Purinogen is not an endogenous substrate used in endothelial cells during substrate deprivation

Ognjen CULIC¹, Ulrich K. M. DECKING, Eva BERGSCHNEIDER and Jürgen SCHRADER

Institut für Herz- und Kreislaufphysiologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany

Porcine aortic endothelial cells (PAEC) are known to be metabolically robust. They are capable of surviving extended periods of complete lack of exogenous substrate, and purine release has been shown to be significantly up-regulated. The endogenous substrates used during substrate deprivation, as well as the sources responsible for the increased purine release, have not been completely identified. We tested the possibility that a phosphoglyceroyl-ATP-containing polymer, purinogen, might support PAEC hibernation induced by lack of exogenous substrate. This involved isolation of the acid-insoluble fraction of PAEC, which was presumed to contain purinogen, and analysis by HPLC and ³¹P NMR. No evidence supporting the presence of triphosphate-containing compounds (purinogen) was found. Similar results were obtained in the rat heart. The majority of the products in the acid-insoluble, alkaline-treated fraction were

identified as RNA degradation products (2'- and 3'-nucleoside monophosphates). A [¹⁴C]adenosine labelling experiment showed that incorporation of adenosine into the acid-insoluble fraction was almost completely prevented after inhibition of RNA synthesis with actinomycin D. Furthermore, RNA isolated from PAEC and subsequently treated with alkali showed a profile that was almost identical with the HPLC profile of the acid-insoluble fraction. Finally, substrate-free incubation of the cells did not quantitatively or qualitatively influence the distribution of acid-insoluble derivatives. We conclude that PAEC survival during the absence of exogenous substrate is not supported by purinogen but rather by some other, yet-to-be-identified, endogenous substrate.

Key words: HPLC, ³¹P NMR, RNA.

INTRODUCTION

In a previous study we showed that porcine aortic endothelial cells (PAEC) can survive at least 2 h of perfusion with a substrate-free medium [1]. Although their intracellular adenine nucleotide content, as well as energy turnover, was drastically decreased during this phase, they were capable of fully recovering upon reperfusion with a glucose-containing medium [1,2]. Furthermore, purine nucleoside and nucleobase release of PAEC, under conditions of exogenous substrate lack, was significantly up-regulated. The increased *de novo* synthesis of purines, which contributed to the increase in purine release, did not fully explain these findings. No substantial energy reserve in the form of phosphocreatine or glycogen could be detected [2]. Most likely, survival of cells, as well as purine release, during substrate deprivation is supported by certain endogenous substrates which remain elusive at present.

Labelling of rat hearts with [¹⁴C]adenosine revealed the existence of a rapidly labelled trichloroacetic acid-insoluble nucleotide pool [3]. It has been reported that, quantitatively, this pool corresponds to approx. 25–55% of the total acid-soluble adenine nucleotide pool [4,5]. Preparative biochemistry combined with diverse analytical methods showed that a phosphate derivative of adenosine is a main building block of this polymeric adenine nucleotide, named purinogen [3,6]. It has been proposed that purinogen might play an important role as an energy reserve in the heart and also in other tissues such as liver and kidney [5–8].

In view of these findings we explored whether purinogen is present in endothelial cells and whether its presence could explain the remarkable ability of this cell type to survive extended

periods of endogenous substrate deprivation and/or for the increased purine release during this phase.

METHODS AND MATERIALS

Cell culture and preparation of acid-soluble and acid-insoluble fractions

PAEC were isolated by scraping of pig aortae obtained from the local slaughterhouse. The cell-culture medium used was M199 supplemented with antibiotics and 20% (v/v) newborn calf serum. Cells were cultured and characterized as described previously [1].

Typically, we used one 100 mm cell-culture dish with confluent PAEC for isolation of the purinogen fraction. After removal of the cell-culture medium and washing twice with 10 ml of PBS, cells were harvested by scraping the plates with a rubber policeman after the addition of 2 × 1 ml of trichloroacetic acid (1 M)/methanol (25%, v/v). The cells were centrifuged (10 min; 10000 g), and the supernatant was decanted into a fresh tube. The supernatant (acid-soluble fraction) was neutralized with K₃PO₄ and centrifuged. The pellet obtained after trichloroacetic acid precipitation was washed once with trichloroacetic acid/methanol, once with acetone and finally three times with diethyl ether. The pellet obtained after centrifugation was then dried and resuspended in KOH (4 M)/EDTA (10 mM) by thorough vortex-mixing. While being incubated at 37 °C, this solution was sonicated three times for 5 min (every 15 min) in an ultrasound water bath. Finally, this material was centrifuged for 10 min at 10000 g and the supernatant was neutralized with concentrated

Abbreviations used: PAEC, porcine aortic endothelial cells; FID, free induction decay.

¹ To whom correspondence should be addressed (e-mail ognjen@herzkreis.uni-duesseldorf.de).

HCl. After centrifugation the supernatant was retained and used for HPLC (NMR) analysis.

³¹P NMR spectroscopy

The acid-insoluble fraction, prepared as described above, was used for ³¹P NMR. Extracts prepared from eight 150 mm confluent cell dishes (about 120 million cells) in a final volume of 10 ml were freeze-dried and taken up in 2.5 ml of ²H₂O. Spectra were acquired on a Bruker AMX400 spectrometer coupled to a vertical-bore 9.4 T magnet. A 10 mm broad-band probe (Bruker) with 25 µs $\pi/2$ pulse length at 161.98 MHz was used for ³¹P measurements. The free induction decay (FID) consisted of 8192 data points in the time domain and was acquired accumulating 16000 or 4000 scans with a pulse delay of 3 s, a pulse length of 25 µs and a spectral width of 30.2 p.p.m. Bi-level (16 dB/24 dB) waltz16 proton-decoupling was employed where indicated. The FID was processed by exponential multiplication (1 Hz line broadening) and Fourier transformation. Phosphocreatine was added as internal standard. Resonance assignment was achieved by comparison with reference data and spiking with appropriate standards (see below).

HPLC measurements

Samples were injected on to a reverse-phase C₁₈ column (4 µm particle size, 300 mm length; Bondapak, Waters) and a linear elution gradient was used to separate the purine and pyrimidine nucleotides, changing from tetrabutylammonium sulphate/KH₂PO₄, pH 5.4, to 70 % methanol. HPLC peaks were identified by comparing the retention times with those of the standards and quantified by comparison of the integrated peak areas with those of the standards after interactive baseline correction. Additionally, enzyme-shift assays were performed (alkaline phosphatase, 5'-nucleotidase, 3'-nucleotidase and phosphodiesterase I). As a control (when possible), standard nucleotides were also treated with the relevant enzyme, using conditions identical with those of the samples. Where applicable, products of the enzyme-shift assay were identified in a separate HPLC analysis, as previously described [1,2]. Nucleotide content was related to the protein content measured by the Lowry method [9].

RNA isolation

RNA was isolated from cultured cells by a single-step isolation method (acid guanidinium thiocyanate/phenol/chloroform extraction) as described by Chomczynski and Sacchi [10].

[¹⁴C]Adenosine labelling

Confluent PAEC grown on a 100 mm cell dish were incubated with RPMI 1640 [with or without glucose (10 mM)] with or without actinomycin D (10 mM, ethanol stock solution, final concn. 10 µM) and [¹⁴C]adenosine (sp. radioactivity 500 mCi/mmol). At the end of the incubation period, cell-culture medium was removed and cells were washed with 3 × 5 ml of PBS. Acid-soluble and acid-insoluble fractions were then prepared as described above. Radioactivity of cell culture medium, acid-soluble and -insoluble fractions were measured in liquid-scintillation counter (Packard) after mixing of the material with scintillation fluid.

Distribution of radioactive label in the acid-insoluble fraction was determined by HPLC (200 mm; C₁₈ column). Fractions were collected (0.5 ml) every 30 s and used for liquid-scintillation counting.

Materials

All nucleoside 5'-, 2'- and 3'-phosphates, 5'-nucleotidase [from rattlesnake (*Crotalus atrox*) venom], 3'-nucleotidase [from ryegrass (*Lolium perenne*)] and phosphodiesterase I (from calf spleen) were purchased from Sigma (Deisenhofen, Germany) and were of the highest available purity. Nucleosides (cytidine, adenosine, uridine and guanosine) and actinomycin D were from Fluka (Deisenhofen, Germany). Alkaline phosphatase (molecular biology grade) was from Boehringer (Mannheim, Germany). HPLC chemicals were bought from Merck (Darmstadt, Germany) and were of highest available purity. [¹⁴C]Adenosine was obtained from Amersham (Braunschweig, Germany). Cell-culture media and media supplements were obtained from Gibco-BRL (Paisley, Renfrewshire, Scotland, U.K.).

RESULTS

PAEC grown to confluency were extracted with trichloroacetic acid/methanol as described by Mowbray et al. [4,5] and subsequently analysed by HPLC. Analysis of the trichloroacetic acid-soluble fraction of PAEC revealed the presence of ATP, ADP, GTP and GDP, as shown in Table 1. In comparison with the adenine nucleotide pool, the cells contained quantitatively a relatively large guanine nucleotide pool. The total nucleotide content of the cells was 14.10 ± 3.74 nmol/mg of protein.

Acid-insoluble pellets from the same experiments were dissolved in KOH/EDTA and then used for HPLC analysis. A typical chromatogram of this fraction is shown in Figure 1(A). In order to identify nine major fractions (HPLC peaks), each of the peaks was isolated by preparative HPLC and subsequently used for enzyme-shift assays. Alkaline phosphatase shift showed that all peaks were phosphates, since a shift to a product with a shorter retention time was always obtained when the material was treated with this enzyme. In addition, all peaks were resistant to 5'-nucleotidase enzyme treatment, excluding the possibility that any of the peaks was a 5'-nucleoside monophosphate. In a separate HPLC analysis, alkaline phosphatase dephosphorylation products of each fraction (peak) were identified as follows: cytosine for peaks #1 and #2, guanosine and uridine for peak #3, guanosine for peak #4 and adenosine for peaks #5 and #6. Alkaline phosphatase treatment of peaks #7, #8 and #9 resulted in dephosphorylation products which could not be identified.

On the basis of results of enzyme-shift assays as well as of spiking with 2'- and 3'-XMP standards, peaks were finally identified as indicated in Figure 1(A). Total 2'- and 3'-AMP (obtained from the acid-insoluble fraction) was 45.6 ± 16.8 nmol/

Table 1 Nucleotide content of PAEC acid-soluble fraction

Cells were treated with trichloroacetic acid/methanol as described in the Materials and methods section. Supernatant obtained after centrifugation was neutralized and analysed by HPLC. Results given are mean values $\pm$ S.D. obtained from four experiments. Abbreviation: n.d., not detected.

Nucleotide	Content (nmol/mg of protein)
ATP	8.97 ± 2.5
ADP	0.25 ± 0.1
AMP	n.d.
GTP	3.88 ± 3.00
GDP	1.00 ± 0.45
GMP	n.d.

Figure 1 HPLC analysis of the alkali-treated cellular fractions from PAEC

(A) HPLC analysis of an acid-insoluble fraction. Shown is a representative chromatogram. The acid-insoluble fraction of PAEC was obtained as a pellet after precipitation with trichloroacetic acid (1 M)/methanol (25%, v/v), followed by centrifugation. This fraction was treated with KOH/EDTA as described in the Materials and methods section. After neutralization with HCl followed by centrifugation, the supernatant fraction was isolated and injected on to a 300 mm reversed-phase HPLC C_{18} column. Detection was at 254 nm. Peaks were identified by spiking with different standards combined with enzyme-shift assays, as described in the Materials and methods section. (B) HPLC analysis of RNA isolated from PAEC. RNA was prepared as described in the Materials and methods section and was subsequently treated with alkali, similar to the acid-insoluble fraction. Shown is a typical chromatogram. Abbreviation: A.U., A_{254} unit.

mg of protein ($n = 6$). Comparison of this pool with total cytoplasmic adenine pool [acid-soluble fraction (see Table 1)] revealed a ratio of approx. 5:1. The molar ratio of the peaks which could be distinguished as pure nucleoside monophosphates (peaks #1, #2, #4, #5 and #6) was determined. On the basis of three experiments the molar ratio 3'-CMP/2'-CMP/2'-GMP/3'-AMP/2'-AMP was 1.0:1.3:5.7:1.7:1.4. Unidentified peaks #7 and #8 were shown to be shifted by 3'-nucleotidase. Peak #9 was resistant to 3'-nucleotidase derivatization, but otherwise it was shifted by both alkaline phosphatase and phosphodiesterase I (results not shown).

Figure 2 ^{31}P -NMR spectra of the alkali-treated acid-insoluble fraction of PAEC

Shown is a representative, partially relaxed ^{31}P spectrum of material isolated from approx. 120 million cells as described in the Materials and methods section. A total of 16 000 scans were accumulated in 13.5 h; for details see the Materials and methods section. The following peaks and regions are indicated: PME, phosphomonoester region; P_i , inorganic phosphate; PDE, phosphodiester region; α -, β - and γ -NTP, α -, β - and γ -phosphate regions of the nucleoside triphosphates. PCr, phosphocreatine was added as internal standard. The inset shows the PME region (5.0–3.5 p.p.m.) only. To better identify the individual peaks, a proton-decoupled ^{31}P spectrum (4000 scans) was obtained from the same sample. The following phosphomonoesters were detected: 3A, 3'-AMP; 3G, 3'-GMP; 3U, 3'-UMP; 3C, 3'-CMP; 2U, 2'-UMP; 2C, 2'-CMP; 2G, 2'-GMP; 2A, 2'-AMP.

Parallel ^{31}P -NMR analysis of the total acid-insoluble cell fraction (dissolved in KOH/EDTA) revealed signals which were mainly located in the phosphomonoester region of the NMR spectrum. A relatively broad signal was also observed in the phosphodiester region (Figure 2). Importantly, no indication of the presence of signals in the α -, β - or γ -nucleoside triphosphate region was seen ($n = 3$). When studying the phosphomonoester region more closely in a proton-decoupled ^{31}P spectrum combined with spiking with different 2'- and 3'-nucleoside monophosphates, it became apparent that this region almost exclusively consisted of 2'- and 3'-nucleoside monophosphates, specifically AMP, GMP, UMP and CMP (Figure 2, inset).

2'- and 3'- Nucleoside monophosphates are known to be degradation products of alkaline hydrolysis of RNA [11,12]. Therefore we isolated RNA from PAEC by the guanidinium isothiocyanate/phenol/chloroform method [10]. HPLC of the total isolated RNA revealed one major peak (results not shown). Subsequent treatment of isolated RNA with KOH/EDTA led to

Figure 3 HPLC analysis of radioactivity incorporation in acid-insoluble PAEC fraction

Cells were incubated with [^{14}C]adenosine (sp. radioactivity 500 mCi/mmol). The concentration of actinomycin D, when present, was 5 μM . After incubation for 180 min at 37 $^{\circ}\text{C}$, cell medium was removed, and the cells washed and subsequently treated with trichloroacetic acid/methanol. Details of the experimental procedure are given in the Materials and methods section. Separation was performed on a 200 mm reverse-phase C_{18} column. HPLC effluent was collected every 30 s and the radioactivity of each fraction was measured in a liquid-scintillation counter.

the HPLC pattern shown in Figure 1(B). This chromatogram is virtually identical with that of the acid-soluble fraction of PAEC that was prepared by the method of Mowbray et al. [4,5] (see Figure 1A for comparison).

In order to distinguish the cellular RNA pool from other possible cellular acid-insoluble pools, adenosine labelling experiments were undertaken. Labelling of PAEC with extracellularly added [^{14}C]adenosine (sp. radioactivity 500 mCi/mmol, final concentration 40 $\mu\text{mol/l}$) showed a time-dependent accumulation of radioactivity in the acid-soluble pool, as well as in the acid-insoluble pool (about 55 and 15% of total radioactivity input after 180 min respectively). The acid-soluble pool was labelled much faster than the acid-insoluble pool (results not shown). Distribution of the label in acid-insoluble fraction, as determined by HPLC, is shown in Figure 3. Five radioactive peaks were identified. Retention times of peaks #1, #2, #3 and #4 (results not shown) were identical with the retention times of 3'-GMP, 2'-GMP, 3'-AMP and 2'-AMP respectively (basically comparable with peaks #3, #4, #5 and #6 respectively shown in Figure 1A). Similarly, radioactively labelled peak #5 (Figure 3) corresponds to the region of peaks #7, #8, #9 in Figure 1(A). Most importantly, the incorporation of radiolabel in the acid-insoluble fraction was almost completely prevented by addition of the RNA synthesis inhibitor actinomycin D (Figure 3).

We also addressed the proposed biological significance of purinogen, namely its role as ATP store (endogenous energy reserve) that could be used during a period of substrate deprivation. We investigated whether incubation of PAEC in medium with no exogenous substrate (PBS) changed the composition of the acid-insoluble fraction as compared with a control incubated in the presence of glucose. Acid-insoluble fractions of the cells incubated for 2 h with or without glucose were analysed by HPLC. Both chromatograms resembled the pattern shown in Figure 1(A). Moreover, a lack of glucose for 2 h neither quantitatively nor qualitatively influenced the acid-insoluble fraction of cells (results not shown).

Since we were unable to detect purinogens in PAEC, we applied the same purinogen isolation procedure, originally

Figure 4 HPLC analysis of acid-insoluble fraction of the rat heart

Heart tissue was homogenized and the acid-insoluble fraction was prepared according to the same procedure that was used for PAEC (see the Materials and methods section). Acid-insoluble fraction was treated for 1 h at 37 $^{\circ}\text{C}$ with KOH (4 M)/EDTA (10 mM) and then neutralized with conc. HCl. Following centrifugation the supernatant was used for a subsequent HPLC analysis (as described in the Materials and methods section). Abbreviation: A.U., A_{254} unit.

described by Mowbray's group [4,5], to the saline-perfused isolated rat heart. After solubilization of the rat heart acid-insoluble fraction in KOH (4 M)/EDTA (10 mM), followed by neutralization, this fraction was analysed by both NMR and HPLC. Similar to NMR spectrum of the PAEC acid-insoluble fraction, NMR analysis of the heart acid-insoluble fraction showed no indication of ATP-containing compounds (results not shown). Results of the parallel HPLC analysis are shown in Figure 4. With exception of the peaks #7, #8 and #9 (which were only seen in PAEC), the chromatogram was nearly identical with that obtained from PAEC (shown in Figure 1A).

DISCUSSION

Purinogen has been suggested to play an important role as an energy reserve in the rat heart [3]. To eventually prove the presence of purinogen in endothelial cells could explain their extraordinary ability to survive under conditions of exogenous substrate deprivation. Nevertheless, in the present work, four different lines of evidence are presented that suggest the absence of purinogen from PAEC.

(1) HPLC analysis of the acid-soluble PAEC fraction dissolved in KOH/EDTA showed that quantitatively the majority of detected substances are 2'- and 3'-nucleoside monophosphates. Use of phosphoglyceroyl-ATP as a standard was unfortunately not possible in our experiments, owing to a lack of a commercially available standard. Peaks #7, #8 and #9 were not fully identified, but information obtained through various enzyme-shift assays are not compatible with phosphoglyceroyl-ATP structure. Since the HPLC separation method used in the present study was different from the one used by Mowbray et al. [4,5], it is theoretically possible that phosphoglyceroyl-ATP did not bind to the HPLC column under the experimental conditions applied. This seems rather unlikely, since the HPLC system used has been shown to be capable of detecting practically all kinds of nucleoside monophosphates, diphosphates, triphosphates or dinu-

cleotide polyphosphates [2]. Further evidence disputing the lack of phosphoglyceroyl-ATP binding to HPLC column is obtained from the NMR experiment, in which the whole acid-insoluble fraction was analysed.

(2) ^{31}P -NMR data of the whole isolated acid-insoluble fraction of PAEC revealed no α -, β - or γ -NTP phosphate signals, indicating the absence of purinogen in PAEC. Major signals were found instead in the phosphomono-ester and -diester region of the spectrum. The phosphomonoester region consisted almost exclusively of nucleoside monophosphates (AMP, CMP, GMP and UMP) in an apparently equimolar mixture of 2'- and 3'-phosphates. These data further corroborate our finding that most of the HPLC-detected compounds in acid-insoluble fraction are a monophosphate mixture. The NMR pattern shown in the present study was clearly different from the NMR spectrum of the rat heart purinogen fraction reported by Hutchinson et al. [6], despite a comparable number of scans and a better signal-to-noise ratio in our study. Since we did not find any indication of a nucleoside triphosphate (e.g. ATP)-containing substance in the acid-insoluble fraction of PAEC, we compared it with the acid-insoluble fraction of rat hearts. This fraction was also prepared as described by Mowbray's group [4,5]. NMR analysis of the acid-insoluble fraction of the rat heart showed no indication of the presence of ATP-containing substances. In contrast with previously reported data in the same species [6], we observed no α -, β - or γ -NTP signals, but again eight different 2'- and 3'-monophosphates were observed (results not shown). Parallel HPLC analysis revealed a pattern which was very similar to the acid-insoluble fraction isolated from PAEC (Figure 4 versus Figure 1A). Interestingly, peaks #7, #8 and #9 were not seen in the acid-insoluble fraction of the rat heart. NMR data obtained from PAEC and rat heart, combined with the fact that peak #9, as measured by HPLC, was not identified in the heart acid-insoluble fraction, make the possibility that peak #9 could present phosphoglyceroyl-ATP very unlikely.

(3) HPLC analysis revealed that RNA isolated from PAEC, if not treated with alkali, was eluted as a single peak (results not shown). In contrast, RNA after treatment with alkali showed a pattern strikingly similar to that of the isolated acid-insoluble fraction. These results support the finding that cellular RNA is indeed very unstable under (applied) alkaline conditions. Alkaline degradation of RNA has often been used as a basic principle for quantitative determination of total RNA [10–12].

(4) The evidence supporting the presence of purinogen was originally based on adenosine-labelling experiments of the perfused rat heart [3]. It is known that endothelial cells in the heart are quantitatively primarily responsible for adenosine uptake [13]. The present adenosine-labelling experiments showed that, up to 3 h incubation, there was no significant incorporation of the radioactive label into acid-insoluble fraction that could not be inhibited by the RNA synthesis inhibitor actinomycin D at a concentration of $5\text{ }\mu\text{M}$. Similar experiments by Heyworth et al. performed in the perfused hearts revealed that $2\text{ }\mu\text{M}$ actinomycin D decreased the incorporation of radioactive uridine and adenosine by approx. 60% [4]. Unfortunately, there have been no reports on the effect of higher concentrations of actinomycin D.

PAEC incubated for 3 h with labelled adenosine led to quantitatively almost equal distribution of radioactivity in fractions previously identified as 2'-AMP, 3'-AMP, 2'-GMP and 3'-GMP. This is further evidence supporting our finding that acid-insoluble fraction of PAEC contains mainly, if not exclusively, RNA-degradation products.

The exact structure of peaks #7, #8 and #9 remains unknown, but adenosine-labelling experiments (Figure 3) indicate that they most probably contain adenosine and/or guanosine. If these peaks represent purinogen, they would quantitatively correspond to about 60% of the total adenine content stored in RNA (peaks #5 + #6). The molar ratio of the RNA adenine nucleotide pool to the total soluble adenine nucleotide pool in endothelial cells is estimated to be 5:1, which is apparently much higher than the same ratio estimated for heart [6]. Interestingly, Siddiq et al. showed up to a 5-fold difference in the RNA/mg protein ratio between heart and liver [14]. Recently, it has been postulated that purinogen in heart may not serve as an energy store, but instead act as a rapidly accessible reservoir of P_i [15].

In conclusion, in order to investigate the possible presence of purinogens in PAEC, we used the isolation procedure identical with that previously published by Mowbray's group [4] and found no evidence (HPLC, NMR and labelling experiments) for the existence of phosphoglyceroyl-ATP (purinogen). It is therefore unlikely that purinogen could be an important energy source responsible for the increased purine release observed in endothelial cells during lack of substrate. Other alternative substrates must be considered responsible for the metabolic robustness of PAEC.

We thank Dr. Thomas Stumpe and Ulrich Flögel for their help and valuable suggestions, and Mrs. Adele Brandt for technical help in manuscript preparation. This research was funded in part by the Deutsche Forschungsgemeinschaft (DFG) (DE 487/4-1) and by the Center for Biological and Medical Research (Biomedizinisches Forschungszentrum) of the Heinrich Heine University, Düsseldorf.

REFERENCES

- Culic, O., Gruwel, M. L. H. and Schrader, J. (1997) *Am. J. Physiol.* **273**, C205–C213
- Culic, O., Decking, U. K. M. and Schrader, J. (1998) *Am. J. Physiol.*, in the press
- Mowbray, J., Hutchinson, W. L., Tibbs, G. R. and Morris, P. G. (1984) *Biochem. J.* **223**, 627–632
- Heyworth, P. G., Hutchinson, W. L. and Mowbray, J. (1987) *Biochim. Biophys. Acta* **927**, 158–162
- Patel, B., Sarcina, M. and Mowbray, J. (1994) *Eur. J. Biochem.* **220**, 663–669
- Hutchinson, W. L., Morris, P. G. and Mowbray, J. (1986) *Biochem. J.* **234**, 623–627
- Patel, B., Costi, A., Hardy, L. and Mowbray, J. (1991) *Biochem. J.* **274**, 275–279
- Hutchinson, W. L., Ratcliffe, P. J. and Mowbray, J. (1986) *Biochem. J.* **240**, 597–599
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Schmidt, G. and Cohen, W. E. (1954) in *Methods in Biochemical Analysis*, vol. 1 (Glick, J. L., ed.), p. 287, Interscience, New York
- Schmidt, G. and Thannhauser, J. S. (1945) *J. Biol. Chem.* **161**, 83–91
- Nees, S., Herzog, V., Becker, B. F., Böck, M., Des Rosiers, Ch. and Garlach, E. (1985) *Basic Res. Cardiol.* **80**, 515–529
- Siddiq, T., Richardson, P. J. and Preedy, V. R. (1993) *Biochem. Med. Metabol. Biol.* **49**, 149–163
- Patel, B., Berhane, Y., Petrovic, A. and Mowbray, J. (1998) *Eur. J. Biochem.* **254**, 75–80