney slices

Slices were prepared from organs removed from anaesthetized (ether) mice 30 min after intravenous injections of formaldehyde-treated ^{125}I -labelled bovine serum albumin. Incubations were at 37°C in 5 ml of modified (bicarbonate-free) Krebs-Ringer phosphate solution, pH 5.2. Values for kidney slices are means $\pm$ S.E.M. for at least four experiments. Values shown were determined as illustrated in Table 1. Data for liver-slice experiments have been adjusted to the nearest whole numbers: numbers of experiments are given in parentheses.

Conditions	Concn. (mm)	Percentage of total radio-activity converted into acid-soluble form during 60 min incubation at 37°C		
		Liver		
		Kidney	Mean	Range
Control	—	32.8 ± 1.3	34	33–40 (4)
+2,4-Dinitrophenol	9	20.3 ± 1.1	21	11–28 (4)
+NaF	10	30.6 ± 1.4	24	16–39 (4)
Anaerobiosis	—	21.1 ± 1.8	21	20–22 (2)
+Iodoacetamide	2	14.6 ± 0.3	17	13–21 (2)
+NaCN	1	15.0 ± 1.2	22	16–28 (2)
+NaN ₃	100	16.3 ± 0.4	18	13–22 (2)
+NaHAsO ₃	1	21.3 ± 0.8	13	— (1)

Table 3. Effects of metabolic inhibitors on cell integrity during incubations of mouse liver and kidney slices at 37°C

Cell viability was determined in mouse liver and kidney slices by measuring lactate dehydrogenase activities in the incubation media as described in the Materials and Methods section. Each value represents the average of two determinations. Concentrations of inhibitors were the same as those shown in Table 2.

Inhibitor	Percentage of intact cells remaining after 60 min incubation	
	Kidney	Liver
None	93.4	64.9
NaCN	87.5	62.4
NaN ₃	86.6	72.8
NaAsO ₂	87.0	48.8
NaF	92.6	72.0
Iodoacetamide	86.8	64.7
2,4-Dinitrophenol	88.6	58.7
Anaerobiosis	89.0	34.0

In the present study, an energy requirement has been demonstrated for the degradation of an exogenous protein within intact cells of mouse liver and kidney. This protein is taken up from the bloodstream by endocytosis into cells of the reticuloendothelial system after intravenous injection. In the cell, the protein appears in endocytosis vesicles, which aggregate to

form phagosomes (Novikoff, 1961; Becker *et al.*, 1967; Gordon, 1973). Phagosomes become phagolysosomes after fusing with lysosomes, which provide the proteinases required for degradation of the protein (Straus, 1964; Gordon, 1973). Phagolysosomes may be sedimented from cell-free tissue homogenates by centrifugation in appropriate media and proteolysis may be measured in these organelles during incubation at 35°C (Mego, 1973a). These particles are liable to osmotic disruption and must be protected by non-penetrating substances, such as sucrose or salts, in relatively high concentrations (Mego, 1973a; Mego *et al.*, 1967). The total salt concentration in the modified Krebs-Ringer phosphate solution used in the experiments reported in the present study was about 0.13M, which is not sufficient for optimal maintenance of osmotic integrity of phagolysosomes, although it may have provided some protection. The homogenization of tissue slices in modified Krebs-Ringer phosphate solution therefore resulted in a relatively high rate of osmotic lysis of phagolysosomes containing labelled protein and cathepsins. Proteolysis would be inhibited in these preparations by dilution of enzymes and labelled protein in the relatively large volume of medium (12ml).

Of the metabolic inhibitors tested for effects on intralysosomal proteolysis in tissue slices, only iodoacetamide, arsenite and arsenate inhibit intralysosomal proteolysis in cell-free suspensions of albumin-containing phagolysosomes (Mego, 1973a). These substances are known inhibitors of cathepsins (Misaka & Tappel, 1971). Cyanide, azide, 2,4-dinitrophenol, anaerobiosis and fluoride had no effects (J. L. Mego, unpublished work; Mego, 1973a). However, 2,4-dinitrophenol abolished the stimulatory effects of ATP in these cell-free suspensions (Mego, 1975). The inhibitory effects of anaerobiosis, azide, cyanide, fluoride and 2,4-dinitrophenol on the degradation of intravenously injected labelled albumin in tissue slices therefore was not directly on cathepsin activity in lysosomes, but on some process closely related to lysosome function. Since these substances are classic inhibitors or uncouplers of mitochondrial electron transport or of glycolysis and hence of ATP formation, their effects must be on an energy requirement for lysosome function. This energy may be required for incorporation of protein into lysosomes, for maintenance of lysosomal integrity or for some aspect of hydrolase function such as maintenance of intralysosomal acidity. A period of at least 20min after intravenous injection is required for maximum uptake of labelled albumin into osmotically active particles capable of degrading the protein in mouse liver (Mego & McQueen, 1967). Therefore the energy requirement for maximum proteolysis in tissue slices did not appear to be to facilitate incorporation of protein into lysosomes, since maximum uptake had already occurred before

the death of the animals. Although the presence of ATP in cell-free suspensions containing albumin-filled phagolysosomes did indeed appear to prevent excessive phagolysosome disruption during incubations at 35°C, the primary effect was on rates of intralysosomal proteolysis (Mego *et al.*, 1972). It appears likely therefore that the energy requirement for intralysosomal proteolysis in tissue slices was also not primarily for maintenance of phagolysosome integrity. Reijngoud & Tager (1973) and Henning (1975) have shown that intralysosomal pH is approx. 1 unit lower than the incubating media. Since intracellular pH is about 7, the pH within lysosomes in the cell should therefore be near 6. At this pH, crude mouse liver lysosomal proteinase activity is about 50% lower than it is at pH5 (Mego, 1971). This is approximately the maximum inhibition of intralysosomal proteolysis observed with metabolic inhibitors such as cyanide or azide in tissue slices reported in the present study. An ATP-energized system that maintains the intralysosomal acidity required for optimal hydrolase activity (pH5) therefore appears to be a logical function of the energy requirement reported in the present studies.

This work was supported by grants GM21829 and ES00591 from the National Institutes of General Medical Sciences and Environmental Health Science, National Institutes of Health, U.S. Public Health Service.

References

- Becker, N. H., Novikoff, A. B. & Zimmerman, H. M. (1967) *J. Histochem. Cytochem.* **15**, 160-165
- Brostrom, C. O. & Jeffay, H. (1970) *J. Biol. Chem.* **245**, 4001-4008
- Dean, R. T. (1975a) *Nature (London)* **257**, 414-416
- Dean, R. T. (1975b) *Eur. J. Biochem.* **58**, 9-14
- Dean, R. T. (1975c) *Biochem. Biophys. Res. Commun.* **67**, 604-609
- Etlinger, J. D. & Goldberg, A. L. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 54-58
- Goldberg, A. L. (1976) *Annu. Rev. Biochem.* **45**, 747-803
- Goldberg, A. L., Olden, J. & Prouty, W. F. (1975) in *Intracellular Protein Turnover* (Schimke, R. T. & Katunuma, N., eds.), pp. 17-55, Academic Press, New York and London
- Gordon, A. H. (1973) in *Lysosomes in Biology and Pathology* (Dingle, J. T., ed.), vol. 3, pp. 89-134, North-Holland Publishing Co., Amsterdam and London
- Henning, R. (1975) *Biochim. Biophys. Acta* **401**, 307-316
- Hershko, A. & Tomkins, G. M. (1971) *J. Biol. Chem.* **246**, 710-714
- Hopgood, M. F., Clark, M. G. & Ballard, F. G. (1977) *Biochem. J.* **164**, 399-407
- Mego, J. L. (1971) *Biochem. J.* **122**, 445-452
- Mego, J. L. (1973a) in *Lysosomes in Biology and Pathology* (Dingle, J. T., ed.), vol. 3, pp. 138-168, North-Holland Publishing Co., Amsterdam and London

- Mego, J. L. (1973b) in *Lysosomes in Biology and Pathology* (Dingle, J. T., ed.), vol. 3, pp. 527–537, North-Holland Publishing Co., Amsterdam and London
- Mego, J. L. (1975) *Biochem. Biophys. Res. Commun.* **67**, 571–575
- Mego, J. L. & McQueen, J. D. (1967) *J. Cell. Physiol.* **70**, 115–120
- Mego, J. L., Bertini, F. & McQueen, J. D. (1967) *J. Cell Biol.* **32**, 699–707
- Mego, J. L., Farb, R. M. & Barnes, J. (1972) *Biochem. J.* **128**, 763–769
- Misaka, E. & Tappel, A. L. (1971) *Comp. Biochem. Physiol. B* **38**, 651–662
- Mortimore, G. E., Neely, A. N., Cox, J. R. & Guinivan, R. A. (1973) *Biochem. Biophys. Res. Commun.* **54**, 89–95
- Neely, A. N. & Mortimore, G. E. (1974) *Biochem. Biophys. Res. Commun.* **59**, 680–687
- Novikoff, A. B. (1961) in *The Cell: Biochemistry, Physiology, Morphology* (Brachet, J. & Mirsky, A. E., eds.), vol. 2, pp. 423–488, Academic Press, New York and London
- Pine, M. J. (1975) in *Intracellular Protein Turnover* (Schimke, R. T. & Katunuma, N., eds.), pp. 65–76, Academic Press, New York and London
- Poole, B. & Wibo, M. (1973) *J. Biol. Chem.* **248**, 6221–6226
- Reijngoud, D. J. & Tager, J. M. (1973) *Biochim. Biophys. Acta* **297**, 174–178
- Reijngoud, D. J. & Tager, J. M. (1975) *FEBS Lett.* **54**, 76–79
- Reijngoud, D. J., Oud, P. S. & Tager, J. M. (1976) *Biochim. Biophys. Acta* **448**, 290–302
- Simpson, M. V. (1953) *J. Biol. Chem.* **202**, 143–154
- Straus, W. (1964) *J. Cell Biol.* **20**, 497–507