Nucleotide catabolism and nucleoside cycles in human thymocytes

Role of orthophosphate

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The catabolism of GTP and ATP in intact human thymocytes was studied, and the effect of intracellular P_i concentrations on various catabolic reactions was evaluated. Induction of nucleotide catabolism was performed with either NaN_3 or deoxyglucose. NaN_3 induced an increase in intracellular P_i , whereas deoxyglucose induced a decrease in P_i in human thymocytes. At elevated P_i concentrations, an intracellular accumulation of GMP, IMP and AMP was observed, and the entry of these nucleotides into the nucleoside cycles was diminished. In contrast, at lowered P_i concentrations, there was no accumulation of nucleoside monophosphates, but the production of purine bases and nucleosides was increased as a result of enhanced nucleotide entry into the nucleoside cycles. The dephosphorylation of nucleotides thus seems to be regulated by intracellular P_i concentrations. ATP catabolism proceeded mainly via adenylate deamination at both elevated and lowered P_i concentrations. Guanylate formed during GTP catabolism was mainly dephosphorylated, but significant amounts of GMP were also deaminated to yield IMP.

The discovery of the purine nucleoside cycles, i.e. the inosine cycle and the guanosine cycle (Barankiewicz *et al.*, 1982), raised the question of the significance and the possible physiological role(s) of these cycles. These cycles are completed by the action of three enzymes: 5'-nucleotidase (EC 3.1.3.5), purine nucleoside phosphorylase (EC 2.4.2.1) and hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8).

P_i is an important factor in the operation of both nucleoside cycles. Thus P_i is a product of IMP and GMP dephosphorylation by 5'-nucleotidase, and is a substrate for the subsequent catabolic step catalysed by purine nucleoside phosphorylase. In addition, P_i may have regulatory functions in the operation of these cycles. It has been shown that the dephosphorylation of intracellular nucleoside monophosphates is catabolized by a cytoplasmic 5'-nucleotidase (Newby, 1980; Carson *et al.*, 1981; Schütz *et al.*, 1981; Bagnara & Hershfield, 1982; Bontemps *et al.*, 1983), an enzyme which is effectively inhibited by P_i (Van den Berghe *et al.*, 1977; Itoh, 1981a,b; Worku & Newby, 1982). It has been also suggested that the activity of purine nucleoside phosphorylase from human erythrocytes may be limited by the availability of P_i (Planet & Fox,

1976). Finally, the third step of the purine nucleoside cycles, that catalysed by hypoxanthine/guanine phosphoribosyltransferase, is also phosphate-dependent, since P_i affects several steps in the synthesis of 5-phosphoribosyl 1-pyrophosphate from glucose, including ribosephosphate pyrophosphokinase (EC 2.7.6.1) (Passonneau & Lowry, 1962; Rizzo & Eckel, 1966; Fox & Kelley, 1972; Ellison *et al.*, 1975). Thus studies in intact Ehrlich ascites-tumour cells (Barankiewicz *et al.*, 1977) have shown that synthesis of 5-phosphoribosyl 1-pyrophosphate and ribose 1-phosphate increased with increasing P_i concentrations.

Although the catabolism of ATP and GTP has been studied previously in some cells (McComb & Yushok, 1964; Lomax *et al.*, 1975; Henderson *et al.*, 1978; Matsumoto *et al.*, 1980; Van den Berghe *et al.*, 1980; Des Rosiers *et al.*, 1982; Worku & Newby, 1983), the possible roles of P_i are not clear.

In view of the association of purine nucleoside phosphorylase (Giblett *et al.*, 1975) and adenosine deaminase (EC 3.5.4.4) (Giblett *et al.*, 1972) deficiency with immunodeficiency and with T-lymphocyte differentiation in humans (review by Martin & Gelfand, 1981), we have studied possible roles of these purine-catabolic enzymes in GTP

and ATP catabolism and in the operation of nucleoside cycles in human T lymphocytes.

Materials and methods

Chemicals

[8-³H]Guanosine was purchased from ICN Chemical and Radioisotope Division (Irvine, CA, U.S.A.); [8-³H]adenine was purchased from Amersham International (Amersham, Bucks., U.K.). Non-radioactive purine bases, nucleosides, nucleotides and 2-deoxy-D-glucose were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and NaN₃ was from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). Freon TF was obtained from DuPont Canada Inc. (Maitland, Ont., Canada), and Alamine from Henkel Corp. (Kankakee, IL, U.S.A.). RPMI medium was purchased from Ontario Cancer Institute (Toronto, Ont., Canada). Foetal-calf serum was purchased from Flow Laboratories (McLean, VA, U.S.A.). Polyethyleneimine-cellulose t.l.c. sheets were obtained from Fisher Scientific Co. (Don Mills, Ont., Canada), and 13255-cellulose t.l.c. sheets without fluorescent indicator from Eastman Kodak Co. (Rochester, NY, U.S.A.).

Separation of thymocytes

Human thymocytes were separated from thymuses obtained from children with no known immune dysfunction who were undergoing cardiac surgery requiring partial thymectomy. Cells were separated by the Ficoll-Hypaque density-gradient method as previously described (Cohen *et al.*, 1980). Post-Ficoll thymocytes were resuspended in RPMI medium containing 10% heat-inactivated (68°C; 30 min) foetal-calf serum.

Measurement of nucleotide catabolism

Thymocytes (2×10^7 cells) were incubated with 2 μ Ci of [8-³H]guanosine (20 Ci/mmol) or [8-³H]adenine (25 Ci/mmol) to synthesize radioactive GTP and ATP respectively. After 30–60 min incubation, unused precursors were completely washed out and the cells were resuspended in 0.154M-NaCl. Under these conditions, the radioactivity was incorporated mainly into GTP and ATP, and amounts of radioactivity found in nucleotides other than GMP, GDP and GTP, and AMP, ADP and ATP, respectively, were less than 2% of that in these guanine or adenine nucleotides. Also, washing of cells did not alter purine nucleotide concentration. These thymocytes (2×10^6 cells) were incubated for 0–30 min with 5.5 mM-deoxyglucose or 1 mM-NaN₃ to induce GTP and ATP catabolism. In some ATP-catabolism experiments a 30 min preincubation of thymocytes with the adenosine deaminase inhibitor 2'-deoxycoformycin (10 μ M)

was carried out. Thymocytes were incubated with NaN₃ or deoxyglucose for 30 min, and after 0, 5, 10, 20 and 30 min thymocytes (2×10^6) were collected by centrifugation (5 min at 700g; r_{av} 6 cm), and the radioactivity in nucleosides and bases, which were found exclusively in the medium, was measured. No nucleotides were detected in the medium. The cell pellet was extracted with 50 μ l of 0.4M-HClO₄, and extracts were neutralized with Alamine 336/Freon (Khym, 1975). Extracts were then used for nucleotide analysis.

Separation of nucleotides, nucleosides and bases

Analysis of radioactive nucleotides was performed by one-dimensional polyethyleneimine-cellulose t.l.c. in three steps of increasing sodium formate buffer concentrations (Henderson *et al.*, 1974). Separation of the radioactive ribonucleosides and bases (guanosine, guanine, inosine, hypoxanthine and xanthine, produced during GTP catabolism, and adenosine, inosine, hypoxanthine and xanthine, produced during ATP catabolism) was performed by one-dimensional cellulose t.l.c. with aq. 5% (w/v) Na₂HPO₄ as eluent. To separate adenosine from inosine in experiments where 2'-deoxycoformycin was used, plates were developed also in butanol/methanol/water/conc. NH₃ (sp.gr. 0.880) (60:20:20:1, by vol.) (Henderson *et al.*, 1974). Intracellular concentrations of guanine and adenine nucleotides in thymocytes (A. Cohen, J. Barankiewicz, H. M. Lederman & E. W. Gelfand, unpublished work) were taken into account in the calculation of specific radioactivities in the results.

Determination of P_i

To determine intracellular P_i concentrations, thymocytes (20×10^6) were washed once in 10 ml of 0.154M-NaCl and were extracted with 1 ml of 0.4M-HClO₄. After 10 min at 4°C, 0.4 ml of 50% (w/v) water suspension of Norit A charcoal was added to remove nucleotides. After removal of the charcoal by centrifugation (5 min at 4000g; r_{av} 6 cm), 1 ml of supernatant was used for P_i determination (Chen *et al.*, 1956).

Hypoxanthine salvage

To evaluate the purine salvage in human thymocytes treated with NaN₃ or deoxyglucose, thymocytes (1×10^6) were incubated in 0.15M-NaCl with 1 mM-NaN₃ or 5.5 mM-deoxyglucose for 15 min. After incubation, cells were washed once in 0.154M-NaCl and incubated with 1 μ Ci of [8-³H]-hypoxanthine for 20 min. Cells were then extracted with 50 μ l of 0.4M-HClO₄, and extracts neutralized with Alamine 336/Freon. Extracts were spotted on polyethyleneimine-cellulose thin-layer chromatograms. Radioactive nucleosides and bases were washed out into the wick in methanol/water

(Henderson *et al.*, 1974) and the total incorporation of radioactivity into nucleotides was measured.

Results

The intracellular P_i concentrations in human thymocytes incubated with NaN_3 or deoxyglucose are shown in Fig. 1. Deoxyglucose caused a rapid depletion of intracellular P_i in human thymocytes, whereas NaN_3 caused a marked increase in P_i compared with untreated thymocytes.

The kinetics of GTP catabolism are illustrated in Fig. 2. Both NaN_3 (Fig. 2a) and deoxyglucose (Fig. 2c) induced a rapid GTP breakdown; however, the extent of GTP catabolism was more pronounced in the presence of NaN_3 (80% GTP breakdown after 5 min) than in the presence of deoxyglucose (40% breakdown after 5 min). NaN_3 caused a rapid decrease in GTP/GDP ratio and a marked increase in GMP concentrations. In contrast, deoxyglucose had a smaller effect on the GTP/GDP ratio and did not cause significant accumulation of GMP. There was a small but

Fig. 1. Effect of NaN_3 and deoxyglucose on intracellular P_i concentrations

Thymocytes were incubated at 37°C in 0.154M-NaCl with 1mM-NaN_3 (○), $5.5\text{mM-deoxyglucose}$ (△) or 0.154M-NaCl (●). At the end of the incubation, P_i was extracted with 0.4M-HClO_4 and nucleotides were removed by a 50% suspension of activated charcoal. The charcoal was removed by centrifugation, and P_i concentration was determined as described in the Materials and methods section. Values reported are averages of duplicate analyses, and are representative of results obtained in four individual experiments.

detectable accumulation of IMP and XMP in the presence of NaN_3 but not in the presence of deoxyglucose. In general, cells incubated with deoxyglucose (Fig. 2d) secreted larger amounts of purines into the medium than did cells incubated in the presence of NaN_3 (Fig. 2b). The major product of GTP degradation was guanine; however, cells incubated in the presence of deoxyglucose excreted significantly more guanine than did cells incubated in the presence of NaN_3 . Guanosine and inosine were other major products of GTP degradation induced by deoxyglucose, whereas no nucleosides were excreted by cells incubated in the presence of NaN_3 . Both catabolic inducers caused the production of significant amounts of hypoxanthine from GTP, but only minor amounts of xanthine (Figs. 2b and 2d).

Induction of ATP catabolism by NaN_3 and deoxyglucose is depicted in Fig. 3. As with GTP catabolism, NaN_3 caused a rapid decrease in ATP, a decrease in ATP/ADP ratio, and subsequently the accumulation of IMP and AMP (Fig. 3a). The increase in IMP in the presence of NaN_3 was especially pronounced (Fig. 3a); in contrast, no IMP was accumulated when catabolism was induced by deoxyglucose (Fig. 3c). Deoxyglucose caused a smaller decrease in ATP, only a transient accumulation of ADP and AMP, and a relatively small fall in the ATP/ADP ratio. No interconversion into XMP and GMP was observed in the presence of either deoxyglucose or NaN_3 . Whereas both hypoxanthine and inosine are the main purines excreted in the presence of deoxyglucose (Fig. 3d), cells incubated in the presence of NaN_3 excreted only hypoxanthine (Fig. 3b). When thymocytes were preincubated with deoxycytosine (10 μM) and then ATP catabolism was induced by NaN_3 or deoxyglucose, there was only a small adenosine accumulation, never exceeding

Table 1. Hypoxanthine incorporation by human thymocytes in the presence of NaN_3 or deoxyglucose

Thymocytes (10^6) were incubated in 0.154M-NaCl with 1mM-NaN_3 or $5.5\text{mM-deoxyglucose}$ for 15 min. Cells were then washed once and incubated with $[8\text{-}^3\text{H}]\text{hypoxanthine}$ for the next 20 min. Total hypoxanthine incorporation into nucleotides was measured. Values are averages of duplicate analyses and are representative of results obtained in three individual experiments.

Addition	Incorporation	
	(pmol/ 10^6 cells)	(% of control)
None	189.6	100
NaN_3	223.9	120.5
Deoxyglucose	114.2	60.2

Fig. 2. GTP catabolism in human thymocytes

Thymocytes (2×10^6 cells) containing radioactive GTP were incubated in the presence of 1 mM- NaN_3 (a, b) or 5.5 mM-deoxyglucose (c, d) for the indicated times, and the radioactivities in nucleotides (a, c) were analysed in cell extracts: ●, GTP; ○, GDP; △, GMP; □, IMP; ▲, XMP. The radioactivities in nucleosides and bases (b, d) were analysed in the incubation medium: ●, guanine; ○, guanosine; △, hypoxanthine; ▲, inosine; ■, xanthine. Data shown are representative of results obtained from five experiments. For additional details see the Materials and methods section.

10% of the total purine excretion observed (results not shown).

When thymocytes containing radioactive GTP or ATP were incubated in RPMI medium without NaN_3 or deoxyglucose for 0–4 h, only small amounts of radioactive guanine, hypoxanthine or other radioactive metabolites were found in the incubation medium, and no significant changes in nucleotide concentrations were observed (results not shown).

Effects of NaN_3 and deoxyglucose on the incorporation of hypoxanthine into nucleotide pools are shown in Table 1. The results show that hypoxanthine salvage in human thymocytes is markedly decreased by deoxyglucose, but not by NaN_3 .

Discussion

In these studies, deoxyglucose and NaN_3 were employed to induce GTP and ATP catabolism in human thymocytes. Under these conditions regeneration of nucleoside triphosphates is impaired, and rapid breakdown of GTP and ATP takes place. Deoxyglucose caused ATP utilization for the formation of the slowly metabolized deoxyglucose 6-phosphate, and results in the depletion of intracellular P_i (Sauer, 1978). In contrast, NaN_3 inhibits oxidative phosphorylation, causing breakdown of nucleoside triphosphates, and as a result intracellular P_i increases (Fig. 1). Therefore the comparison of nucleotide degradation in the presence of deoxyglucose or NaN_3 allows us to study

Fig. 3. ATP catabolism in human thymocytes

Thymocytes (2×10^6 cells/ml) containing radioactive ATP were incubated for the indicated times in the presence of 1 mM- NaN_3 (a, b) or 5.5 mM-deoxyglucose (c, d). The radioactivities in nucleotides (a, c) were analysed in cell extracts: ●, ATP; ○, ADP; △, AMP; ▲, IMP. The radioactivities in nucleosides and bases (b, d) were determined in incubation medium: ○, inosine; ●, hypoxanthine; ▲, adenosine. Data shown are representative of results obtained in five experiments. For details see the Materials and methods section.

the relationship between intracellular P_i concentrations and GTP and ATP catabolism.

The induction of GTP and ATP catabolism in the presence of high P_i concentrations caused a marked accumulation of intracellular nucleoside monophosphates (Figs. 2a and 3a). These results

agree with data reported by Lomax *et al.* (1975) showing that in Ehrlich ascites-tumour cells about 70% of products have been found as nucleoside monophosphates during dinitrophenol-induced nucleoside triphosphate catabolism. The accumulation of nucleoside monophosphates is probably

caused by the inhibition of their dephosphorylation by increased intracellular P_i . The inhibition of cytosol 5'-nucleotidase by P_i has been previously reported for purified enzymes (Van den Berghe *et al.*, 1977; Itoh, 1981a,b; Worku & Newby, 1982). In the presence of high intracellular P_i concentrations, the free purine bases guanine (Fig. 2b) or hypoxanthine (Fig. 3b) were exclusively excreted, indicating phosphorolysis of the respective nucleosides formed during catabolism by purine nucleoside phosphorylase.

At low intracellular P_i concentrations nucleoside monophosphates are dephosphorylated, resulting in enhanced purine efflux (Figs. 2d and 3d). It has been previously reported that decrease in the concentration of P_i caused significant increase of nucleotide dephosphorylation by cytosol 5'-nucleotidase (Itoh, 1981b). Substantial amounts of the purines excreted by cells in the presence of deoxyglucose remained in the nucleoside form (guanosine and inosine in Figs. 2d and 3d), indicating a marked decrease of the purine nucleoside phosphorylase reaction by low intracellular P_i concentrations (Gotto *et al.*, 1964). Thus these results indicate that in human thymocytes at low intracellular P_i concentrations purine nucleoside phosphorylase may become the rate-limiting enzyme in nucleotide degradation.

Nucleoside monophosphate dephosphorylation and nucleoside phosphorolysis take part in the active nucleoside cycles described by Barankiewicz *et al.* (1982). Under normal physiological conditions the excretion of purines from thymocytes is low because degradation of nucleosides is not accelerated and metabolic flow through the nucleoside cycles is high (Barankiewicz *et al.*, 1982). At high P_i concentration, phosphoribosylation of purine bases is increased (Table 1) by enhanced 5-phosphoribosyl 1-pyrophosphate synthesis (Barankiewicz *et al.*, 1977). This indicates that nucleoside cycles operate when nucleotide catabolism is induced by NaN_3 , and saturation of the phosphoribosylation reaction results in efflux of purine bases (Figs. 2c and 3c). In contrast, at low P_i concentrations phosphoribosylation of purine bases is decreased (Table 1), probably as a result of a decrease in 5-phosphoribosyl 1-pyrophosphate synthesis. Thus at low P_i concentrations the operation of the nucleoside cycles is diminished not only at the purine nucleoside phosphorylase reaction but also at the purine-phosphoribosylation step. This results in enhanced efflux of purine bases and nucleosides (Fig. 2d and 3d).

It seems that intracellular P_i concentrations may represent an important factor in regulation of nucleotide catabolism. The critical rate-limiting step seems to be the dephosphorylation of IMP and GMP, which is inhibited at high P_i concentrations.

The next P_i -sensitive step in nucleotide degradation is nucleoside phosphorolysis, which is limited at low intracellular P_i concentration. Finally, the salvage of purine bases is regulated by the availability and concentration of 5-phosphoribosyl 1-pyrophosphate, which depend also on intracellular P_i concentrations (Barankiewicz *et al.*, 1977).

Some of the GMP formed during GTP catabolism (Fig. 2) is deaminated to IMP by guanylate reductase (EC 1.6.6.8), an enzyme present in cell extracts of erythrocytes and calf thymus (MacKenzie & Sorensen, 1973; Stephens & Whittaker, 1973). The formation of significant amounts of hypoxanthine and inosine indicates that reductive deamination of GMP is a quantitatively important pathway in GMP catabolism by human thymocytes. Intracellular P_i concentrations in thymocytes have no effect on the reductive deamination of intracellular GMP. It should be noted that in human thymocytes activity of GMP reductase was measurable when GTP catabolism was induced, but not when GTP was synthesized from guanine (results not shown).

The small adenosine accumulation in the presence of deoxycoformycin suggests that most of the AMP formed during ATP catabolism is directly deaminated to IMP, and only a small portion of the AMP formed is dephosphorylated to adenosine and then deaminated to inosine. A similar conclusion was reached by Smith *et al.* (1977) for fructose-induced degradation of adenine nucleotides. Although it has been shown previously that P_i inhibits adenylate deaminase in cell extracts (Zielke & Suelter, 1971), the accumulation of large amounts of IMP in intact human thymocytes at high intracellular P_i concentrations indicates that the adenylate deamination reaction is sufficiently active even at high P_i concentrations. Therefore the decrease in nucleotide degradation observed at elevated P_i concentrations (Van den Berghe *et al.*, 1980) may be due to inhibition by P_i of nucleoside monophosphate dephosphorylation rather than inhibition of the adenylate deaminase reaction.

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References

- Bagnara, A. S. & Hershfield, M. S. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2673-2677
- Barankiewicz, J., Battell, M. L. & Henderson, J. F. (1977) *Can. J. Biochem.* **55**, 834-840
- Barankiewicz, J., Gelfand, E. W., Issekutz, A. & Cohen, A. (1982) *J. Biol. Chem.* **257**, 11597-11600
- Bontemps, F., Van den Berghe, G. & Hers, H.-G. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2829-2833

- Carson, D. A., Kaye, J. & Wasson, D. B. (1981) *J. Immunol.* **126**, 348–352
- Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758
- Cohen, A., Lee, J. W. W., Dosch, H.-M. & Gelfand, E. W. (1980) *J. Immunol.* **125**, 1578–1582
- Cohen, A., Dosch, H.-M. & Gelfand, E. W. (1981) *Clin. Immunol. Immunopathol.* **18**, 287–290
- Des Rosiers, C., Lalanne, M. & Willemot, J. (1982) *Can. J. Biochem.* **60**, 1101–1108
- Ellison, E. R., Lueck, J. D. & Fromm, H. J. (1975) *J. Biol. Chem.* **250**, 1864–1871
- Fox, I. H. & Kelley, W. N. (1972) *J. Biol. Chem.* **247**, 2126–2130
- Giblett, E. R., Anderson, I. E., Cohen, F., Pollara, B. & Meuwissen, H. J. (1972) *Lancet* **ii**, 1067–1069
- Giblett, E. R., Amman, A. Y., Wara, D. W., Sandman, R. & Diamond, L. K. (1975) *Lancet* **i**, 1010
- Gotto, A. M., Meikle, A. W. & Touster, O. (1964) *Biochim. Biophys. Acta* **80**, 552–561
- Henderson, J. F., Freser, J. H. & McCoy, E. E. (1974) *Clin. Biochem.* **7**, 339–358
- Henderson, J. F., Zombor, G. & Burridge, P. W. (1978) *Can. J. Biochem.* **56**, 474–479
- Itoh, R. (1981a) *Biochim. Biophys. Acta* **657**, 402–410
- Itoh, R. (1981b) *Biochim. Biophys. Acta* **659**, 31–37
- Khym, J. X. (1975) *Clin. Chem.* **21**, 1245–1251
- Lomax, C. A., Bagnara, A. S. & Henderson, J. F. (1975) *Can. J. Biochem.* **53**, 231–247
- MacKenzie, J. J. & Sorensen, L. B. (1973) *Biochim. Biophys. Acta* **237**, 282–294
- Martin, D. W., Jr. & Gelfand, E. W. (1981) *Annu. Rev. Biochem.* **50**, 845–877
- Matsumoto, S. S., Raivio, K. O., Willis, R. C. & Seegmiller, J. E. (1980) *Adv. Exp. Med. Biol.* **122**, 277–282
- McComb, R. B. & Yushok, W. D. (1964) *Cancer Res.* **24**, 198–203
- Newby, A. C. (1980) *Biochem. J.* **186**, 907–918
- Passonneau, J. V. & Lowry, O. H. (1962) *Biochem. Biophys. Res. Commun.* **7**, 10–15
- Planet, G. & Fox, I. H. (1976) *J. Biol. Chem.* **251**, 5839–5844
- Rizzo, S. C. & Eckel, R. E. (1966) *Am. J. Physiol.* **211**, 429–436
- Sauer, L. A. (1978) *Cancer Res.* **38**, 1057–1063
- Schütz, W., Schrader, J. & Gerlech, E. (1981) *Am. J. Physiol.* **240**, H963–H970
- Smith, C. M., Rovamo, L. M. & Raivio, K. O. (1977) *Can. J. Biochem.* **55**, 1237–1240
- Stephens, R. W. & Whittaker, V. K. (1973) *Biochem. Biophys. Res. Commun.* **53**, 975–981
- Van den Berghe, G., Van den Pottelsberghe, C. & Hers, H.-G. (1977) *Biochem. J.* **162**, 611–616
- Van den Berghe, G., Bontemps, F. & Hers, H.-G. (1980) *Biochem. J.* **188**, 913–920
- Worku, Y. & Newby, A. C. (1982) *Biochem. J.* **205**, 503–510
- Worku, Y. & Newby, A. C. (1983) *Biochem. J.* **214**, 325–330
- Zielke, C. J. & Suelter, C. H. (1971) *Enzymes 3rd Ed.* **4**, 47–78