The purification and characterization of glucokinase from the thermophile *Bacillus stearothermophilus*

Christopher R. GOWARD, Roy HARTWELL, Tony ATKINSON and Michael D. SCAWEN

Microbial Technology Laboratory, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wilts. SP4 0JG, U.K.

Homogeneous glucokinase (EC 2.7.1.2) from the thermophile *Bacillus stearothermophilus* was isolated on the large scale by using four major steps: precipitation of extraneous material at pH 5.5, ion-exchange chromatography on DEAE-Sephadex, pseudo-affinity chromatography on Procion Brown H-3R-Sephadex 4B and gel filtration on Ultrogel AcA 34. The purified enzyme had a specific activity of about 330 units/mg of protein and was shown to exist as a dimer of subunit M_r 33000. Kinetic parameters for the enzyme were determined with a variety of substrates. The glucokinase was highly specific for α -D-glucose, and the only other sugar substrate utilized was *N*-acetyl- α -D-glucosamine. The enzyme shows Michaelis–Menten kinetics, with a K_m value of 150 μ M for α -D-glucose. The glucokinase was maximally active at pH 9.0.

INTRODUCTION

Hexokinases (ATP:D-hexose 6-phosphotransferases, EC 2.7.1.1) catalyse the formation of hexose 6-phosphates from hexoses and are widely distributed in micro-organisms, invertebrates and vertebrates. Hexokinases lack specificity towards the carbohydrate substrate. However, glucokinases (ATP: α -D-glucose 6-phosphotransferases, EC 2.7.1.2) specifically catalyse the transfer of the terminal phosphate of ATP to glucose, thus forming glucose 6-phosphate and ADP.

Glucokinase has been isolated from *Bacillus stearothermophilus* (N.C.I.B. 8924) (Hengartner & Zuber, 1973), *Zymomonas mobilis* (Doelle, 1982; Scopes *et al.*, 1985), *Brevibacterium fuscum* (Saito, 1965), *Streptococcus mutans* (Porter *et al.*, 1980, 1982), *Aerobacter aerogenes* (Kamel *et al.*, 1966) and *Entamoeba histolytica* (Reeves *et al.*, 1967). The present paper reports the purification and characterization of glucokinase from *B. stearothermophilus* (N.C.I.B. 11270), a mutant of N.C.I.B. 8924 cultured on glycerol and selected for glycerokinase production (Comer *et al.*, 1979; Scawen *et al.*, 1983).

Glycerokinase and many other enzymes isolated from this *B. stearothermophilus* exhibit good thermal stability, a factor which is of value in an enzyme used as a reagent in clinical chemistry (Atkinson, 1983). Glucokinase could be of value in the determination of glucose and ATP. For this to be investigated, it was necessary to be able to prepare the enzyme on a large scale. The procedure described for the small-scale purification of glucokinase from *B. stearothermophilus* N.C.I.B. 8924 was unsuitable for application on a larger scale, because a preparative electrophoresis step was included. The method described for the purification of glucokinase from *Streptococcus mutans* was also unsuitable, owing to involvement of affinity chromatography using a relatively labile agarose–hexane–ATP column (Porter *et al.*, 1980, 1982). Scopes *et al.* (1985) report the small-scale purification of a glucokinase from *Zymomonas mobilis* with Procion Scarlet MX-G–Sephadex.

Affinity chromatography can provide a rapid and elegant means of purifying many enzymes. Unfortunately

it is in many cases unacceptable for large-scale working because of the expense and instability of many natural ligands. However, many enzymes have been found to bind immobilized reactive dyes. These same immobilized dyes can be used to purify enzymes (Lowe *et al.*, 1980; Atkinson *et al.*, 1981), e.g. glycerokinase from *B. stearothermophilus* (Scawen *et al.*, 1983), hexokinase from pig heart (Farmer & Easterby, 1982), malate dehydrogenase (EC 1.1.1.37) and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) from *Rhodospseudomonas spheroides* (Scawen *et al.*, 1982) and alcohol dehydrogenase (EC 1.1.1.1) from horse muscle (Roy & Nishikawa, 1979). In all these instances an immobilized dye was found to have a much higher binding capacity than an equivalent nucleotide ligand, as well as being much less expensive and easier to couple to a chromatography matrix. For these reasons a purification procedure for glucokinase based on dye affinity chromatography was developed. This study shows that the dye Procion Brown H-3R could be used to separate glycerokinase and glucokinase differentially from a partially purified extract containing both enzymes, which previously proved difficult to separate by ion-exchange chromatography.

MATERIALS AND METHODS

Materials

Glucose, triethanolamine, ethanolamine, triethylamine, L-histidine hydrochloride, EDTA and NaN_3 were purchased from BDH. Glucose-6-phosphate dehydrogenase, nucleotides, dithiothreitol and phenylmethanesulphonyl fluoride were obtained from Sigma. Calibration proteins, DEAE-Sephadex and Sephadex 4B were purchased from Pharmacia, and Ultrogel AcA 34 was from LKB. Tryptone was obtained from Oxoid and Yeastex from Bovril Food Ingredients. The Procion dyes were a gift from Dr. C. V. Stead, ICI, and all other reagents were purchased from Boehringer Corp. (London). Pyruvate kinase and lactate dehydrogenase were obtained as a mixed suspension in $(\text{NH}_4)_2\text{SO}_4$ containing equal activities of both enzymes.

Culture of cells

Small-scale and production cultures were grown as described previously (Comer *et al.*, 1979; Scawen *et al.*, 1983).

Glucokinase assay

Glucokinase activity was measured by following the decrease in A_{340} at 30 °C with the pyruvate kinase (EC 2.7.1.40)/lactate dehydrogenase (EC 1.1.1.27) assay system, or the increase in A_{340} at 30 °C with the glucose-6-phosphate dehydrogenase (EC 1.1.1.49) assay system, with a Perkin-Elmer 552S spectrophotometer. The glucose-6-phosphate dehydrogenase assay system is specific for glucokinase; the pyruvate kinase/lactate dehydrogenase assay system was employed for the determination of glucokinase activity against substrates other than glucose.

The pyruvate kinase/lactate dehydrogenase-based assay mixture contained 75 mM-triethanolamine/HCl (pH 7.6), 1.5 mM-KCl, 5 mM-MgCl₂, 5 mM-ATP, 0.3 mM-NADH, 6.5 mM-phosphoenolpyruvate, 2.4 mM- α -D-glucose and 8 units of pyruvate kinase/lactate dehydrogenase in a final volume of 1 ml. The reaction was initiated with 1–10 μ l of glucokinase solution (Garland & Randle, 1962).

The glucose-6-phosphate dehydrogenase-based assay mixture contained 92 mM-triethanolamine/HCl (pH 7.6), 1.8 mM-KCl, 5.4 mM-MgCl₂, 0.32 mM-NADP⁺, 1.9 mM-ATP, 2.55 mM- α -D-glucose and 3.5 units of glucose-6-phosphate dehydrogenase in a final volume of 1 ml. The reaction was initiated with 1–10 μ l of glucokinase solution. (This method was modified from a procedure provided by Boehringer Mannheim.)

One unit of glucokinase activity is defined as that which catalyses the formation of 1 μ mol of glucose 6-phosphate/min at 30 °C.

Protein assay

Protein concentrations were determined by using the Coomassie-Brilliant-Blue-binding method of Bradford (1976), with bovine serum albumin as the standard. The protein content of column eluates was also monitored by the A_{280} .

Kinetics

Kinetic parameters were evaluated by the double-reciprocal-plot method.

Screening of Procion dye-Sephadex conjugates

A total of 71 different Procion dye-Sephadex conjugates were screened for their ability to bind glucokinase, but not glycerokinase. Columns (1 ml; 1.6 cm $\times$ 0.9 cm) of the various matrices were equilibrated in 20 mM-potassium phosphate buffer, pH 7.0, and loaded with 4 ml of partially purified enzyme mixture containing 12 units of glucokinase/ml and 16 units of glycerokinase/ml. The columns were washed with 5 ml of buffer and eluted with 5 ml of 5 mM-ATP in equilibration buffer.

Coupling of Procion Brown H-3R to Sephadex 4B

For this, 6.0 litres of Sephadex 4B was suspended in 21.0 litres of demineralized water and 90 g of Procion Brown H-3R, dissolved in 6.1 litres of demineralized water, was added. After 5 min stirring at ambient

temperature, 2.1 litres of 5 M-NaCl was added. After a further 30 min stirring, 156 ml of 5 M-NaOH was added and the mixture incubated at 37 °C for 48 h, with gentle stirring. The coupled gel was washed copiously with water, and then successively with 1 litre of 5 M-urea, then 1 litre of 5 mM-Tris/HCl/5 mM-EDTA/500 mM-KCl/1 M-guanidine hydrochloride. Finally, the coupled gel was washed with water until no further leaching of dye was observed. The degree of substitution was determined spectrophotometrically after hydrolysis of gel samples in 7.5 M-acetic acid for 15 min at 85 °C. The final ligand concentration was 1 μ mol of Procion Brown H-3R/g moist wt. of Sephadex 4B.

SDS/polyacrylamide-gel electrophoresis

This was performed with 10% -acrylamide slab gels run in an LKB Vertical Electrophoresis Unit (Laemmli, 1970). Proteins were stained by Coomassie Brilliant Blue R-250.

Determination of isoelectric point

The pI of the enzyme was determined with an LKB Multiphor Apparatus and broad-pH-range gels (3.5–9.5) followed by narrow-pH-range gels (4–6.5); appropriate Pharmacia calibration kits were used. The thin-layer polyacrylamide gels were ready-prepared containing ampholytes (LKB Ampholine PAG Plates). Densitometer gel scans were used to assess the pI.

RESULTS

Screening of Procion dye-Sephadex conjugates

Of the 71 dye-Sephadex conjugates examined, only 10 bound glucokinase. Of these only one, Procion Brown H-3R, did not bind glycerokinase, so was chosen for the subsequent purification.

Enzyme purification

Purification was conducted at 4 °C. Column dimensions are expressed as bed height $\times$ internal diameter. The purification described below was a typical preparation based on 28 kg of bacterial cell paste.

Disruption of cells. Cells (28 kg) were thawed overnight at 4 °C in 40 litres of 100 mM-potassium phosphate buffer, pH 8.0, containing 1 mM-EDTA, 10 mM-2-mercaptoethanol, 0.1 mM-phenylmethanesulphonyl fluoride and 0.5 μ g of deoxyribonuclease/ml. The thawed suspension was disrupted with two 15M-8BA Manton-Gaulin homogenisers at 550 kg/cm². The homogenate was centrifuged at 17000 *g* at 8 °C and a flow rate of 50 litres/h in a Sharples AS-26 continuous-flow centrifuge. It was then mixed with an equal volume of 100 mM-KH₂PO₄; the increased ionic strength prevented the enzyme adsorbing on to the precipitate in the subsequent step. The diluted extract was adjusted to pH 5.5 with 3.2 M-H₃PO₄ and centrifuged at 17000 *g* as above. The supernatant was then adjusted to pH 8.0 with 10 M-KOH.

Ion-exchange chromatography. The supernatant was applied to a 48-litre DEAE-Sephadex column (45 cm $\times$ 37 cm) equilibrated with 100 mM-potassium phosphate buffer, pH 8.0, containing 0.1 mM-phenylmethanesulphonyl fluoride and 10 mM-2-mercapto-

Fig. 1. Pseudo-affinity chromatography on Procion Brown H-3R-Sepharose 4B

After loading of the enzyme, the column was eluted with (A) 10 litres of 10 mM-potassium phosphate buffer, pH 7.5, and (B) 10 litres of potassium phosphate buffer, pH 7.5, containing 5 mM-ATP. ●, Protein (mg/ml); ▲, glucokinase (units/ml).

Fig. 2. Gel filtration on Ultrogel AcA 34

●, A_{280} ; ▲, glucokinase (units/ml).

ethanol. The enzyme was eluted with a 300-litre linear gradient of 100–400 mM-potassium phosphate, pH 8.0, collected in 4-litre fractions.

Pseudo-affinity chromatography. The main active fractions from the ion-exchange column were pooled and concentrated with an Amicon DC50 ultrafiltration unit fitted with five H10P10 hollow-fibre cartridges. The ionic strength was lowered to that of 10 mM-potassium phosphate, pH 7.5, by dilution and re-concentration. The pool was applied to a 5.2-litre Procion Brown H-3R-Sepharose 4B column (55 cm × 11 cm) previously equilibrated with 10 mM-potassium phosphate, pH 7.5, con-

taining 0.1 mM-dithiothreitol, 0.1 mM-phenylmethanesulphonyl fluoride and 0.02% NaN_3 . The column was washed with 10 litres of buffer and eluted with 10 litres of 5 mM-ATP in the same buffer, collected in 500 ml fractions (Fig. 1).

Gel-filtration chromatography. Active fractions from the Procion dye-Sepharose column were pooled, concentrated to 100 ml with Amicon PM10 membranes and applied to a 5.7-litre Ultrogel AcA 34 column (90 cm × 9 cm), equilibrated with 50 mM-potassium phosphate, pH 7.6, containing 0.1 mM-dithiothreitol, 0.1 mM-phenylmethanesulphonyl fluoride and 0.02% NaN_3 . The eluate was collected in 30 ml fractions. Glucokinase-containing fractions were pooled on the basis of activity and electrophoretic homogeneity (Fig. 2).

The results of this purification are summarized in Table 1. The enzyme was shown to be homogeneous, giving a single protein band on SDS/polyacrylamide-gel electrophoresis.

M_r of native enzyme and subunits

The subunit M_r was shown to be 33000 by SDS/polyacrylamide-gel electrophoresis. Gel filtration on Ultrogel AcA 34 produced anomalous data, with the apparent M_r varying between 70000 and 130000 with elution buffers of 50 mM-potassium phosphate, pH 7.6, 50 mM-sodium phosphate, pH 7.6, or 50 mM-Tris/500 mM-NaCl, pH 7.6. This behaviour occurred when the enzyme solution was loaded in the presence or absence of

Table 1. Purification of glucokinase from 28 kg of *Bacillus stearothermophilus* cells

Step	Volume (ml)	Protein (mg)	Glucokinase (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	60000	1898200	3608000	1.9	—	100
pH 5.5 supernatant	57000	641400	2886400	4.5	2.4	80
DEAE-Sepharose	60000	79500	1876200	23.6	12.4	52
Procion Brown H-3R-Sepharose 4B	5000	7400	1695800	229.0	121.0	47
Ultrogel AcA 34	2100	4100	1371000	334.0	176.0	38

Fig. 3. Cross-linking of glucokinase by glutaraldehyde

This was performed as follows. Glucokinase was denatured with 0.05% SDS for 5 min at 100 °C. The enzyme concentration was 3.5 mg/ml in 50 mM-potassium phosphate, pH 7.5. The subunits were cross-linked by addition of 25% (v/v) glutaraldehyde to a final concentration of 100 mM. The reaction was quenched after 10 h by adding ethanolamine to a final concentration of 300 mM, and the cross-linked enzyme was dialysed against 25 mM-Tris/190 mM-glycine buffer, pH 8.3. Glucokinase subunit and cross-linked subunit were compared with protein standards by SDS/polyacrylamide-gel electrophoresis. Densitometer scans show: (a) 1, glucokinase subunit; (b) 1, cross-linked glucokinase subunit; (c) 1, phosphorylase *b* (M_r 94000); 2, bovine serum albumin (M_r 67000); 3, ovalbumin (M_r 43000); 4, carbonic anhydrase (M_r 30000). The gel boundary is designated a and the dye marker b.

2 mM-MgATP or 2 mM-D-glucose at 4, 25 and 55 °C. The M_r was therefore studied by alternative techniques, namely high-performance gel filtration and electrophoresis after cross-linking of the protein subunit with glutaraldehyde. H.p.l.c. was performed with a Waters Protein Pak 300SW column. The mobile phase was 100 mM-potassium phosphate buffer, pH 7.0. The standards were carbonic anhydrase (M_r 30000), ovalbumin (M_r 43000), bovine serum albumin (M_r 67000), lactate dehydrogenase (M_r 150000), aldolase (M_r 158000), catalase (M_r 232000), and thyroglobulin (M_r 669000). The M_r of native enzyme by h.p.l.c. was 78000. The results of the glutaraldehyde-cross-linking experiments show a broad band of cross-linked protein between M_r 32000 and 38000 corresponding to the monomer, and another band of M_r 70000–80000 corresponding to the native dimer. There was no evidence of higher oligomers (Fig. 3). The discrepancy in M_r on gel filtration on the acrylamide-agarose co-polymer cannot be explained, but may be due to different hydration states of the molecule or conformational deformation.

Isoelectric point

The pI of the glucokinase was shown to be 5.65–5.70 by using narrow-range (pH 4–6.5) LKB Ampholine PAG plates.

Stability

At 70 °C the enzyme is fully inactivated after 70 min even in the presence of dithiothreitol. Dithiothreitol was shown to be protective towards the enzyme at 70 °C, whereas urea and SDS rapidly inactivated the enzyme (Fig. 4).

Effect of metal ions and inhibitors

Glucokinase was shown to require the presence of Mg^{2+} ions (with a nucleoside triphosphate) for activity, by using the glucose-6-phosphate dehydrogenase assay system. Other bivalent cations can substitute for Mg^{2+} . Co^{2+} and Mn^{2+} show 68 and 45% activity respectively, but the large ions Ca^{2+} and Zn^{2+} show no activity.

N-ethylmaleimide, $AgNO_3$, *p*-chloromercuribenzoic acid, *p*-hydroxychloromercuribenzoate and $HgCl_2$ completely inhibited glucokinase activity at pH 7.6, and $Pb(NO_3)_2$, 1,10-phenanthroline and iodacetamide were less effective inhibitors, giving 68, 65 and 40% inhibition respectively.

Effect of pH

Glucokinase showed activity over a broad pH range, and was optimally active at pH 9.0, as shown in Fig. 5. The assays were unreliable above pH 11.0, because Mg^{2+} was precipitated out of solution.

Substrate specificity

The specificity of glucokinase with a variety of carbohydrates was investigated at pH 7.6 and at pH 9.0, where the enzyme is maximally active in the pyruvate kinase/lactate dehydrogenase assay system. Carbohydrates included in the investigation were α -D-glucose, *N*-acetyl- α -D-glucosamine, D-glucosamine hydrochloride, D-xylose, D-sucrose, D-cellobiose, D-lactose, 2-deoxy-D-glucose, D-mannose, D-melibiose, D-ribose, D-maltose, 3-*O*-methyl-D-glucopyranose, 1-*O*-methyl D-glucopyranoside, D-sorbitol, D-galactose, D-raffinose and β -D-fructose. At pH 7.6 *N*-acetyl-D-glucosamine gave

Fig. 4. Denaturation of glucokinase

Glucokinase was assayed at 70 °C, pH 7.6, in the presence of: 0.1 mM-dithiothreitol (●); 8 M-urea (■); 0.1% SDS (○); no addition (control; ▲).

Fig. 5. pH/activity profile of glucokinase

The enzyme was assayed immediately with the glucose-6-phosphate dehydrogenase assay system. Buffers: pH 5.0–7.0, L-histidine/HCl; pH 6.5–8.5, triethanolamine; pH 8.5–10.5, ethanolamine. The profile was similar whether the buffer molarity was constant at 100 mM or the ionic strength constant at 0.1.

Fig. 6. Inhibition of glucokinase with carbohydrates

The enzyme was assayed by the pyruvate kinase/lactate dehydrogenase method described in the text, in the presence of various concentrations of α -D-glucose (□). The concentrations of D-maltose (●), glucose 6-phosphate (△) and *N*-acetyl- α -D-glucosamine (■) were varied at a fixed concentration of α -D-glucose of 100 mM.

50% of the activity with α -D-glucose. However, at pH 9.0 the activity was decreased to 5% of that with α -D-glucose. All other carbohydrates induced no activity at either pH. These results indicate that the hydroxyl group at position 1 has to be in the α conformation and the hydroxyl groups at positions 2 and 4 have to be in the equatorial conformation.

Kinetic studies

The apparent K_m for α -D-glucose was determined as 150 μ M at 30 °C. Mixed inhibition of glucokinase activity was shown in all instances where carbohydrates were introduced into the assay system in addition to α -D-glucose (Fig. 6). The inhibition constants (K_i) for *N*-acetyl- α -D-glucosamine, glucose 6-phosphate and D-maltose were 280, 270 and 250 μ M respectively.

As the temperature was increased, the maximum velocity of the enzyme reaction increased, to reach a peak at 60 °C, with either α -D-glucose or ATP as the variable substrate, with a 4.6-fold increase from 30 to 60 °C.

Kinetic data were used to produce an Arrhenius plot over the range 30–55 °C and to calculate the activation energy of the enzyme, 41.4 kJ/K per mol. The plot was a straight line, indicating a single activation energy. Since apparent activation of the enzyme was observed after preincubation in the presence of glucose or ATP at increasing temperatures up to 55 °C, one may expect a biphasic Arrhenius plot if values above 55 °C were included. Unfortunately the additional enzymes required in the assay systems were inactivated above this temperature.

DISCUSSION

The strain of *B. stearotherophilus* employed appears to be unusual in that glycerol as a carbon source gives up to 6-fold greater glucokinase yields compared with culture on glucose. Enzyme was purified 176-fold overall with a yield of 38% by the protocol adopted, which successfully separated glucokinase from glycerokinase. The specific activity increased 10-fold on pseudo-affinity chromatography. The specific activity of the homogeneous enzyme was 330 units/mg of protein with the pyruvate kinase/lactate dehydrogenase assay system and 300 units/mg of protein with the glucose-6-phosphate dehydrogenase system. The previous purification of glucokinase from *B. stearotherophilus* (Hengartner & Zuber, 1973) attained a yield of 19.8% with a specific activity of 304 units/mg of protein. The purification procedure described allows the economical purification of glucokinase from kilogram quantities of *B. stearotherophilus* cells. The crucial step involved pseudo-affinity chromatography with Procion Brown H-3R coupled to Sepharose 4B. The elution of glucokinase from the Procion Brown H-3R with ATP alone contrasts with the elution of glycerokinase from Procion Blue MX-3G, which requires Mg^{2+} -ATP (Scawen *et al.*, 1983). Glucokinase has been purified from *S. mutans* OMZ70 by affinity chromatography on agarose-hexane-ATP, but only on a small scale involving about 113 units of enzyme. The 3.6×10^6 units of glucokinase from 28 kg of bacterial cell paste have been purified by the pseudo-affinity step, which would have proved extremely expensive if an adenine-nucleotide-substituted gel had been used.

Glucokinase from *B. stearotherophilus* demonstrated a requirement for a bivalent cation, as observed for the enzyme from other sources (Fromm & Zewe, 1962; Saito, 1965; DeMoss, 1968; Porter *et al.*, 1980). However, the enzyme from *S. mutans* OMZ70 differed in its cation requirement, in that Mn^{2+} and Co^{2+} were more effective than Mg^{2+} , giving 173 and 264% activity respectively, and Zn^{2+} and Ca^{2+} also induced glucokinase activity, of 56 and 34% respectively.

The other sugars investigated all exhibited mixed competition for the glucose-binding site, but were not phosphorylated. The enzyme was specific in catalysing the phosphorylation only of α -D-glucose at pH 9.0.

The enzyme was shown to be very thermostable, exhibiting maximum activity at pH 9.0 and 60 °C. The thermostability was markedly increased in the presence of dithiothreitol.

Conflicting gel-filtration data indicated the possibility of the native glucokinase existing as trimer/tetramer. H.p.l.c. and glutaraldehyde cross-linking, however, subsequently confirmed the enzyme's existence as dimer. This supports the work of Hengartner & Zuber (1973), where the enzyme originated from the same strain of *B. stearotherophilus* but was prepared by preparative electrophoresis.

This work was supported in part by a grant from the Department of Trade and Industry.

REFERENCES

- Atkinson, A., Hammond, P. M., Hartwell, D. R., Hughes, P., Scawen, M. D., Sherwood, R. F., Small, D. A. P., Bruton, C. J., Harvey, M. J. & Lowe, C. R. (1981) *Biochem. Soc. Trans.* **9**, 290-293
- Atkinson, T. (1983) *Philos. Trans. R. Soc. London Ser. B* **300**, 399-410
- Bradford, M. (1976) *Anal. Biochem.* **72**, 348-354
- Comer, M. J., Bruton, C. J. & Atkinson, T. (1979) *J. Appl. Biochem.* **1**, 259-270
- DeMoss, R. D. (1968) *J. Bacteriol.* **95**, 1692-1697
- Doelle, H. W. (1982) *Eur. J. Appl. Microb. Biotechnol.* **14**, 241-246
- Farmer, E. E. & Easterby, J. S. (1982) *Anal. Biochem.* **123**, 373-377
- Fromm, H. J. & Zewe, V. (1962) *J. Biol. Chem.* **237**, 1661-1667
- Garland, P. B. & Randle, P. J. (1962) *Nature (London)* **196**, 987-988
- Hengartner, H. & Zuber, H. (1973) *FEBS Lett.* **37**, 212-216
- Kamel, M. Y., Allison, D. P. & Anderson, R. L. (1966) *J. Biol. Chem.* **241**, 690-694
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Lowe, C. R., Small, D. A. P. & Atkinson, A. (1980) *Int. J. Biochem.* **13**, 33-40
- Porter, E. V., Chassy, B. M. & Chester, E. H. (1980) *Biochim. Biophys. Acta* **611**, 289-298
- Porter, E. V., Chassy, B. M. & Chester, E. H. (1982) *Biochim. Biophys. Acta* **709**, 178-186
- Reeves, R. E., Montalvo, F. & Sillero, A. (1967) *Biochemistry* **6**, 1752-1760
- Roy, S. K. & Nishikawa, A. H. (1979) *Biotechnol. Bioeng.* **21**, 775-785
- Saito, N. (1965) *J. Biochem. (Tokyo)* **57**, 363-371
- Scawen, M. D., Darbyshire, J., Harvey, M. J. & Atkinson, T. (1982) *Biochem. J.* **203**, 699-705
- Scawen, M. D., Hammond, P. M., Comer, M. J. & Atkinson, T. (1983) *Anal. Biochem.* **132**, 413-417
- Scopes, R. K., Testolin, V., Stoter, A., Griffiths-Smith, K. & Alger, E. M. (1985) *Biochem. J.* **228**, 627-634