Studies on Alkaline Phosphatase

INHIBITION BY PHOSPHATE DERIVATIVES AND THE SUBSTRATE SPECIFICITY

By H. N. FERNLEY AND P. G. WALKER

Department of Biochemistry, Institute of Orthopaedics, Stanmore, Middlesex

(Received 24 January 1967)

1. The kinetics of inhibition of calf-intestinal alkaline phosphatase by inorganic phosphate, fluorophosphate, inorganic pyrophosphate, β -glycerophosphate and adenosine 5'-triphosphate in the range pH 8–10 were investigated. The reference substrate was 4-methylumbelliferyl phosphate. 2. The inhibitions were 'mixed' in that both K_m and V were affected, but the competitive element was by far the stronger. 3. In each case the pH profile for the competitive K_i was similar to the pH profile for K_m . Since the K_m and K_i values both change 100-fold over the pH range 8–10, it is concluded that the inhibitors compete with the substrate for the same active site. 4. It was also found that the enzyme preparation hydrolysed fluorophosphate, pyrophosphate and adenosine 5'-triphosphate as readily as it hydrolysed 4-methylumbelliferyl phosphate and β -glycerophosphate. Each pH-activity curve, however, had a different shape, but with the exception of pyrophosphate the activity approached the same maximum value at high pH. 5. Attempts to separate the phosphomonoesterase and pyrophosphatase activities by column chromatography were not successful, and the results of other experiments listed suggest that the two activities are a property of the same enzyme. 6. The effect of Mg^{2+} ions is briefly mentioned: the phosphomonoesterase activity is enhanced whereas the pyrophosphatase and adenosine triphosphatase activities are strongly inhibited in the presence of excess of Mg^{2+} ions.

Alkaline phosphatase is inhibited by its specific reaction product, P_i , a phenomenon first described by Martland & Robison (1927) and since confirmed for a wide variety of alkaline phosphatases [e.g. calf-intestinal-mucosal enzyme (Morton, 1955); human-placental enzyme (Ahmed & King, 1960); *Escherichia coli* enzyme (Garen & Levinthal, 1960)]. The inhibition is strictly competitive [calf-intestinal enzyme (Lazdunski & Ouellet, 1962); *E. coli* enzyme (Garen & Levinthal, 1960)]. However, P_i is not simply a competitive inhibitor but under suitable conditions is also a substrate [mammalian enzyme (Stein & Koshland, 1952; Engström, 1961*b,c*, 1964); *E. coli* enzyme (Schwartz & Lipmann, 1961; Engström, 1962; Schwartz, 1963)], the enzyme incorporating P_i or catalysing ^{18}O exchange between P_i and $H_2^{18}O$, or both.

PP_i is also an inhibitor of alkaline phosphatase (Morton, 1955) and has been regarded as a substrate [mammalian enzyme (Schmidt & Thannhauser, 1943); *E. coli* enzyme (Heppel, Harkness & Hilmoe, 1962)], though the consensus of opinion at the present time is that the purified enzyme is inactive towards PP_i (Stadtman, 1961; Dixon & Webb,

1964). On the other hand, there are several reports that suggest that alkaline phosphatase is a pyrophosphatase. Perkins & Walker (1958) found that, in rachitic rats, the cartilage pyrophosphatase and phosphomonoesterase activities at pH 7.4 were the same. Fleisch & Bisaz (1962) found that alkaline phosphatase destroyed a blood plasma inhibitor of bone calcification, identified as PP_i . In human hypophosphatasia, Russell (1965) found there was an increased excretion of PP_i . More direct evidence comes from Cox & Griffin (1965) and Moss, Eaton, Smith & Whitby (1967), who reported that alkaline phosphatases from a variety of mammalian tissues are all active pyrophosphatases.

Other phosphomonoesterases have been described as possible pyrophosphatases. For instance, Nordlie & Arion (1964) consider that a liver microsomal glucose 6-phosphatase is also a pyrophosphatase, and Mayer, Campbell, Smith & McKinney (1961) found that a soya-bean acid phosphatase is probably also a pyrophosphatase. Purified potato acid phosphatase has considerable pyrophosphatase activity (Lora-Tamayo, Alvarez & Andreu, 1962). Other plant phosphatases with claims to pyro-

phosphatase activity include phosphoesterases from *Pisum sativum* seedlings (Pierpoint, 1957), from white-lupin seedlings (Newmark & Wenger, 1960) and from rice ears and apricot kernels (Ikawa, Nisizawa & Miwa, 1964).

In the present work we have investigated the effect of pH on the inhibition of a purified alkaline phosphatase from calf-intestinal mucosa by P_i , PP_i , ATP, monofluorophosphate and β -glycerophosphate. The effect of pH on the inhibition by P_i is of interest because the inhibitor constant K_i contains a much smaller velocity term (Stein & Koshland, 1952) than does K_m and so might be expected to reveal the nature of the binding between enzyme and inhibitor or substrate. We have found that the enzyme preparation hydrolyses PP_i , fluorophosphate and ATP as rapidly as it hydrolyses a normal phosphomonoester. Evidence that the same enzyme is responsible for all these activities is presented and discussed. A preliminary report of this work has already appeared (Fernley & Walker, 1966).

MATERIALS AND METHODS

The preparation and use of the 2-amino-2-methylpropane-1,3-diol (ammediol)-HCl buffer system, MUP* and calf-intestinal alkaline phosphatase (EC 3.1.3.1) (preparation AP-1 15436; about 15% pure; C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) were described in an earlier paper (Fernley & Walker, 1965). A suspension of *E. coli* alkaline phosphatase in $(NH_4)_2SO_4$ solution (preparation BAP-C; 42 units/mg.; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was dialysed against distilled water for 24 hr. (four changes) before use. ATP (disodium salt) (99% pure; Sigma Chemical Co., St Louis, Mo., U.S.A.), disodium monofluorophosphate (containing 2% of P_i ; Koch-Light Laboratories Ltd., Colnbrook, Bucks.), disodium β -glycerophosphate (grade 1; Sigma Chemical Co.), Na_2HPO_4 and $Na_4P_2O_7$ (both analytical-grade reagents) were used without further purification. For the column chromatography, DEAE-cellulose (Serva Ltd., Heidelberg, Germany) and Sephadex G-100 and G-200 (Pharmacia, Uppsala, Sweden) were employed. With the first compound the column was equilibrated with 0.01 *M* ammediol-HCl buffer, pH = p*K*, at 2° and a solution of the calf-intestinal enzyme in the same buffer was added. With the Sephadex columns (0.7 cm. $\times$ 50 cm.) the gel was equilibrated and elution carried out with the above 0.01 *M* ammediol-HCl buffer.

Assay procedures. Where fluorescence of released 4-methylumbelliferone was to be measured, buffer, water, inhibitor and substrate were mixed, brought to 30° and enzyme was added. Reaction was stopped by adding an equal volume of 1.0 *M*- K_2HPO_4 -KOH reagent, pH 10.4, containing 0.01 *M*-EDTA (to prevent possible precipitation of magnesium phosphate). The fluorescence of the solution was measured directly in a Locarte fluorimeter, as described previously (Fernley & Walker, 1965).

* Abbreviation: MUP, 4-methylumbelliferyl phosphate monoester.

Where P_i determinations were required, enzyme was generally used without any added albumin, the assay volume was 1.0 ml. and reaction was stopped by adding 2.0 ml. of $CuSO_4$ -acetate buffer, pH 4.0, 0.4 ml. of molybdate reagent and 0.4 ml. of metol reagent (Delsal & Manhour, 1958). Where albumin was present, 0.5 ml. of 25% (w/v) trichloroacetic acid was added to a 2.0 ml. assay mixture, and after centrifuging, 1.5 ml. was treated with 2.5 ml., 0.5 ml. and 0.5 ml. respectively of the Delsal-Manhour reagents.

RESULTS

Inhibition of alkaline phosphatase by inorganic phosphate and other phosphate derivatives. For this series of experiments, MUP was the reference substrate. The effects of varying the substrate concentration at fixed P_i concentration and vice versa are shown in Fig. 1. Plots are of $[S]/[P]$ against $[S]$ and of $[I]/[P]$ against $[I]$, where $[S]$ is the initial substrate concentration, $[I]$ the initial inhibitor concentration and $[P]$ the final concentration of product (4-methylumbelliferone) under the standard assay conditions. Data for the other phosphate derivatives are given in Table 1. It is shown below that in all cases except for P_i the inhibitor is hydrolysed at a rate comparable with that for the substrate, but under the experimental conditions the enzyme activity is so low that the initial concentrations are very little changed during the assay. It was determined that the time-course of reaction was linear in the presence of each inhibitor, provided that 10% total hydrolysis was not exceeded.

It can be seen that in all cases K_m is higher in the presence of inhibitor (K'_m) and V is lower (V'). The plots of $[I]/[P]$ against $[I]$ were all linear, indicating that only one inhibitor molecule is combining with the enzyme. The rate equation may be written:

$$v = \frac{V}{1 + K_m/[S](1 + [I]/K_i) + [I]/K'_i}$$

where v is the observed rate of substrate hydrolysis, V is the maximum rate of substrate hydrolysis in the absence of inhibitor, K_m is the Michaelis constant and K_i and K'_i are inhibition constants. The parameters K'_m and V' obtained in the presence of a fixed concentration of inhibitor are related to the true K_m and V values, as follows:

$$K'_m = \frac{K_m(1 + [I]/K_i)}{V} V'$$

$$V' = \frac{V}{1 + [I]/K'_i}$$

The inhibition constants K_i (acting on K_m) and K'_i (acting on V) can thus be evaluated.

Effect of pH on the inhibition of alkaline phosphatase. In this series of experiments MUP was

Fig. 1. Inhibition of calf-intestinal alkaline phosphatase by P_i at pH 9.25. Enzyme in 0.02*I* ammonium-HCl buffer containing albumin (0.01%) was incubated with substrate (MUP) and inhibitor for 30 min. at 30° (volume of assay medium 1.0 ml.). (a) Varying substrate concentration in the absence (●) and presence (○) of $50 \mu M$ P_i ; 1.0 mμg. of enzyme was used. (b) Varying P_i concentration in the presence of $20 \mu M$ substrate and 2.0 mμg. of enzyme. Other details are given in the Materials and Methods section. The K_m , K'_m , V and V' values listed in (a) are derived statistically (Wilkinson, 1961) from the data given. Velocities are expressed as μM -methylumbelliferone produced by the enzyme during the assay. The K_i value (b) is determined graphically by using the K_m value given in (a).

again the reference substrate and the procedure employed was to vary the substrate concentration in the presence and absence of a fixed concentration of each inhibitor at each of seven pH values in the range pH 8.0–9.8. From a statistical analysis of the data (Wilkinson, 1961) it was possible to derive both K_i and K'_i values in each instance, but the K'_i values could not be determined accurately, because there was not sufficient difference between the V and V'

values. In all cases the K'_i values lay well above the inhibitor concentrations employed, whereas the K_i values were usually lower than the inhibitor concentrations. Plots of K_i against pH are given in Fig. 2 and K_m values for MUP are included for reference. All the pH profiles are the same shape, but there are differences in detail. With P_i , the K_i values are numerically similar to K_m for MUP over the pH range 8–10, where both K_m and K_i change 100-fold. The pH profiles for PP_i and ATP inhibitions are very similar to that for P_i . With fluorophosphate the K_i values are higher than K_m (MUP) by a constant factor 3, and with β -glycerophosphate the factor varies from about 20 at pH 9.8 to 6 at pH 8.0.

It is noteworthy that PP_i has an ionizing group with $pK 9.1$ at 0.02*I*. This ionization does not appear in the plot of pK_i against pH, which is similar to the plot with ATP.

Hydrolysis of phosphates by alkaline phosphatase. The pH-activity curves for MUP, PP_i , ATP, fluorophosphate and β -glycerophosphate with the calf-intestinal preparation are shown in Fig. 3. The curves for ATP, fluorophosphate and β -glycerophosphate are similar to that for MUP, whereas with PP_i the curve shows a maximum at about pH 9.2. The question at issue is whether all these activities are catalysed by the same enzyme. For comparison, the pH-activity curves for MUP, PP_i and ATP with *E. coli* alkaline phosphatase under similar conditions are shown in Fig. 4. Here also PP_i shows a falling-off in activity at high pH.

Identity of phosphomonoesterase and pyrophosphatase activities. With regard to these two activities of the calf-intestinal preparation, the rates of heat inactivation at 55° were identical (k 0.024 min.⁻¹ at pH 9.25; 0.02*I* ammonium-hydrochloric acid buffer, measured at 30°, and 0.01% albumin). Both were strongly inhibited by 10 mM-L-histidine and 20 mM-DL-phenylalanine, the latter a specific inhibitor for intestinal alkaline phosphatase (Fishman, Green & Inglis, 1962). Mixed-substrate experiments (Table 2) showed that PP_i inhibited the monoesterase activity and MUP inhibited the pyrophosphatase activity roughly in accordance with their respective constants (Table 1, assuming that in each case $K_m = K_i$). Here K_m (MUP) is $17 \mu M$ and K_i (PP_i) is $29 \mu M$, and one would predict inhibitions of 37% and 62% respectively (actual values 44% and 64%).

The K_m values for pyrophosphate at pH 9.5–9.8 are given in Fig. 5 and they compare reasonably well with the K_i values given in Fig. 2. Agreement would normally be expected where an inhibitor is a competing substrate.

Attempts were made to separate the two activities by ion-exchange chromatography on DEAE-cellulose and gel filtration through Sephadex G-100

Table 1. *Inhibition of calf-intestinal alkaline phosphatase by phosphate derivatives at pH 9.2 and 30°*

The experimental conditions were similar to those described in Fig. 1. K_m , K'_m , V and V' values were determined as in Fig. 1(a) and in each case refer to the concentration of inhibitor given in the Table. Standard errors are given in parentheses. K_i values were determined graphically as in Fig. 1(b) by using the K_m values listed.

Inhibitor	Concn. (μ M)	pH of assay	K_m	K'_m	V	V'	K_i (μ M)
Fluorophosphate	200	9.25	20.0 (2.0)	84.2 (5.4)	2.02 (0.06)	1.87 (0.07)	68
PP _i	100	9.19	17.1 (1.2)	62.2 (3.3)	1.78 (0.04)	1.50 (0.04)	29
β -Glycerophosphate	1000	9.25	23.8 (1.1)	74.0 (4.5)	2.69 (0.04)	2.22 (0.07)	250
ATP	50	9.25	20.5 (0.6)	59.6 (2.8)	2.39 (0.02)	1.78 (0.04)	19

Fig. 2. Effect of pH on the inhibition of alkaline phosphatase. pK values refer to a 0.02 *I* ammediol-HCl buffer system containing albumin (0.01%) at 30°. Inhibition constants were determined by the method outlined in the text. ●, MUP (pK_m); ○, PP_i (pK_i); ▲, ATP (pK_i); △, β -glycerophosphate (pK_i); ■, inorganic fluorophosphate (pK_i); □, P_i (pK_i).

and G-200. In each case the fractions highest in monoesterase activity were also the highest in pyrophosphatase activity (Fig. 6), but the ratios of the activities showed small significant differences after the various treatments both with respect to each other and also to the adenosine triphosphatase activity (Table 3).

Fig. 3. Hydrolysis of phosphate esters and other derivatives by calf-intestinal alkaline phosphatase. Enzyme (50 μ g.) in 0.02 *I* ammediol-HCl buffer was incubated with each substrate for 20 min. at 30° in a 1.0 ml. volume of assay medium. Activities are expressed as μ M-substrate hydrolysed by the enzyme during the assay. ●, 1 mM-MUP; ○, 1 mM-PP_i; ▲, 1 mM-ATP; △, 5 mM- β -glycerophosphate; ■, 1 mM-fluorophosphate. Other details are given in the Materials and Methods section.

Effect of magnesium chloride on the activities. As is well known, Mg^{2+} ions enhance the phosphomonoesterase activity of alkaline phosphatase. The effect of Mg^{2+} ions on the pyrophosphatase and adenosine triphosphatase activities, however, is mainly inhibitory (Fig. 7). At low magnesium chloride concentrations there is a slight activation with PP_i and ATP, but in the presence of excess of magne-

Fig. 4. Hydrolysis of MUP, PP_i and ATP by *E. coli* alkaline phosphatase. The conditions employed were similar to those of Fig. 3. Enzyme (1 μ g.) was incubated with 1 mM-substrate. ●, MUP; ○, PP_i; ▲, ATP.

Table 2. Hydrolysis of 4-methylumbelliferyl phosphate and sodium pyrophosphate by calf-intestinal alkaline phosphatase

Enzyme (40 μ g.) was incubated with MUP (1 mM) or with PP_i (1 mM) or with both (MUP, 1 mM; PP_i, 1 mM) at pH 9.19 (0.02 *I* ammediol-HCl buffer) for 30 min. at 30°. Mixtures were analysed for P_i and free 4-methylumbelliferone. Activities are expressed as μ moles of product/mg. of enzyme/min. and refer to a 1 ml. volume of assay medium.

Substrate	Activity	
	P _i	MUP
MUP	96	95
PP _i	211	—
MUP + PP _i	130	53

sium chloride a strong inhibition is observed, especially with PP_i. At pH 9.2 in the presence of 10 mM-magnesium chloride the monoesterase activity is unaffected by 1 mM-PP_i, suggesting that PP under these conditions is neither an inhibitor nor a substrate.

DISCUSSION

As noted in the introduction, the substrate specificity of alkaline phosphatase has not yet been clearly established. It is of some importance, as the enzyme has a wide distribution in mammalian tissues, though we know little of its metabolic role. It has been stated by Morton (1955) that the pure calf-intestinal enzyme does not hydrolyse PP_i. His experimental conditions included a large excess of Mg²⁺ ions (20-fold), which in our experience would be highly inhibitory, and little or no activity would be expected. Using an enzyme preparation similar in activity to Morton's preparation, Portmann (1957) also found no activity against PP_i. In a later paper, Portmann, Rossier & Chardonnens (1960) list a variety of conditions under which they detected no pyrophosphatase activity. With our enzyme preparation we were able to detect appreciable pyrophosphatase activity under the conditions of Portmann *et al.* (1960). One would be justified in concluding that the pyrophosphatase activity in our preparation must be due to some impurity, were it not for the fact that an exactly similar situation has arisen with regard to *E. coli* alkaline phosphatase (see Garen & Levinthal, 1960; Heppel *et al.* 1962), and the evidence here favours the common identity of the phosphomonoesterase and pyrophosphatase activities.

Our own data for calf-intestinal alkaline phosphatase on the whole support the idea that the phosphomonoesterase, pyrophosphatase, adenosine triphosphatase and fluorophosphatase activities are associated with the same enzyme. The pH profiles of *K_i* for PP_i, ATP and fluorophosphate are very similar to the *K_m* profile for MUP. The simplest satisfactory explanation of these observations is that all the compounds are substrates for the same enzyme and compete for the same active site. The occurrence of *K_i* would be explained in terms of inhibition by excess of (second) substrate, which is known to occur with phenyl phosphate (Morton, 1957) and PP_i (Schmidt & Thannhauser, 1943) separately. The result of the mixed-substrates experiment (Table 2) is also consistent. The heat-inactivation data and the inhibitions by histidine and phenylalanine also support the one-enzyme theory. The column chromatography techniques failed to achieve any gross separation of the activities, though some evidence of heterogeneity was obtained. The microheterogeneity of calf-intestinal alkaline phosphatase is a well-established fact (Engström, 1961a) that probably reflects differences in charge and other properties within a population of enzyme molecules. One might expect that different distributions of enzyme molecules in the various chromatographic fractions would show differences in their behaviour towards the various

Fig. 5. K_m determinations for PP_i and calf-intestinal alkaline phosphatase. Enzyme in 0.02 I ammediol-HCl buffer was incubated with substrate for 30 min. at 30° in a 1.0 ml. assay. Velocities are expressed as μM -phosphate produced by the enzyme during the assay. (a) pH 9.54, 10 μg . of enzyme; (b) pH 9.80, 40 μg . of enzyme.

Fig. 6. Fractionation of calf-intestinal enzyme on DEAE-cellulose. Enzyme (50 μg .) in 1 ml. of buffer was applied to a 0.8 cm. $\times$ 12 cm. column and eluted with a linear gradient (0.01–0.10 I) of ammediol-HCl buffer, pH = pK . Fractions (2.0 ml.) were analysed for phosphomonoesterase activity (full line) with 100 μM - p -nitrophenyl phosphate, pH 8.9 (0.1 I ammediol-HCl and 30°), and pyrophosphatase activity ($\circ$) with 1 mM- PP_i , pH 9.2 (0.02 I ammediol-HCl and 30°). Activities are given as a percentage of the maximum. The actual values can be calculated from the data in Table 3.

Table 3. Activities of calf-intestinal alkaline-phosphatase preparation after various fractionation procedures

Fractions showing highest phosphomonoesterase activity were incubated with 1 mM-MUP, 1 mM- PP_i or 1 mM-ATP for 30 min. at pH 9.25 [2.0 ml. of assay medium, 0.02 I ammediol-HCl buffer containing albumin (0.01%) and 30° . Activities are expressed as μM -substrate hydrolysed during the assay.

Fractionation procedure	Activities with		
	MUP	PP_i	ATP
Original preparation (0.1 μg .)	121	134	178
Cellulose-DEAE*	103	79	116
Sephadex G-100	95	67	125
Sephadex G-200	117	66	118

* 50 μl . of fraction no. 39 (Fig. 6).

substrates at a particular pH value. In other words, it is possible that one is observing kinetic micro-heterogeneity and not partially separating two or more distinct enzymes.

In a similar study, Moss *et al.* (1967) failed to separate the phosphomonoesterase and pyrophosphatase activities in human intestinal alkaline phosphatase by gel filtration, ion-exchange chromatography or starch-gel electrophoresis. The last problem in this context relates to the differences in pH optimum at high substrate concentration. Of particular relevance is the qualitatively similar picture that obtains with *E. coli* alkaline phosphatase.

Fig. 7. Effect of Mg^{2+} ions on the activity of calf-intestinal alkaline phosphatase. Enzyme (100 μg .) in 0.02 *M* ammonium-HCl buffer, pH 9.24, was incubated with 1 *mM* substrate for 20 min. at 30° in 1.0 ml. of assay medium. Activities are given as a percentage of each control with no added MgCl_2 . Control activities are given as μM -substrate hydrolysed by the enzyme during the assay. ●, MUP, control 146 μM ; ○, PP_i, control 142 μM ; ▲, ATP, control 198 μM .

tase in that the three activities (monoesterase, pyrophosphatase and adenosine triphosphatase) differ with the same enzyme molecule. With the exception of PP_i, the substrates for the calf enzyme approach the same limiting activity at high pH. The actual values shown in Fig. 3 are lower than the true values at high pH because of decreasing saturation of the enzyme by substrate (assuming $K_i = K_m$). The conclusion is that the same enzyme is responsible for all these activities and that there is a common rate-determining step at high pH. It is not easy to explain the behaviour of PP_i. The ionization at pH 9.1 that did not appear in the K_i curve is possibly occurring in the enzyme-substrate complex such as to prevent reaction of the fully ionized substrate with the enzyme. If this is the case, an important conclusion would be that there is no change in pK on binding, and this makes the whole

process of binding very difficult to understand in terms of an electrostatic interaction.

In a previous paper (Fernley & Walker, 1965) it was postulated that alkaline phosphatase undergoes a conformation change during the hydrolysis cycle and that one form of the enzyme combined with substrate whereas the second form reacted to give products. This relatively simple picture cannot explain all the features discussed in the present paper. For instance, K_i (P_i) has a similar pH-dependence to K_m (MUP), and because K_i must be an equilibrium constant it follows that K_m cannot include rate terms apart from k_2 (rate of transformation of the Michaelis complex) that do not appear in K_i . Yet the evidence suggests that there might be a change in rate-limiting step at about pH 9.0: below this pH there is a fairly wide variation in k_0 for different substrates, whereas at higher pH k_0 tends to be independent of the nature of the substrate. The relationship between K_m and V found for MUP (Fernley & Walker, 1965) also exists with ATP, in spite of the fact that the pH-dependences of K_m and V are different, but with fluorophosphate and β -glycerophosphate plots of K_i/V against K_i are not linear.

This work was supported by a grant from the National Fund for Research into Poliomyelitis and Other Crippling Diseases. The technical assistance of Mr D. A. Byers is gratefully acknowledged.

REFERENCES

- Ahmed, Z. & King, E. J. (1960). *Biochim. biophys. Acta*, **45**, 581.
 Cox, R. P. & Griffin, M. J. (1965). *Lancet*, ii, 1018.
 Delsal, J.-L. & Manhoury, H. (1958). *Bull. Soc. Chim. biol., Paris*, **40**, 1623.
 Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., p. 223. London: Longmans, Green and Co.
 Engström, L. (1961a). *Biochim. biophys. Acta*, **52**, 36.
 Engström, L. (1961b). *Biochim. biophys. Acta*, **52**, 49.
 Engström, L. (1961c). *Biochim. biophys. Acta*, **54**, 179.
 Engström, L. (1962). *Ark. Kemi*, **19**, 129.
 Engström, L. (1964). *Biochim. biophys. Acta*, **92**, 71.
 Fernley, H. N. & Walker, P. G. (1965). *Biochem. J.* **97**, 95.
 Fernley, H. N. & Walker, P. G. (1966). *Biochem. J.* **99**, 39 p.
 Fishman, W. H., Green, S. & Inglis, N. I. (1962). *Biochim. biophys. Acta*, **62**, 363.
 Fleisch, H. & Bisaz, S. (1962). *Nature, Lond.*, **195**, 911.
 Garen, A. & Levinthal, C. (1960). *Biochim. biophys. Acta*, **38**, 470.
 Heppel, L. A., Harkness, D. R. & Hilmoe, R. J. (1962). *J. biol. Chem.* **237**, 841.
 Ikawa, T., Nisizawa, K. & Miwa, T. (1964). *Nature, Lond.*, **203**, 939.
 Lazdunski, M. & Ouellet, L. (1962). *Canad. J. Biochem. Physiol.* **40**, 1619.
 Lora-Tamayo, M., Alvarez, E. F. & Andreu, M. (1962). *Bull. Soc. Chim. biol., Paris*, **44**, 501.
 Martland, M. & Robison, R. (1927). *Biochem. J.* **21**, 665.

- Mayer, F. C., Campbell, R. E., Smith, A. K. & McKinney, L. L. (1961). *Arch. Biochem. Biophys.* **94**, 301.
- Morton, R. K. (1955). *Biochem. J.* **61**, 232.
- Morton, R. K. (1957). *Biochem. J.* **65**, 674.
- Moss, D. W., Eaton, R. H., Smith, J. K. & Whitby, L. G. (1967). *Biochem. J.* **102**, 53.
- Newmark, M. Z. & Wenger, B. S. (1960). *Arch. Biochem. Biophys.* **89**, 110.
- Nordlie, R. C. & Arion, W. J. (1964). *J. biol. Chem.* **239**, 1680.
- Perkins, H. R. & Walker, P. G. (1958). *J. Bone Jt Surg.* **40B**, 333.
- Pierpoint, W. S. (1957). *Biochem. J.* **65**, 67.
- Portmann, P. (1957). *Hoppe-Seyl. Z.* **309**, 87.
- Portmann, P., Rossier, R. & Chardonnens, H. (1960). *Helv. physiol. Acta*, **18**, 414.
- Russell, R. G. G. (1965). *Lancet*, ii, 461.
- Schmidt, G. & Thannhauser, S. J. (1943). *J. biol. Chem.* **149**, 369.
- Schwartz, J. H. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 871.
- Schwartz, J. H. & Lipmann, F. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1996.
- Stadtman, T. C. (1961). In *The Enzymes*, 2nd ed., p. 58. Ed. by Boyer, P. D., Lardy, H. A. & Myrbäck, K. New York: Academic Press Inc.
- Stein, S. S. & Koshland, D. E. (1952). *Arch. Biochem. Biophys.* **39**, 229.
- Wilkinson, G. N. (1961). *Biochem. J.* **80**, 324.