Identification of Cysteine as the Reactive Group in Pyruvate Kinase Alkylated by 5-Chloro-4-oxopentanoic Acid

By R. ALAN CHALKLEY and DAVID P. BLOXHAM

Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.

(Received 8 March 1976)

4-Hydroxypentanoic acid alanine thioether was synthesized and characterized by n.m.r. spectroscopy. This derivative corresponded to the modified amino acid obtained by allowing 5-chloro-4-oxo[3,5-³H]pentanoic acid to react with rabbit muscle pyruvate kinase. Performic acid oxidation of 4-oxo[3,5-³H]pentanoic acid alanine thioether in pyruvate kinase gave [³H]succinate (67%) and [³H]carboxymethylcysteine (33%) as expected. Evidence is presented to show that NaBH₄ reduction followed by periodate oxidation and analysis of radioactive formaldehyde production may provide a convenient method for distinguishing between thiol and amino alkylation by halogenomethyl ketone compounds. Peptide 'mapping' confirms that the modification by 5-chloro-4-oxopentanoic acid occurs primarily at one region of pyruvate kinase.

In the preceding paper (Bloxham & Chalkley, 1976), 5-chloro-4-oxopentanoic acid was shown to specifically alkylate rabbit muscle pyruvate kinase at a group which might be located close to the 'phosphoryl overlap' region of the active site (Reynard *et al.*, 1961). The next stage in the study of this reaction was to identify the nature of the functional group at the active site. The report describes the synthesis of a standard reference amino acid (4-hydroxypentanoic acid alanine thioether, I) and the use of performic acid oxidation to analyse the labelled amino acid. Degradation by performic acid oxidation should be particularly useful in this system, since the proposed structure of the inhibitor amino acid adduct

$$\begin{array}{c} \text{O} \\ || \\ (\text{X}-\text{CH}_2-\text{C}-\text{CH}_2-\text{CH}_2-\text{CO}_2\text{H}) \end{array}$$

means that the compound should be split into two chemically identifiable intermediates (see below for detailed explanation). Although performic acid oxidation has been used previously to produce carboxymethyl-amino acids from more complex amino acids (Petra *et al.*, 1965; Husain & Lowe, 1968), the structure of the degraded derivative allowed only the carboxymethyl-amino acid to be analysed, and this meant that complete information on the mechanism of this reaction for protein-bound intermediates was not obtained. The present communication shows that there is a high degree of preference in the cleavage reaction. Finally, it is suggested that periodate oxidation may be advantageous in the preliminary screening to identify the amino acid.

Materials and Methods

Pyruvate kinase modification

The exact conditions for the bulk modification of pyruvate kinase were described in the previous paper

(Bloxham & Chalkley, 1976). In all these experiments the reaction was terminated by adjusting the pH to 7.0 with 1 M-acetic acid before 70% loss of activity to eliminate any problems due to protein aggregation. For the experiments involving amino acid analysis or periodate oxidation the protein was reduced with 5 mg of NaBH₄. All the 5-chloro-4-oxopentanoate-labelled pyruvate kinase samples were exhaustively dialysed against water at 4°C and finally freeze-dried.

Chromatographic analysis of modified amino acids

Labelled pyruvate kinase (25 mg) was hydrolysed in 10 ml of 6 M-HCl at 110°C for 18 h. The hydrolysate was flash-evaporated and then vacuum-dried twice from 5 ml of water. High-voltage electrophoresis was performed with either a Gilson high-voltage electrophorator model D or Locarte electrophoresis apparatus. The buffers used were pH 1.9 (acetic acid/formic acid/water; 27:25:888, by vol.), pH 3.5 (pyridine/acetic acid/water; 1:10:289, by vol.), pH 4.7 (pyridine/acetic acid/water; 1:1:48, by vol.), and pH 6.5 (pyridine/acetic acid/water; 25:1:475, by vol.). Amino acids were located as either ninhydrin-positive spots or by radioactivity. For initial location of radioactive spots a Camlab radiochromatogram scanner was used. Subsequently the paper was cut into 1 cm strips, soaked in 1 ml of water for 1 h and then counted for radioactivity. Amino acid analyses of hydrolysed samples were performed with a JEOL JLC-6AH amino acid analyser. The column for acidic and neutral amino acids contained JEOL LCR2 resin. For radioactive samples, a stream splitter was used before the ninhydrin-analysing system and approx. 10% of the eluate from the column was collected in a fraction collector. The entire sample was counted for radioactivity.

Performic acid oxidation

Performic acid was prepared by the addition of H_2O_2 (30%, v/v) to formic acid (Hirs, 1956). In a typical experiment, 25 mg of 5-chloro-4-oxo[3,5- ^3H]pentanoate-labelled material was dissolved in 0.5 ml of formic acid, cooled on ice for 30 min, and then 1 ml of performic acid solution was added. After 2.5 h at 0°C , the sample was diluted with 25 ml of water and freeze-dried. The sample was hydrolysed and analysed by electrophoresis. *N*-3-Carboxymethylhistidine and ϵ -carboxymethyl-lysine (Gundlach *et al.*, 1959) were synthesized by Dr. G. Rasool of this department.

Periodate oxidation

The NaBH_4 -reduced 5-chloro-4-oxo[3,5- ^3H]pentanoate-labelled samples were oxidized with 100 mg of NaIO_4 in 10 ml of satd. NaHCO_3 containing 10 mg of carrier formaldehyde. After 16 h the mixture was adjusted to pH 6 with 50% (w/v) acetic acid, and formaldehyde was isolated as the dimer complex (Zaman *et al.*, 1973).

5-Amino-4-hydroxy[5- $^3\text{H}_2$]pentanoic acid was donated by Dr. P. M. Jordan of this department.

Results and Discussion

Synthesis and characterization of 4-hydroxy[3,5- ^3H]pentanoic acid alanine thioether (I)

The first task in identifying the amino acid in pyruvate kinase modified by 5-chloro-4-oxopentanoate was to synthesize an authentic reference amino acid. For this purpose we chose 4-hydroxypentanoic acid alanine thioether (I), since the alkylation reaction was easily monitored by 5,5'-dithiobis-(2-nitrobenzoate) titration (Ellman, 1959). Further, glutathione presents a useful *N*-blocked cysteine derivative, since its adducts are readily precipitated as the barium salt, which aids purification.

Glutathione (0.3 mmol) was allowed to react with 5-chloro-4-oxo[3,5- ^3H]pentanoate (0.42 mmol; 2.5×10^3 d.p.m./nmol) in water, pH 8 (maintained with 1M-NaOH) for 2 h at room temperature (20°C). About 80% of the reaction was complete within 10 min as judged by both 5,5'-dithiobis-(2-nitrobenzoate) titration and alkali uptake. The carbonyl group in the adduct was then reduced with NaBH_4 and after 30 min an excess of barium acetate (4 ml, 1M) was added. The barium salt was precipitated with 4 vol. of ice-cold ethanol and collected by centrifugation. After washing three times with ice-cold ethanol and twice with diethyl ether the product was dried *in*

vacuo. After acid hydrolysis, the amino acid analyser showed the presence of glutamate, glycine and a new amino acid, which was presumably compound I (Fig. 1). To confirm this, we purified the amino acid and identified its structure by n.m.r. spectroscopy. Fortunately, the mixture of amino acids was easily purified by high-voltage electrophoresis at pH 3.5. After performing this chromatographic purification twice, the modified amino acid gave only a single ninhydrin-positive and radioactive peak in the amino acid analyser. The n.m.r. spectrum of this compound was recorded in $^2\text{H}_2\text{O}$ by using a Varian XL100-12 machine operated in the CW mode at 100 MHz with a ^{19}F lock and external trimethylsilyl reference. The hydrogen resonances observed were as follows; δ 1.8–2.8 p.p.m., 4H, multiplet, $-\text{CH}_2\text{CH}_2-$; δ 2.85 p.p.m., 2H, doublet, $\text{S}-\text{CH}_2\text{C}$;

δ 3.05 p.p.m., 2H, triplet, $\text{C}-\text{CH}_2-\text{S}$; δ 4.6–4.9

p.p.m., 1H, multiplet, $-\underset{\text{N}^2\text{H}_2}{\text{CH}}-$. This spectrum accounted for all the expected resonances. The methylene group adjacent to the amino group

($-\underset{\text{N}^2\text{H}_2}{\text{CH}}-\text{CH}_2-\text{S}-$) in compound I is not a simple

Fig. 1. Chromatography of 4-hydroxy[3,5- ^3H]pentanoic acid alanine by using the amino acid analyser

This Figure shows the chromatography of 4-hydroxy[3,5- ^3H]pentanoic acid alanine thioether, which was produced either synthetically from glutathione or by alkylation of pyruvate kinase. The trace shows the elution profile from only the acidic and neutral column of the amino acid analyser. O, Ninhydrin-positive trace when 5-chloro-4-oxo[3,5- ^3H]pentanoate-labelled glutathione was analysed. ●, Distribution of radioactivity when 5-chloro-4-oxo[3,5- ^3H]pentanoate-labelled pyruvate kinase was analysed.

doublet, but is more complex. This may be due to the fact that it is adjacent to an asymmetric centre which was derived from the L-cysteine in glutathione. This may be contrasted with the methylene group adjacent to the hydroxyl group ($\text{S}-\text{CH}_2-\text{CH}-$), which is a

simple doublet, reflecting that the NaBH_4 reduction of the carbonyl produces a racemic mixture.

Having satisfactorily demonstrated that the synthetic amino acid corresponds to 4-hydroxypentanoic alanine thioether, it was compared with the modified amino acid derived from 5-chloro-4-oxopentanoate-labelled pyruvate kinase. Fig. 1 shows that after acid hydrolysis of labelled pyruvate kinase, a single radioactive peak was obtained which corresponds exactly to 4-hydroxypentanoic acid alanine thioether. The identity of the modified amino acid was confirmed by high-voltage electrophoresis, which showed that in four solvents the synthetic amino acid and the modified amino acid derived from pyruvate kinase had identical chromatographic properties. When electrophoresis was carried out at 5 kV and 15 mA for 2 h, the observed mobilities towards the cathode were as follows; pH 1.9, 7.5 ± 0.5 cm; pH 3.5, 5.5 ± 0.5 cm; pH 4.7, 3.2 ± 0.4 ; pH 6.5, 1.4 ± 0.4 . Both the synthetic amino acid and the derivative from pyruvate kinase failed to move towards the anode at pH values greater than 3.5 (approximate pK_a of CO_2H). This suggests that compound I may be isolated as the lactone with one less negative charge than expected. Note that carboxymethylcysteine does migrate towards the anode at pH 3.5.

Performic acid oxidation of the 5-chloro-4-oxopentanoate-modified amino acid in pyruvate kinase

Although the initial experiment gives strong evidence in favour of cysteine as the reactive group on pyruvate kinase, the result is ambiguous because the lysyl 5-chloro-4-oxopentanoate adduct was not prepared. Attempts to prepare an authentic sample of this compound by using either polylysine or α -N-acetyl-lysine failed to give the authentic compound. We chose to use performic acid oxidation (Baeyer-Villiger reaction; House, 1972) to degrade the modified amino acid. The rationale behind this experiment is illustrated in Scheme 1.

The reaction involves treatment with performic acid, and, as illustrated, while the initial structure is intact, acid-catalysed enolization causes loss of ^3H to the medium so that a low final recovery of ^3H is anticipated. Performic acid causes cleavage of either C—C bond adjacent to the carbonyl. Usually the oxidation shows a distinct preference in the cleavage reaction according to which alkyl group migrates most readily (House, 1972). If route (a) is followed then the eventual products will be [^3H]succinate (^3H

derived from position 3 of 5-chloro-4-oxopentanoate) and [^3H]formaldehyde. This route gives no information on the nature of the amino acid modified. In contrast, route (b) leads to the formation of the [^3H]carboxymethyl-amino acid (^3H derived from position 5 of 5-chloro-4-oxopentanoate), which can be identified by comparison with standards. [^3H]Hydroxypropionate formed by route (b) will be volatile and not analysed. It follows that analysis of the distribution of isotope between succinate and the carboxymethyl-amino acid will enable both the identification of the labelled amino acid and also the migration preferred in the reaction mechanism. Performic acid oxidation was performed on 5-chloro-4-oxo[3,5- ^3H]pentanoate, the 5-chloro-4-oxo[3,5- ^3H]pentanoate glutathione adduct and 5-chloro-4-oxo[3,5- ^3H]pentanoate-labelled pyruvate kinase. The first two samples served as controls. Under our experimental conditions, there was only a 5–6% recovery of radioactivity as involatile material after performic acid oxidation and acid hydrolysis. Loss of radioactivity was presumably due to both acid-catalysed detritiation of the ketone before oxidation and loss of volatile products. The involatile products were analysed by electrophoresis in pH 3.5 buffer at 3.5 kV and 50 mA for 45 min. Under these conditions the mobilities of standards were: carboxymethyl-lysine, -3.4 ± 0.5 cm; N-3-carboxymethylhistidine, -2.0 ± 0.4 cm; succinate, $+1.50 \pm 0.4$ cm; carboxymethylcysteine, $+6.5 \pm 0.5$ cm. The following observations were made on the degradation procedure. (1) 5-Chloro-4-oxo[3,5- ^3H]pentanoate (1.5×10^4 d.p.m.) only gave rise to [^3H]succinate (1.5×10^4 d.p.m.), and there were no radioactive peaks in the vicinity of any of the standard carboxymethyl-amino acids. (2) Both 5-chloro-4-oxo[3,5- ^3H]pentanoate glutathione adduct and the 5-chloro-4-oxo[3,5- ^3H]pentanoate-modified amino acid from pyruvate kinase gave rise to [^3H]carboxymethylcysteine as the sole radioactive carboxymethylamino acid. This confirms unambiguously that the amino acid modified by this compound is cysteine and eliminates both histidine and lysine. (3) When the distribution of radioactivity between succinate and carboxymethylcysteine was compared, 5-chloro-4-oxo[3,5- ^3H]pentanoate glutathione gave 1.3×10^4 d.p.m. in [^3H]succinate and 2×10^3 d.p.m. in [^3H]carboxymethylcysteine. Similarly, the 5-chloro-4-oxo[3,5- ^3H]pentanoate-modified amino acid from pyruvate kinase gave 1×10^4 d.p.m. in [^3H]succinate and 5×10^3 d.p.m. in [^3H]carboxymethylcysteine. The formation of substantially higher amounts of [^3H]succinate shows that reaction pathway a (Scheme 1) occurs preferentially. The preference is of the order of 70–80%, which is similar to the specificity of performic acid oxidation in other reactions (House, 1972). This result clearly demonstrates that the successful application of performic acid degradation

Scheme 1. Performic acid degradation of 5-chloro-4-oxo[3,5-³H]pentanoate-modified pyruvate kinase

The scheme shows the general pathway for performic acid degradation of a 5-chloro-4-oxo[3-5-³H]pentanoate-labelled amino acid. X may be Cys, Lys or His. The asterisk indicates the presence of ³H.

depends on the exact nature of the alkylating group for the protein and its influence on migration preference (pathway *a* versus *b*, Scheme 1). A high migration preference of this type might explain why performic acid oxidation of 1,3-dibromoacetone-linked histidine and cysteine in papain gave rise to only carboxymethylhistidine (Husain & Lowe, 1968) rather than an equal mixture of both carboxymethylcysteine and carboxymethylhistidine.

Periodate oxidation: a simple method for distinguishing between thiol and amino alkylation by halogenomethyl ketone compounds

The previous experiments have proved unambiguously that the group modified in pyruvate kinase by

5-chloro-4-oxopentanoate is a cysteine thiol. The methods used for establishing this reaction are rigorous, but they are also time-consuming and quite complicated. One can readily imagine circumstances where the structure of the halogenomethyl ketone may be sufficiently complex to preclude the synthesis of the inhibitor amino acid adduct. Indeed, it is probable that the authentic lysyl adduct cannot be made by the simple reaction between the amine and halogeno-

Scheme 2. NaBH_4 reduction followed by periodate oxidation as a general method for identifying the functional group modified by a halogenomethyl ketone alkylating reagent

The scheme shows the reaction of a general chloromethyl ketone with either a protein-bound thiol (R-SH , route *c*) or amino (R-NH_2 , route *d*). For 5-chloro-4-oxo[3,5- ^3H]pentanoate, R^1 is $\text{CO}_2\text{-CH}_2\text{-CH}_2$. The asterisk indicates an isotopic label which could be either ^{14}C or ^3H . Note that for 5-chloro-4-oxo[3,5- ^3H]pentanoate if the reaction proceeds through route *d*, then only ^3H at C-5 (50%) will be liberated by this procedure. Normally, the labelled protein would be hydrolysed before the periodate oxidation. Analysis is finally achieved by measuring the isotope liberated as formaldehyde.

proceeds preferentially through the Schiff base (D. Evans, I. Climie & M. Akhtar, personal communication). Further, as we have demonstrated quite clearly, the success of the application of the performic acid degradation depends on the good fortune with regard to the rate of proton exchange adjacent to the carbonyl group and the preference obtained in the migration reaction.

Since halogenomethyl ketone modifications of proteins will continue to be a common procedure in enzyme chemistry, we felt that it might be appropriate to point out a convenient method that allows some distinction between whether thiol or amino groups are reacting with the halogenomethyl ketone group. The method simply involves NaBH_4 reduction followed by periodate oxidation (Scheme 2). The method is based on the fact that periodate oxidation will occur only if a cyclic ester is formed as follows (Bunton, 1965)

where $\text{X} = \text{O}, \text{S}, \text{NH}$ or *N*-alkyl.

From Scheme 2 it is clear that intermediate III has the correct configuration for periodate oxidation, whereas intermediate II should not react. The

failure of compounds of structure type II to give labelled formaldehyde is shown by Expts. 1-4 (Table 1) where both synthetic cysteine 5-chloro-4-oxopentanoate adducts and pyruvate kinase 5-chloro-4-oxopentanoate adducts were tested. The observation that the pyruvate kinase 5-chloro-4-oxopentanoate adduct gave no [^3H]formaldehyde is consistent with cysteine as the group in pyruvate kinase alkylated by 5-chloro-4-oxopentanoate. The first four experiments quoted in Table 1 give negative results and would only be verified by successful degradation of a compound of structure type (III). We have been unable to synthesize the authentic 5-chloro-4-oxopentanoate lysine adduct, but we had in our laboratory a sample of a related analogue 5-amino-4-hydroxy[5- ^3H]pentanoic acid. This was obtained from biosynthetic experiments with δ -aminolaevulinatase synthetase to convert [$^3\text{H}_2$]glycine and succinyl-CoA (carboxypropionyl-CoA) into 5-amino-4-oxo[5- ^3H]pentanoic acid, which was reduced with NaBH_4 (Zaman *et al.*, 1973). It is apparent that, as expected, this type III structure gives a high yield of [^3H]formaldehyde (Expt. 5, Table 1). As final proof of the validity of this method, Meloche (1973) has shown that periodate oxidation of compound IV leads to the production of [^3H]formaldehyde in excellent yield (shown as Expt. 6, Table 1 for convenience). We feel that these experiments demonstrate that NaBH_4 reduction of the amino acid radioactive inhibitor adduct followed

Table 1. *Periodate degradation of labelled amino acids*

The details of the periodate oxidation and isolation of formaldehyde as the dimedone are described in the Materials and Methods section. For Expts. 1-4 the samples were reduced with NaBH₄ before oxidation of the intermediate used in the periodate oxidation. The percentage recovery in formaldehyde in these experiments was corrected for the fact that only 50% of ³H-labelled 5-chloro-4-oxopentanoate (COP) is at position 5 which gives rise to formaldehyde. The data in Expt. 6 are taken from Meloche (1973).

Expt. no.	Sample degraded	Total ³ H (d.p.m.)	³ H in formaldehyde (d.p.m.)	% recovery in formaldehyde
1	[3,5- ³ H]COP glutathione adduct	2.6 × 10 ⁶	3.7 × 10 ⁴	2.85
2	4-Hydroxy[3,5- ³ H]pentanoic acid alanine thioether	1.5 × 10 ⁵	1.5 × 10 ³	2.0
3	[3,5- ³ H]COP pyruvate kinase adduct (tryptic digestion)	1.22 × 10 ⁵	2 × 10 ³	3.27
4	[3,5- ³ H]COP pyruvate kinase adduct (total acid hydrolysis)	3.11 × 10 ⁵	1.8 × 10 ³	1.15
5	5-Amino-4-hydroxy[5- ³ H]pentanoic acid	5 × 10 ⁴	4.7 × 10 ⁴	94
6	Compound IV	5.5 × 10 ³	5 × 10 ³	90.0

by periodate oxidation and analysis of [³H]formaldehyde production provides a most useful initial procedure in the identification of the labelled amino acid in a protein. As a note of caution it must be pointed out that this procedure does not distinguish between alkylation reactions with either cysteine or imidazole. Therefore this procedure will probably have its maximum utility, provided there are good reasons to expect that either a cysteine or lysine has been modified.

Preliminary analysis of 5-chloro-4-oxopentanoate-labelled peptides

5-Chloro-4-oxo[3,5-³H]pentanoate-labelled pyruvate kinase (80mg; 15 × 10⁶ d.p.m.; 1.2mol of inhibitor per mol of protomer) was dissolved in 10ml of 50mM-Tris/HCl, pH8.2, containing 6M-urea. First, the carbonyl group was stabilized by reduction with NaBH₄ (5mg; 1h). The protein was reduced (80mg of dithiothreitol; 30°C; 3h), and carboxymethylated (iodoacetic acid, 400mg; NaHCO₃, 200mg; 4h; 30°C). The protein was exhaustively dialysed against 6 × 5 litres of water (72h at 4°C). Virtually all the radioactivity was retained. Tryptic digestion was performed for 5h at 37°C in 16ml of 50mM-NH₄HCO₃ containing 2mg of diphenylcarbamoyl chloride-treated trypsin. At the end of digestion, trypsin was denatured at 90°C and removed by centrifugation. A suitable sample (5mg of protein) was freeze-dried, dissolved in water and

analysed by two-dimensional chromatography. The sample was subjected to descending chromatography in butan-1-ol/acetic acid/water (4:1:5, by vol., upper phase) for 21h followed by electrophoresis in pyridine/acetic acid/water (1:10:280, by vol.) at 2000V and 120mA for 75min. After ninhydrin staining 56 peptides were located out of a predicted 66 based on the number of lysine and arginine residues (Cottam *et al.*, 1969). Each peptide was cut out, soaked in 0.5ml of water for 1h and analysed for radioactivity. Out of all the peptides, three contained radioactivity of which the majority (74%) was located in a peptide region that migrated 6.5cm and 13.5cm by chromatography and electrophoresis respectively. This result substantiates that the site of action of 5-chloro-4-oxopentanoate appears to be directed selectively towards one region of the protein.

An initial attempt has been made to purify this peptide further. After tryptic digestion, peptides were resolved from undigested material by chromatography on a column (100cm × 2cm) of Sephadex G-10. The peptides were then fractionated on a column (17cm × 2.5cm) of DEAE-cellulose by using a 500ml gradient from 25mM- to 1M-NH₄HCO₃. The major radioactive peak (80%) was eluted with 100mM-NH₄HCO₃. This was further purified by descending chromatography for 24h in butan-1-ol/acetic acid/water (4:1:5, by vol., upper phase). Of the eight peptides located by ninhydrin only one peptide contained all of the radioactivity (mobility 7cm). The overall yield of this peptide was only 3% which was

inadequate for sequence work with the facilities available in this laboratory. The amino acid composition was shown to be Arg (1.00), Asp (1.13), Ser (0.71), Glu (0.61) and 4-hydroxy[3,5-³H]pentanoic acid-alanine thioether, (0.58). This indicates that the peptide produced by the trypsin treatment is only a small fragment.

We are grateful to Professor M. Akhtar for his invaluable advice on this project. R. A. C. acknowledges the award of an M.R.C. studentship. Mr. I. Climie performed the n.m.r. analysis.

References

- Bloxham, D. P. & Chalkley, R. A. (1976) *Biochem. J.* **159**, 201–211
- Bunton, C. A. (1965) in *Oxidation in Organic Chemistry, Part A* (Wiberg, K. G., ed.), pp. 367–407, Academic Press, New York and London
- Cottam, G. L., Hollenberg, P. F. & Coon, M. J. (1969) *J. Biol. Chem.* **244**, 1481–1489
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Gundlach, H. G., Stein, W. H. & Moore, S. (1959) *J. Biol. Chem.* **234**, 1754–1760
- Hirs, C. H. W. (1956) *J. Biol. Chem.* **219**, 611–621
- House, H. O. (1972) in *Modern Synthetic Reactions*, pp. 321–330, W. A. Benjamin, Menlo Park, CA
- Husain, S. S. & Lowe, G. (1968) *Biochem. J.* **108**, 855–859
- Meloche, H. P. (1973) *J. Biol. Chem.* **248**, 6945–6951
- Petra, H. P., Cohen, W. & Shaw, E. (1965) *Biochem. Biophys. Res. Commun.* **21**, 612–618
- Reynard, A. M., Hass, L. F., Jacobsen, D. D. & Boyer, P. D. (1961) *J. Biol. Chem.* **236**, 2277–2283
- Zaman, Z., Jordan, P. M. & Ahktar, M. (1973) *Biochem. J.* **135**, 257–263