Biochimica et Biophysica Acta, 613 (1980) 410—419

BBA 68986

AN INVESTIGATION INTO THE APPARENT INHIBITION BY ARGININE PHOSPHATE OF THE ACTIVITY OF *CARCINUS MAENAS* TYPE-M PYRUVATE KINASE

PETER C. POAT, IAN G. GILES * and KENNETH A. MUNDAY

School of Biochemical and Physiological Sciences, University of Southampton, Southampton, SO9 3TU (U.K.)

(Received October 18th, 1979)

Key words: Arginine phosphate; Pyruvate kinase; (Carcinus maenas)

Summary

An enzymic synthesis utilising arginine kinase for preparing arginine phosphate in a high state of purity is described.

The dissociation constant of magnesium arginine phosphate, determined by gel filtration, was 30.0 ± 0.9 mM. That for potassium arginine phosphate was calculated to be 63.0 ± 4.0 mM measured by the effect of potassium on the apparent magnesium dissociation constant.

The effect of KCl on the reaction catalysed by the type-M pyruvate kinase from *Carcinus maenas* (the common shore crab) pincer and leg muscle was investigated.

No effect was seen on the *C. maenas* pyruvate kinase activity, apart from that due to alteration of the K^+ concentration, on adding up to 70 mM potassium arginine phosphate to the reaction medium.

The less pure form of arginine phosphate was found to give an apparent non-competitive inhibition of the enzyme when phospho*enol*pyruvate was the varied substrate. This apparent inhibition can be accounted for by the removal of ADP from the assay medium in a side reaction involving arginine kinase and arginine phosphate.

These results are discussed in terms of the possible physiological control of the type-M pyruvate kinase from *C. maenas*.

* To whom correpondence should be addressed.
Abbreviation: EGTA, ethylene glycol bis(aminoethyl ether)-*N*,*N*′-tetraacetic acid.

Introduction

Analysis of the concentration of glycolytic intermediates in a number of tissues has indicated that the reaction catalysed by pyruvate kinase (ATP: pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) is greatly displaced from equilibrium and so may be a control site [1]. A further observation is that the maximal activity of pyruvate kinase in an extract is much higher than that of phosphofructokinase, the key rate-limiting enzyme of glycolysis. This implies that the enzyme must be severely inhibited in vivo.

Investigations into the properties of pyruvate kinase in vitro have shown that at least two types of enzyme exist. These can be distinguished on chromatographic, electrophoretic, immunological, kinetic and chemical properties. The type-L enzymes are found in gluconeogenic tissues and, characteristically, are activated by the glycolytic intermediate fructose 1,6-bisphosphate and the substrate phospho*enol*pyruvate [2–5]. Furthermore, ATP and alanine will inhibit the enzyme at physiological concentrations [6,7]. These observations and those of Engstrom's laboratory (for recent review see [8]) that a reversible phosphorylation/dephosphorylation reaction, mediated by a cyclic AMP-dependent protein kinase, can modulate the activity of the type-L pyruvate kinase can be used to postulate a plausible model for the control of this enzyme.

The second distinct form of pyruvate kinase, the type-M isoenzyme, is widely distributed and is present in very high amounts in skeletal muscle. The activity of this isoenzyme is not influenced by fructose 1,6-bisphosphate. The only physiologically significant modulator of its activity that has been reported is the phosphagen creatine phosphate [9]. This may represent an important in vivo control mechanism.

In invertebrate species a variety of phosphagens are found; in crustaceans and mollusca a common one is arginine phosphate. It has been reported that the partially purified enzyme from the tail muscle of *Oplophorus gracilirostris* is inhibited by arginine phosphate. The type of inhibition appeared to be similar to that of creatine phosphate on the mammalian enzyme [10]. In the snail, *Helix pomatia*, it was found that inhibition by arginine phosphate of the enzyme in a crude extract was not competitive and it was suggested that the inhibition is modulated by a protein in the extract that transfers either the phosphagen or a phosphate group onto the enzyme [11]. Subsequent studies on crude preparations of the enzyme from *Mytilus edulis* [12] lead to the suggestion that the apparent effect of arginine phosphate on pyruvate kinase is possibly due to a side reaction involving arginine kinase present in the pyruvate kinase preparation.

The common shore crab, *C. maenas*, possesses two distinct pyruvate kinases similar to mammalian species. The enzyme isolated from hepatopancreas has properties characteristic of a type-L enzyme [13] and is subject to modulation by several metabolites, including fructose 1,6-bisphosphate and L-alanine. The enzyme purified from the leg muscle of this crustacean, however, is typical of the type-M isoenzymes. As the detailed kinetic properties of this extensively purified enzyme are known (Newton, C.J., Poat, P.C. and Giles, I.G., unpublished data) a study of the effects of arginine phosphate on the enzyme was conducted.

Experimental

Materials

The ion-exchange resins Dowex 1-X8, (200—400 mesh) and Dowex 50-X8 (200—400 mesh), Trizma base, EGTA and the enzymes glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), 3-phosphoglycerate kinase (EC 2.7.2.3), hexokinase (EC 2.7.1.1), adenylate kinase (EC 2.7.4.3) and aldolase (EC 4.1.2.13) were obtained from Sigma (London) Chemical Co. Ltd. (Poole, U.K.).

ADP, ATP, NADH, phospho*enol*pyruvate (tricyclohexylamine salt) and pig heart lactate dehydrogenase (EC 1.1.1.27) were supplied by Boehringer Corporation (London) (Lewes, U.K.) ATP (disodium salt) and phospho*enol*-pyruvate (monocyclohexylamine salt), both for arginine phosphate synthesis, and all remaining chemicals were obtained from B.D.H. Chemicals (Poole, U.K.).

Enzymes

Arginine kinase (EC 2.7.3.3) was prepared from lobster tail muscle according to the method of Pradel and Kassab [14]. The enzyme had a final specific activity of 350 μmol ADP formed/min per mg protein at 25°C. Protein was estimated by the biuret method [15] using bovine serum albumin as the primary standard.

Rabbit muscle pyruvate kinase was prepared according to the method of Tietz and Ochoa [16] and had a final specific activity in excess of 200 units/mg protein at 25°C.

Pyruvate kinase from the leg muscle of *C. maenas* was prepared as previously described by Newton [17]. The enzyme used in the current study has a specific activity of 200 units/mg protein at 25°C. This preparation contained no detectable arginine kinase activity.

Standard kinetic assay of pyruvate kinase

In order to compare the rate of reaction obtained in experiments on different days and/or different batches of enzyme the activity was normalised by measurement under a standard set of conditions. The reaction mixture contained 0.15 μmol NADH, 0.50 μmol ADP, 0.5 μmol phospho*enol*pyruvate, 8.0 μmol Mg^{2+} added as $MgCl_2$, 1.0 μmol EGTA, 200 μmol KCl, 25 μmol Tris-HCl (pH 7.4) and 16 units lactate dehydrogenase in a final volume of 1 ml. The reaction was initiated by the addition of pyruvate kinase after the reaction mixture had been equilibrated to 25°C. Oxidation of NADH was followed at 340 nm in a Pye-Unicam SP1800 spectrophotometer.

Experimental assay of pyruvate kinase

The initial rate of reaction of the enzyme at various ADP and phospho*enol*-pyruvate concentrations, as specified in the text, in the presence and absence of arginine phosphate, were obtained as described above. The arginine phosphate stock solution was carefully adjusted to pH 7.4 using dilute HCl. The pH of this solution, and that of the final reaction medium, were regularly monitored to ensure that the enzyme was assayed at pH 7.4. Solutions of substrates and co-

factors were prepared daily and their concentrations estimated immediately before use.

The amount of $MgCl_2$ required to maintain the Mg^{2+} concentration constant was obtained by calculation, allowing for its chelation with ADP, phospho*enol*-pyruvate and EGTA using the dissociation constants given by MacFarlane and Ainsworth [18] and Sillen and Martell [19]. In addition the magensium chelation by arginine phosphate was calculated using the dissociation constants determined in the present study. These calculations were accomplished with the aid of a Hewlett-Packard 9810 desk-top computer using a modified Fibonacci search procedure.

No effect of arginine phosphate on lactate dehydrogenase could be demonstrated directly. Neither was any change in the initial rate of the reaction observed at any time on doubling the amount of lactate dehydrogenase added to the reaction cuvette.

Determination of substrates

Free L-arginine was estimated using the linked enzyme system arginine kinase/pyruvate kinase/lactate dehydrogenase. Arginine phosphate was determined using the coupled reactions of arginine kinase, hexokinase and glucose-6-phosphate dehydrogenase. The total phosphate content was determined by a slight modification of the method of Summer [20]. The free inorganic phosphate in the arginine phosphate was determined using the coupled reactions of glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase and hexokinase as described by Scopes [21]. The glyceraldehyde 3-phosphate required in this assay was prepared in situ using fructose 1,6-bisphosphate (1.5 mM) and aldolase (2 units/ml). Magnesium was determined by EDTA titration using Eriochrome Black-T as indicator [22]. Barium was determined either by the back titration of a standard EDTA solution with $MgSO_4$ [22] or by using the indicator dye *o*-cresolphthalein complexone [23]. The remaining substrates were determined by standard enzymic techniques: AMP and ADP [24], ATP [25]; phospho*enol*pyruvate [26] and pyruvate [27]. Potassium was determined by flame photometry.

Determination of the dissociation constant of magnesium arginine phosphate

This was measured by the gel-filtration method first described by Hummel and Dreyer [28]. A sample of arginine phosphate (approx. 20 μmol in 1 ml of equilibrating buffer) was applied to a column of Sephadex G-10 (1.75 X 20 cm) equilibrated with 25 mM Tris-HCl (pH 7.4) and known concentrations of $MgCl_2$ and KCl. The column was then eluted with the equilibrating buffer and 1.5 ml fractions collected. These were assayed for both arginine phosphate and magnesium. From the excess magnesium found in the arginine phosphate containing fractions the apparent dissociation constant of magnesium arginine phosphate can be calculated.

Preparation of arginine phosphate

Arginine phosphate was synthesised enzymatically using arginine kinase. Two alternative methods were used to drive the reaction. In one method, the ADP produced was recycled to ATP and AMP using adenylate kinase. In the

second method ATP was regenerated using pyruvate kinase and phospho*enol*-pyruvate. The advantage of the former method is that of cost, but it does require a considerably longer incubation period. This could possibly lead to a less pure preparation; the result of side reactions catalysed by trace impurities in the preparative enzymes. The subsequent purification of the arginine phosphate formed was identical for the two methods and is a modification of that proposed by Marcus and Marrison [29].

Synthesis method 1

A reaction mixture containing 67 mM Tris-HCl (pH 8.0) buffer, 10 mM L-arginine (pH 8.0), 7.5 mM $MgCl_2$, 50 mM KCl, 6.8 mM ATP, 5 mM phospho*enol*pyruvate (monocyclohexylamine salt), 5 mM β-mercaptoethanol, 200 units of rabbit muscle pyruvate kinase and 200 units of arginine kinase in a total volume of 200 ml was incubated at 25°C for 45 min. The arginine phosphate formed was isolated as described below.

Synthesis method 2

The incubation medium used was as described in synthesis method 1 with the following amendments. The pH of the final solution was adjusted to 9.0, the L-arginine concentration was increased to 50 mM, the phospho*enol*pyruvate and pyruvate kinase were omitted and 200 units of adenylate kinase were substituted. The reaction mixture was incubated for 15 h at ambient temperature (20—22°C) after which the arginine phosphate was isolated.

Isolation of arginine phosphate

After the incubation period for the synthetic reaction the reaction mixture was rapidly heated to 80°C and maintained there for 3 min. The solution was then rapidly cooled to 2°C. All subsequent operations were performed at 2°C unless noted.

The water-soluble, alcohol-insoluble barium salt of arginine phosphate was prepared by adjusting the solution to pH 9.0, adding an excess of $BaBr_2$ and then adding 4 vols. of 95% ethanol to the clear supernatant at −10°C containing the water-soluble barium salts. The crude barium arginine phosphate recovered by centrifugation was dissolved in 5 mM Tris-HCl buffer, pH 7.6, and applied to a column containing Dowex 1 (Cl^- form, 25 × 2 cm) equilibrated with 5 mM Tris-HCl, pH 7.6. The resin was washed with at least 2 column vols. of the equilibrating buffer before the arginine phosphate was eluted by including 150 mM KCl in the equilibrating buffer. The peak fractions were pooled and the phosphagen recovered as its barium salt as described above. It was found that although the barium salt could be stored as a dry powder in a vacuum desiccator at −10°C stability was improved by storing it as its potassium salt in 25 mM Tris-HCl, pH 8.0, at 2°C. In these latter conditions less than 2% decay occurred over 15 days. Both synthetic methods gave a yield of 500—600 μmol of arginine phosphate of comparable purity.

Results

Purity of arginine phosphate

The arginine phosphate used in the current investigation was prepared

TABLE I

MAXIMUM IMPURITY LEVELS IN THE PURIFIED POTASSIUM ARGININE PHOSPHATE

Enzymatic analysis for arginine phosphate showed the overall purity of the material to be >98.5%.

Impurity	Content (% mol impurity/mol arginine phosphate)
L-Arginine	0.04
ATP	0.012
ADP	0.003
AMP	0.036
Pyruvate *	0.015
Phospho*enol*pyruvate **	0.01
Inorganic phosphate	0.004
Adenylate kinase **	$<2 \cdot 10^{-6}$ units/μmol arginine phosphate
Pyruvate kinase *	$<2 \cdot 10^{-6}$ units/μmol arginine phosphate
Arginine kinase	$<2 \cdot 10^{-6}$ units/μmol arginine phosphate

* Only assayed for if synthesis method 1 is followed.
**Only assayed for if synthesis method 2 is followed.

enzymatically. This ensured that only the physiologically significant product was formed and it enables the preparation of a highly purified product. Table I shows the analysis of the final isolated material for the potential impurities that could have arisen from the defined reaction medium. The impurities are frequently undetectable using the methods described. For the purposes of this paper, however, maximum possible levels are given. These are calculated assuming that an absorbance change of 0.001 is the minimum that can be detected. In many cases this minimum figure could not be observed experimentally even at concentrations in excess of 50 mM arginine phosphate present in the analytical solution.

The final arginine phosphate prepared contained no detectable adenylate kinase, pyruvate kinase or arginine kinase activity. This result was only achieved after inclusion of the heat-inactivation stage in the purification procedure. In the absence of this step the final potassium arginine phosphate contained a significant amount of arginine kinase (of the order of $5 \cdot 10^{-4}$ units/μmol arginine phosphate).

Analysis of the barium arginine phosphate obtained after Dowex chromatography yielded a total P_i/Ba/arginine phosphate ratio of 0.97 : 1.04 : 1.0. The K/arginine phosphate ratio of the final potassium arginine phosphate was 2.37 ± 0.07 (6): 1 (mean ± S.E. (number of observations)). No barium could be detected in this final solution.

Metal dissociation constants of arginine phosphate

These were determined by gel-filtration as described in the experimental section. A typical elution profile is shown in Fig. 1. In the absence of KCl the dissociation constant of magnesium arginine phosphate in the quoted conditions was 30.3 ± 0.9 mM (6). After the addition of 100 mM KCl to the equilibrating buffer the apparent magnesium dissociation constant increased to 78.4 ± 1.4 mM (18). Assuming that only one K^+ is binding the apparent dissoci-

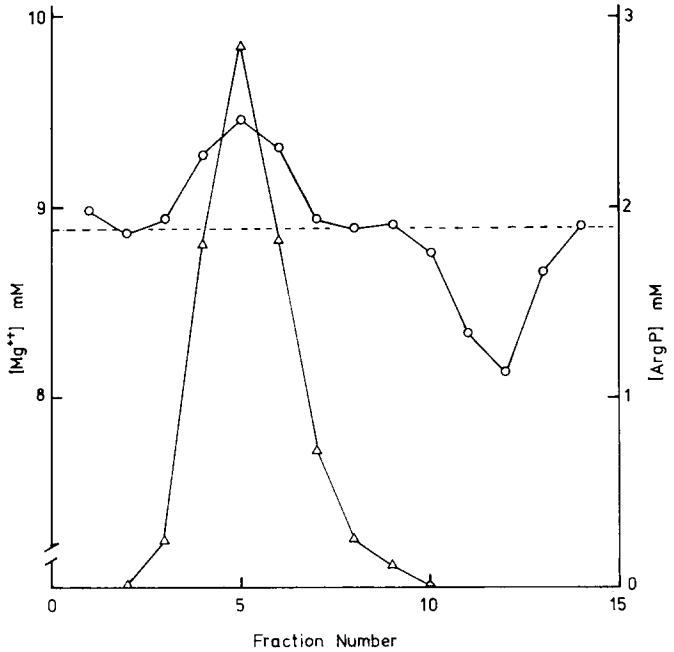


Fig. 1. Measurement of the dissociation constant of magnesium arginine phosphate by gel-filtration. The dissociation constant was measured at 20° C in a 25 mM Tris-HCl buffer, pH 7.4, containing 8.875 mM $MgCl_2$. Fractions were collected (1.5 ml) and analysed for both total Mg^{2+} (○) and arginine phosphate (△). The horizontal dotted line indicates the Mg^{2+} concentration in the equilibrating buffer. Arg*P*, arginine phosphate.

ation constant of potassium arginine phosphate can be calculated as being 63.0 ± 0.4 mM.

Effect of arginine phosphate on C. maenas muscle pyruvate kinase

The analysis of the effect of arginine phosphate on the enzyme is complicated by the counter-ion added with it. In the experiments reported here, this ion was potassium and the mean K/arginine phosphate ratio measured was 2.3 : 1. This cation also has the distinction of being required for catalytic activity of the enzyme [30]. Thus any change in catalytic activity that may occur on adding potassium arginine phosphate must be compared to that seen on adding potassium ions in the absence of arginine phosphate.

A double reciprocal plot of reaction rate against phospho*enol*pyruvate concentrations at a constant ADP concentration and with various concentrations of KCl showed a constant apparent *V* for the reaction. At lower phospho*enol*-pyruvate concentrations, increasing the KCl concentration from 50 mM increased the reaction rate at first, but further increases in the concentration of K^+ caused the reaction rate to fall. This fall in activity on increasing the KCl concentration to high values is probably the result of a redistribution of the substrate species away from the true substrates of the reaction, rather than any direct action on the enzyme. The formation of K · ADP and K · phospho*enol*-pyruvate is well documented [19].

On adding potassium arginine phosphate to the enzyme in the presence of

100 mM KCl, little variation in rate was seen, inspite of the variations in total potassium present. However, calculation indicates that the free K^+ concentration varied from 100 mM in the absence of arginine phosphate to 180 mM at the higher arginine phosphate concentration used. Over this range, very little change in rate due to a potassium effect would be predicted. A similar result was observed when no additional K^+ was added. The overall conclusion to be drawn from these results is that at concentrations up to 70 mM arginine phosphate has no effect on the activity exhibited by *C. maenas* type-M pyruvate kinase.

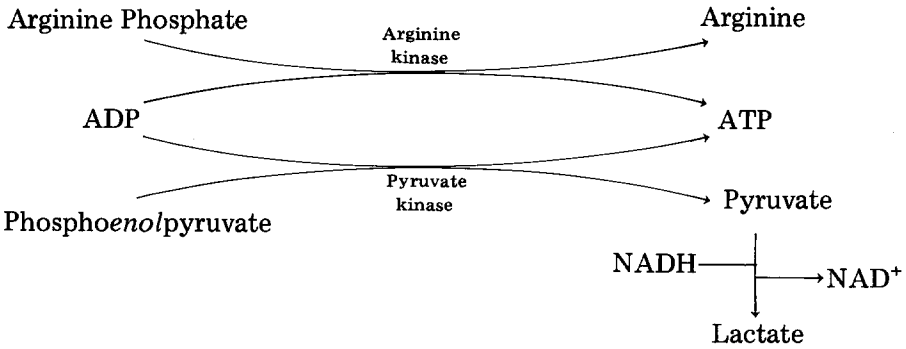
When the preceding experiments were repeated using arginine phosphate that had not been subjected to the heat inactivation step in the purification procedure, an apparent inhibition was seen. The result of one such experiment conducted using arginine phosphate that contained $5 \cdot 10^{-4}$ units of arginine kinase/μmol arginine phosphate gave apparently linear non-competitive inhibition when phospho*enol*pyruvate was the varied substrate. Qualitatively similar patterns were seen at other concentrations of ADP. The apparent K_i value for arginine phosphate calculated from the slope replot was 24.6 mM and from the intercept replot was 18.6 mM.

Discussion

The mass-action ratio of the pyruvate kinase reaction in *C. maenas* indicates that this reaction is far from equilibrium. Furthermore, the amount of pyruvate kinase present, 1.18 units/mg protein is high compared with phosphofructokinase which is present to the extent of only 0.061 units/mg protein [31]. This means that, as in the other species studied, the pyruvate kinase reaction must be severely inhibited in vivo. In common with these other species, no satisfactory explanation has been proposed to account for the extensive inhibition of this enzyme apart from inhibition by the product ATP and the phosphagen.

The results obtained in this study show that at concentrations of up to 70 mM, the phosphagen arginine phosphate has no direct actions on pyruvate kinase from *C. maenas* leg muscle. This is direct confirmation of the suggestion made by De Zwaan and Ebberink [12] that the apparent inhibition of pyruvate kinase (in their case from the mussel *Mytilis edulis*) by arginine phosphate was probably due to a side reaction involving arginine kinase. This explanation was confirmed in the current work when the inclusion of a trace amount of arginine kinase in the reaction medium led to an apparent inhibition of the enzyme.

Several observations in the course of this work would indicate that this inhibition was not of a simple reversible nature. The progress curves for the reaction were markedly non-linear, more so than would be expected for the normal depletion of substrate and accumulation of product. Secondly, the apparent initial rate depended critically on the length of pre-incubation given to achieve thermal equilibrium, before addition of the pyruvate kinase. Finally, the progress curve approached a zero rate after a time that depended on the substrate concentrations used. Addition of ADP at this point resulted in the resumption of the reaction whereas addition of phospho*enol*pyruvate had no effect. All these observations point to a non-reversible type of inhibition occurring, now known to be due to substrate depletion (ADP) via a second reaction involving arginine phosphate and arginine kinase.



When the arginine phosphate used contained $5 \cdot 10^{-4}$ units of arginine kinase/μmol arginine phosphate, 0.005 units of arginine kinase would be present in the 1 ml reaction mixture at 10 mM arginine phosphate. Assuming the enzyme were to continue catalysis at its maximal rate for 6 min, 0.03 μmol ADP would be removed before the reaction was initiated by addition of the pyruvate kinase. This corresponds to a removal of 43% of the ADP added (71.4 nmol). In reality, slightly less than this would be removed as the arginine kinase will not be fully active at this ADP concentration [32]. After addition of the pyruvate kinase the ADP depletion will continue, slowing the progress curve until the reaction ceases when all the ADP has been consumed. At this point it is only the addition of ADP that will allow the pyruvate kinase reaction to continue.

Variations of the concentration of arginine phosphate added will cause a proportional change in the amount of arginine kinase present in the reaction, and hence to the amount of ADP removed per unit time. At relatively low concentrations of arginine phosphate this change in ADP will, to a first approximation, be linear and hence one would expect to find an apparent non-competitive inhibition pattern. This is precisely the effect noted.

No direct inhibition of *C. maenas* pyruvate kinase could be demonstrated using concentrations of arginine phosphate up to 70 mM. Physiologically, arginine phosphate levels varying from 0.48 μmol/g tissue (honey bee flight muscle) to 52.2 μmol/g tissue (scallop snap muscle) have been reported [33]. *C. maenas* leg muscle has been found to contain 18.7 μmol/g tissue, which allowing for an intracellular water space of 0.5 ml/g, corresponds to an overall arginine phosphate concentration in the cell of 37 mM (Giles, I.G. and Poat, P.C., unpublished data). Thus it seems improbable that arginine phosphate has any physiological significance in the regulation of *C. maenas* M-type pyruvate kinase

Ackowledgement

The skilled technical assistance of Mr. M.J. Goldfinch is greatly appreciated.

References

1 Newsholme, E.A. and Start, C. (1973) Regulation in Metabolism, John Wiley and Sons, London
2 Taylor, C.B. and Bailey, E. (1967) Biochem. J. 102, 32c—33c

3 Tanaka, T., Sue, F. and Morimura, H. (1967a) Biochem. Biophys. Res. Commun. 29, 444—449
4 Tanaka, T., Harano, Y., Sue, F. and Morimura, H. (1967b) J. Biochem. 62, 71—91
5 Rozengurt, E., Jiminez de Asua, L. and Carminatti, H. (1969) J. Biol. Chem. 244, 3142—3147
6 Seubert, W., Henning, H., Schoner, W. and L'Age, M. (1968) Adv. Enzyme Reg. 6, 153—187
7 Llorente, P., Marco, R. and Sols, A. (1970) Eur. J. Biochem. 13, 45—54
8 Engstrom, L. (1978) Curr. Top. Cell Regul. 13, 29—51
9 Kemp, R.G. (1973) J. Biol. Chem. 248, 3963—3967
10 Guderley, H., Storey, K.B., Fields, J.H.A. and Hochachka, P.W. (1976) Comp. Biochem. Physiol. 55B, 475—478
11 Wieser, W. and Lackner, R. (1977) FEBS Lett. 80, 299—302
12 De Zwaan, A. and Ebberink, R.H.M. (1978) FEBS Lett. 89, 301—303
13 Giles, I.G., Poat, P.C. and Munday, K.A. (1977) Biochem. J. 165, 97—105
14 Pradel, L.-A. and Kassab, M.R. (1973) C. R. Acad. Sci. Series D, 276, 2843—2846
15 Gornall, A.G., Bardawill, C.S. and Davis, M.M. (1949) J. Biol. Chem. 177, 751—766
16 Tietz, A. and Ochoa, S. (1962) Methods Enzymol. 5, 365—369
17 Newton, C.J. (1977) Ph.D. Thesis, University of Southampton
18 MacFarlane, N. and Ainsworth, S. (1972) Biochem. J. 129, 1035—1047
19 Sillen, L.G. and Martell, A.E. (1971) Stability Constants of Metal-ion Complexes, Supplement, Chem. Soc. Special Publ. No. 25, London
20 Sumner, J.B. (1944) Science 100, 413—414
21 Scopes, R.K. (1972) Anal. Biochem. 49, 88—94
22 Vogel, A.I. (1961) A Text-book of Quantitative Inorganic Analysis Including Elementary Instrumental Analysis, 3rd. edn., p. 434 and pp. 446—447, Longman, London
23 Sarker, B.C.R. and Chauhan, U.P.S. (1967) Anal. Biochem. 20, 155—166
24 Adam, H. (1963) Methods of Enzymatic Analysis, 1st. edn. (Bergmeyer, H.U., ed.), pp. 573—577, Verlag Chemie, Weinheim and Academic Press, New York
25 Lamprecht, W. and Tratschold, I. (1963) Methods of Enzymatic Analysis, 1st. edn. (Bergmeyer, H.U., ed.), 543—551, Verlag Chemie, Weinheim and Academic Press, New York
26 Czok, R. and Eckert, L., (1963) Methods of Enzymatic Analysis, 1st. edn. (Bergmeyer, H.U., ed.), 229—233, Verlag Chemie, Weinheim and Academic Press, New York
27 Bucher, Th., Czok, R. and Latzko, E. (1963) Methods of Enzymatic Analysis, 1st. edn. (Bergmeyer, H.U., ed.), 253—259, Verlag Chemie, Weinheim and Academic Press, New York
28 Hummel, J.P. and Dreyer, W.J. (1962) Biochim. Biophys. Acta 63, 530—532
29 Marcus, F. and Morrison, J.F. (1964) Biochem. J. 92, 429—435
30 Boyer, P.D. (1953) J. Cell Comp. Physiol. 42, 71—77
31 Boulton, A.P. and Huggins, A.K. (1970) Comp. Biochem. Physiol. 33, 491—498
32 Smith, E. and Morrison, J.F. (1971) J. Biol. Chem. 246, 7764—7772
33 Beis, I. and Newsholme, E.A. (1975) Biochem. J. 152, 23—32