Temperature and the Regulation of Enzyme Activity in Poikilotherms

PROPERTIES OF LUNGFISH FRUCTOSE DIPHOSPHATASE

By HANS WERNER BEHRISCH AND PETER W. HOCHACHKA

Department of Zoology, University of British Columbia, Vancouver 8, B.C., Canada

(Received 25 November 1968)

1. The properties of fructose diphosphatase from liver of South American lungfish (*Lepidosiren paradoxa*) were examined. 2. Saturation curves for substrate (fructose diphosphate) and both cofactors (Mn^{2+} and Mg^{2+}) are sigmoidal and Hill plots of these results suggest about 2 interacting substrate and cofactor sites/molecule of enzyme. 3. Mn^{2+} is an efficient positive modulator of the enzyme and K_a for Mn^{2+} is about 20–30-fold lower than the K_a for Mg^{2+} . 4. Lungfish fructose diphosphatase is inhibited by low concentrations of AMP, and the affinity of the enzyme for AMP is insensitive to temperature. 5. The affinities of fructose diphosphatase for fructose diphosphate and Mn^{2+} appear to be dependent on temperature, whereas affinity for Mg^{2+} is temperature-independent. 6. The pH optimum of the enzyme depends on the presence of the particular cofactor. As pH increases, the K_a values of both cations are lowered, maximum velocities are increased and the saturation curves for cofactor become hyperbolic. 7. The possible roles of these ions, pH and substrate in the modulation of fructose diphosphatase and gluconeogenic activity in the lungfish are discussed in relation to aestivation and temperature adaptation.

Both the direction and rate of glucose carbon flow in glycolysis and gluconeogenesis in various animal tissues can be affected by the activity of FDPase* (Weber, Singhal & Srivastava, 1965; Newsholme & Gevers, 1967). In all other systems examined (Newsholme & Gevers, 1967), with the exception of chloroplast FDPase (Morris, 1968), negative AMP modulation of FDPase is generally considered to be the major means of controlling the activity of this reaction. Thus conditions of energy saturation favour the FDPase reaction; energy-depleted conditions (when AMP concentrations are high) tend to inhibit the reaction. We became interested in examining the kinetic and regulatory properties of lungfish liver FDPase since lungfish maintain their liver glycogen contents during starvation and aestivation (Janssens, 1964), presumably through enhancement of gluconeogenesis from amino acid precursors (Janssens, 1964, 1965). The situation is further complicated by the fact that during prolonged starvation (as in aestivation) the energy charge (Atkinson & Walton, 1967) of the cell may be decreased. This would lead to an inhibition of FDPase and would be inconsistent with maintenance of normal or even enhanced rates of gluconeogenesis that are observed during starva-

tion (Weber *et al.* 1965). This suggests that other important control mechanisms are perhaps operative.

In addition, stimulation by pyruvate of liver glycogenesis in the South American lungfish appears to be favoured at temperatures in the upper thermal range of the animal (Hochachka, 1968). Since the kinetic and regulatory properties of FDPases from other organisms are highly temperature-sensitive (Taketa & Pogell, 1965; Behrisch & Hochachka, 1969), it was also decided to investigate the thermal properties of the lungfish enzyme.

METHODS AND MATERIALS

Experimental animals. The fish used in this study, *Lepidosiren paradoxa*, inhabits the non-turbulent waters of swamps and flooded rain forests in South America. The fish is an obligate air breather, surfacing at regular intervals for air. During May and June the Amazon drainage is at high water and much of the rain forest is in flood. At this time the surface temperature of the water is about 27° and shows little or no daily thermal fluctuations. Although there is no thermocline in the open river systems, thermoclines may develop in the still waters of the rain forest (H. Ungermeyer, personal communication), and small (1–2°) thermal gradients have been seen in the surface waters here. In general, however, conditions are stable at this time of year and do not impose a thermal stress on the animal. Later, when the waters recede from the forest, isolated pools are often formed in which the temperature

* Abbreviations: FDPase, fructose diphosphatase (D-fructose 1,6-diphosphatase 1-phosphohydrolase (EC 3.1.3.11); FDP, fructose 1,6-diphosphate.

Table 1. *Effect of temperature on affinity of lungfish FDPase for FDP*

Assays were carried out at pH 7.5. Values of n_H were calculated by a Fortran IV computer programme.

Temp.	1 mM-Mg ²⁺			0.08 mM-Mn ²⁺		
	10 ⁵ K _m for FDP (M)	n _H	Probability of n _H being zero	10 ⁵ K _m for FDP (M)	n _H	Probability of n _H being zero
5°	9	1.4	0.00	7	2.6	0.04
15	9	1.6	0.00	12	1.7	0.00
25	9	2.2	0.03	15	2.1	0.00
35	20	1.7	0.00	58	1.9	0.00

Fig. 1. Substrate saturation curves of lungfish liver FDPase at various temperatures at pH 7.5 in the presence of (a) 1 mM-Mg²⁺ and (b) 0.08 mM-Mn²⁺. Temperatures: ▲, 5°; ○, 15°; ●, 25°; △, 35°.

rises. In some of these H. Ungermeyer (personal communication) has recorded temperatures as high as 38°. Any fish stranded in such pools could come under rather severe stress, made all the more acute by the air-breathing habit, which brings the fish to the warmest surface layers. In periods of extreme drought the lungfish can go into prolonged aestivation.

The South American lungfish, *Lepidosiren*, was obtained from the Museum Goeldi, Belém, Brazil, and was captured in the swamp waters of the area. The animals were in captivity for at least several weeks before these experiments and were starved during this time.

Experimental methods. Lungfish liver homogenates were prepared in three parts of 0.01 M-tris-HCl buffer, pH 7.4, in

a VirTis homogenizer at 1–2°. The homogenates were spun at about 30000g for 30 min. at 4° and the pellets were discarded. The supernatant was brought to 30% saturation with solid (NH₄)₂SO₄ and stirred for at least 3 hr. at 4–5°. The suspension was then centrifuged as above and the pellet discarded. The supernatant was dialysed extensively against 0.01 M-tris-HCl buffer, pH 7.4, at 4°. This procedure effectively separated FDPase from non-specific alkaline phosphatase, which was assayed with *p*-nitrophenyl phosphate (Sigma Chemical Co., St Louis, Mo., U.S.A.) as substrate. The reaction medium contained, in 2.0 ml. of 0.05 M-tris-HCl buffer, various concentrations of FDP, AMP (Sigma Chemical Co.) and Mg²⁺ or Mn²⁺. The reaction was started by adding the FDPase preparation and was assayed by release of P_i. The P_i was determined by the method of Fiske & Subbarow (1925), with the Technicon AutoAnalyzer. Unless otherwise specified all experiments were carried out at pH 7.5. Enzyme activity was linear with time for at least 30 min. and was proportional to the enzyme concentration.

RESULTS

The lungfish liver FDPase has a low affinity for its substrate (FDP) with either Mg²⁺ or Mn²⁺ in the reaction medium. The FDP saturation curves in the presence of either cofactor are sigmoidal and the Hill coefficients, n_H , which were calculated with a Fortran IV computer programme, are about 2 (Table 1). Since n_H is not an elementary kinetic parameter, but is a complex function of both the number of binding sites and the forces of interaction, the number of interacting sites that bind FDP is about two/molecule and cannot be given with any greater precision. In contrast with the FDPases from other organisms investigated (Horecker, Pontremoli, Rosen & Rosen, 1966; Underwood & Newsholme, 1965; Behrisch & Hochachka, 1969; Krebs, 1963), substrate inhibition is not seen in the lungfish enzyme (Fig. 1) in the presence of Mn²⁺, and only about a 10–20% inhibition occurs at 1 mM-FDP in the presence of 1 mM-Mg²⁺ at 25° and 35° (Fig. 1).

Temperature does not appear to affect the affinity of lungfish FDPase for FDP with Mg²⁺ as cofactor between 5° and 25°; however, at 35° there is about a twofold increase in K_m for FDP (Table 1).

Table 2. Activation energies and Q_{10} values of FDPase action with either Mg^{2+} or Mn^{2+} as cofactorAssays were carried out at pH 7.5. Activation energies were calculated from $v_{opt.}$

Temp. range	1 mM- Mg^{2+}		0.08 mM- Mn^{2+}	
	E_a (kcal./mole)	Q_{10}	E_a (kcal./mole)	Q_{10}
5-15°	11.0	2.0	9.1	1.8
15-25	10.5	1.8	3.8	1.2
25-35	17.6	2.6	29.0	4.9

Fig. 2. Arrhenius plots of lungfish FDPase in the presence of optimum amounts of Mg^{2+} (O) and Mn^{2+} (●) at pH 7.5. The points are calculated from values of $v_{opt.}$ Fig. 3. Inhibition of lungfish FDPase by AMP at various temperatures. Assays were carried out in the presence of 1 mM- Mg^{2+} with 1 mM-FDP at pH 7.5. Temperatures: ▲, 15°; ○, 25°; △, 35°.

Between 5° and 25°, at saturating FDP concentrations, values of Q_{10} are about 2; between 25° and 35° the Q_{10} increases to 2.6 (Table 2). When Mn^{2+} is present in the reaction medium, the affinity for

FDP appears to display a more complex dependency on temperature (Fig. 1). Thus the apparent K_m for FDP rises from $7 \times 10^{-5} M$ at 5° to $58 \times 10^{-5} M$ at 35°. This large increase in K_m for substrate in the presence of Mn^{2+} is accompanied by about a fivefold increase in $V_{max.}$ and a three- to eight-fold increase in the apparent activation energy (Table 2). Between 15° and 25° and with Mn^{2+} as cofactor the Q_{10} is less than 2.

The identity of the cationic cofactor also determines the activation energy of the enzyme over a particular temperature range (see Fig. 2). Thus when 1 mM- Mg^{2+} is present in the reaction medium the activation energy is approx. 11 kcal./mole of enzyme between 15° and 25°, and about double this between 25° and 35°. On the other hand when optimum concentrations of Mn^{2+} are present in the reaction medium the enzyme appears relatively insensitive to temperature between 15° and 25°, but appears to be markedly stimulated between 25° and 35°. This result indirectly supports the finding by Hochachka (1968) that temperatures in the upper thermal range of the lungfish favour glycogenesis.

Inhibition by AMP. An important effector of FDPase is AMP (Taketa & Pogell, 1963, 1965; Underwood & Newsholme, 1965; Wallace & Newsholme, 1967). The modulation of activity of FDPase by AMP has been shown to be highly dependent on temperature in the liver of rat (Taketa & Pogell, 1965), ox (Geller, 1967) and fish (Hochachka, 1967; Behrlich & Hochachka, 1969). Within the thermal range of the lungfish and in the presence of optimum concentrations of Mg^{2+} the inhibition of FDPase by AMP appears to be almost totally independent of temperature (Fig. 3), so that from between 15° to 40° the apparent K_i of AMP is about $3.5 \times 10^{-5} M$, some threefold higher than that for the trout FDPase (Behrlich & Hochachka, 1969). In addition, Hill coefficients of inhibition by AMP are about 1 at all temperatures (Table 3), indicating negligible co-operation between the AMP-binding sites. This finding provides further support for the hypothesis that AMP inhibition of FDPase activity in the lungfish is relatively insensitive to temperature. This is in complete contrast

Table 3. *Effect of temperature on inhibition of lungfish FDPase by AMP*Activities were measured in the presence of 1 mM-Mg²⁺ with 1 mM-FDP at pH 7.5.

Temp.	10 ⁵ K _i for AMP (M)	n _H	Probability of n _H being zero
15	1.5	0.85	0.00
25	3.5	0.91	0.00
35	3.4	0.58	0.00
40	3.4	0.87	0.00

Fig. 4. Saturation curves for Mg²⁺ (a) and Mn²⁺ (b) of lungfish FDPase at various temperatures. Assays were carried out with 1 mM-FDP at pH 7.5. Temperatures: ▲, 5°; ○, 15°; ●, 25°; △, 35°.

with inhibition by AMP of the trout enzyme in which the ability of AMP to act as an allosteric effector is highly dependent on temperature (Hochachka, 1967; Behrisc & Hochachka, 1969).

Cation requirements and cation control. Like all FDPases previously examined (Horecker *et al.* 1966) lungfish FDPase has an absolute requirement for a bivalent cation, which may be satisfied by either Mg²⁺ or Mn²⁺. The saturation curve for Mg²⁺ is sigmoidal in the presence of optimum concentrations of FDP (Fig. 4) at pH 7.5, and the *K_a* of Mg²⁺ does not appear to change appreciably with temperature. From the Hill coefficient (Table 4) the apparent number of interacting sites that bind Mg²⁺ is about two/molecule of enzyme. The cation

requirements of the enzyme may be fulfilled by concentrations of Mn²⁺ that are about 10–30-fold lower (Table 4) than those of Mg²⁺. The saturation curve for Mn²⁺ is sigmoid (Fig. 4), in contrast with the hyperbolic saturation curve for Mn²⁺ of the rainbow-trout FDPase (Behrisc & Hochachka, 1969). As with Mg²⁺ the values of the Hill coefficients for Mn²⁺ binding approximate to 2, and this value appears to be independent of temperature.

Lungfish FDPase has a distinct alkaline pH optimum in the presence of optimum concentrations of Mg²⁺ (Fig. 5). However, at optimum concentrations of Mn²⁺ the pH-activity profile changes markedly (Fig. 5). There is a broad peak of maximum or near-maximum activity extending from pH 7.5 to pH 9.5. Replacement of Mg²⁺ with Mn²⁺ appears to stimulate activity near the neutral end of the pH range, a finding not obtained with the rainbow-trout enzyme (Behrisc & Hochachka, 1969). The affinity of lungfish FDPase for its cofactors seems to be dependent on the pH (Table 5), with a rise in pH causing a decrease in the *K_a* values of Mg²⁺ and Mn²⁺ (Fig. 6) and a marked increase in *V_{max.}*. At high pH (pH 9.0) the sigmoidicity of the saturation curves for Mn²⁺ and Mg²⁺ disappears and the saturation curves become hyperbolic, a finding that is quite similar to the effect of pH on the saturation curves for Mg²⁺ of rainbow-trout FDPase (Behrisc & Hochachka, 1969).

DISCUSSION

It is generally accepted that the major means of regulation of FDPase is by negative modulation by AMP (see Atkinson, 1966; Horecker *et al.* 1966). During prolonged starvation or aestivation in the lungfish energy charge of the liver cell may be expected to be depressed, and, since the *K_i* for AMP is quite low, this condition would tend to maintain FDPase in a 'shut-off' conformation. However, in aestivation or starvation maintenance and regulation of liver glucose and glycogen contents would seem to be a condition of survival (Janssens, 1964) requiring normal or indeed relatively enhanced FDPase activity (Weber *et al.* 1965). Hence one might predict a requirement,

Table 4. *Effect of temperature on affinity of lungfish FDPase for cation cofactor*

Assays were carried out with 1 mM-FDP at pH 7.5.

Temp.	$10^4 K_a$ for Mg^{2+} (M)	n_H	Probability of n_H being zero	$10^6 K_a$ for Mn^{2+} (M)	n_H	Probability of n_H being zero
5°	2.5	0.6	0.21	16	1.7	0.00
15	2.0	1.3	0.00	7	1.3	0.03
25	2.8	1.4	0.02	7	1.7	0.00
35	2.5	1.4	0.00	9	1.9	0.00

Fig. 5. Effect of pH on activity of lungfish FDPase in the presence of 0.1 mM-Mg²⁺ (○) and 0.01 mM-Mn²⁺ (●). Assays were carried out with 1 mM-FDP at 25°.

under these conditions, for mechanisms of activation of FDPase or a means of releasing the system from control by AMP inhibition.

It is known from previous studies (Underwood & Newsholme, 1965; Wallace & Newsholme, 1967) that Mg²⁺ can reverse AMP inhibition, but in fish systems the changes in concentration of Mg²⁺ required are quite high (Behrlich & Hochachka, 1969) and this would not seem to be a plausible control feature. Another means of regulating FDPase involves H⁺. As is evident in Fig. 6, increasing pH can lead to very dramatic changes in the K_a for Mg²⁺ (or Mn²⁺) as well as in V_{max} . Moreover, the pH-activity profile displays a steep section in the neutral range in the presence of Mg²⁺ or Mn²⁺. Hence small changes in pH in this range can lead to large changes in activity, a characteristic feature of many efficient regulatory metabolites (Atkinson, 1966). A very active phosphofructokinase could lead to local acidification, since H⁺ is a product of the reaction. Conversely a low rate of glycolysis and phosphofructokinase activity, such as might be expected during lungfish aestivation, would favour a rise in pH and so lead to a significant activation of FDPase.

Although previous studies of FDPase have considered the pH-activity profile of the enzyme in some depth (Horecker *et al.* 1966), the importance

of this type of control has been largely overlooked. Preiss, Biggs & Greenberg (1967) have shown that the pH requirements of the FDPase from spinach leaves can be strongly affected by changes in concentration of Mg²⁺ and likewise indicate a rather steep section in the pH-activity profile. Similar results have been obtained with trout liver FDPase (Behrlich & Hochachka, 1969). In this connexion, Trivedi & Danforth (1967) observed profound effects of pH on the phosphofructokinase reaction, which may couple, in the manner indicated above, with the effect of H⁺ on activity of FDPase. Such a coupling of the control of phosphofructokinase and FDPase is analogous to the coupling of adenylate control of these two enzymes: conditions that favour activity of phosphofructokinase favour inhibition of FDPase, and conditions that inhibit phosphofructokinase tend to activate FDPase (Atkinson, 1966).

A third mechanism for activation of FDPase may involve the substrate, FDP. In previous studies FDP has been implicated in the control of the inter-conversion of FDP and fructose 6-phosphate by the complementary mechanisms of substrate inhibition of FDPase and product activation of phosphofructokinase (see Atkinson, 1966). However, Newsholme & Gevers (1967) argue that substrate inhibition of FDPase is probably not of physiological significance because the concentrations of FDP required are high and the percentage inhibition is relatively low. Recent studies (Preiss *et al.* 1967; Behrlich & Hochachka, 1969) indicate that at least under certain conditions the FDP saturation curve is sigmoidal, suggesting co-operative interaction between substrate-binding sites. Since the apparent K_m for FDP for the lungfish and trout enzymes appears to fall well within the physiological concentration ranges for the substrate (Williamson, Cheung, Coles & Herczeg, 1967), it appears that FDP can serve as a positive modulator as well as substrate of FDPase.

Often it has been suggested that in poikilotherms evolutionary adaptation to temperature may involve selection for enzymes of greater 'efficiency' than in warm-blooded animals. In earlier studies the most commonly used measure of 'efficiency' was the activation energy of the given enzyme-

Table 5. *Effect of pH on affinity of FDPase for cation cofactor*

Assays were carried out with 1 mM-FDP at 27°.

pH	$10^4 K_a$ for Mg^{2+} (M)	n_H	Probability of n_H being zero	$10^6 K_a$ for Mn^{2+} (M)	n_H	Probability of n_H being zero
7.5	2.2	2.3	0.00	24	1.9	0.00
8.2	2.0	1.5	0.00	11	1.7	0.00
9.0	1.6	1.3	0.00	8	1.6	0.00

Fig. 6. Effect of pH on affinity for Mg^{2+} (a) and Mn^{2+} (b) of lungfish FDPase. Assays were carried out with 1 mM-FDP at 27°. ●, pH 7.5; ○, pH 8.2; Δ, pH 9.0.

catalysed reaction, the implication being that enzymes with lower activation energies would be favoured in environments of low thermal energy. In our comparative analyses we have been unable to find such a correlation consistently (Hochachka & Somero, 1968; Somero & Hochachka, 1968). Moreover, for a large number of enzymes, including FDPases from trout (Behrlich & Hochachka, 1969), salmon (H. W. Behrlich, unpublished work) and the lungfish (Fig. 2), Arrhenius plots are often

complex and can be affected by substrates or modulators (Lowry, Schulz & Passonneau, 1964; Helmreich & Cori, 1964; Massey, Curti & Ganther, 1966). For these reasons (for further discussion of this matter in relation to poikilothermy see Hochachka & Somero, 1968) it appears that in evolutionary adaptation to unique thermal environments enzyme 'efficiency' as measured by activation energy does not appear to be a fundamental site of natural selection. Rather, the sites most sensitive to selective pressure appear to include (1) enzyme affinity for unique substrate (Somero & Hochachka, 1968) and (2) enzyme affinity for cofactors or modulators or both (Atkinson & Walton, 1967; Behrlich & Hochachka, 1969).

In a physiological sense the effect of temperature-dependent changes in enzyme-ligand affinities appears to be a decrease in the Q_{10} of the reaction, whereas control mechanisms are held normally effective at all temperatures encountered in Nature. Thus with trout pyruvate kinase (Somero & Hochachka, 1968) the effect of a fall in temperature on reaction rate can be compensated (at physiological substrate concentrations) by a concomitant increase in enzyme affinity for substrate; on the other hand control of the reaction by feed-forward activation and feedback inhibition is largely temperature-insensitive. To some extent lungfish FDPase resembles trout pyruvate kinase in its temperature characteristics. Thus a decrease in temperature leads to an increase in affinity for FDP, whereas the K_i for AMP is largely independent of temperature. In these organisms, then, evolutionary 'strategy' appears to be directed towards a gain of independence from thermal vagaries of the outer environment.

An alternative evolutionary 'strategy', to take advantage of certain temperature variations, is implicit in the work on pyruvate kinases and lactate dehydrogenases (Somero & Hochachka, 1968; Hochachka & Somero, 1968). At the lower temperature extremes the K_m values for substrates for both enzymes fall dramatically as temperature rises. At normal tissue concentrations of these substrates a small increase in thermal energy in this temperature range can lead to a large increase in the reaction rate. By this means a substantial

enzyme activity is achieved at temperatures only a few degrees above the lower lethal limit for the organism. A similar situation is not found with lungfish liver FDPase. Possibly one would not expect it, for the lungfish, as an evolving species, has not been faced with serious falls in temperature in its environment for many geological ages, if ever.

This work was supported by the National Research Council and Fisheries Research Board of Canada. The lungfish tissues were collected during Cruise B of the Amazon Expedition (1967) of the R/V Alpha Helix (University of California, San Diego); the participation of P. W. H. on this cruise was supported by the National Research Council of Canada and the National Science Foundation, U.S.A.

REFERENCES

- Atkinson, D. E. (1966). *Annu. Rev. Biochem.* **35**, 85.
- Atkinson, D. E. & Walton, G. M. (1967). *J. biol. Chem.* **242**, 3239.
- Behrlich, H. W. & Hochachka, P. W. (1969). *Biochem. J.* **111**, 287.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Geller, A. M. (1967). Ph.D. Thesis: Duke University, Durham, N.C.
- Helmreich, E. & Cori, C. F. (1964). *Proc. nat. Acad. Sci., Wash.*, **52**, 647.
- Hochachka, P. W. (1967). In *Molecular Mechanisms of Temperature Adaptation*, p. 117. Ed. by Prosser, C. L. Washington, D.C.: American Academy of Science Press.
- Hochachka, P. W. (1968). *Comp. Biochem. Physiol.* **25**, 107.
- Hochachka, P. W. & Somero, G. N. (1968). *Comp. Biochem. Physiol.* **27**, 659.
- Horecker, B. L., Pontremoli, S., Rosen, O. & Rosen, S. (1966). *Fed. Proc.* **25**, 1521.
- Janssens, P. A. (1964). *Comp. Biochem. Physiol.* **11**, 105.
- Janssens, P. A. (1965). *Comp. Biochem. Physiol.* **16**, 317.
- Krebs, H. A. (1963). In *Advances in Enzyme Regulation*, vol. 1, p. 385. Ed. by Weber, G. Oxford: Pergamon Press Ltd.
- Lowry, O., Schulz, D. W. & Passonneau, J. V. (1964). *J. biol. Chem.* **239**, 1947.
- Massey, V., Curti, B. & Ganther, H. (1966). *J. biol. Chem.* **241**, 2347.
- Morris, I. (1968). *Biochim. biophys. Acta*, **162**, 467.
- Newsholme, E. A. & Gevers, W. (1967). *Vitam. & Horm.* **25**, 1.
- Preiss, J., Biggs, M. L. & Greenberg, E. (1967). *J. biol. Chem.* **242**, 2292.
- Somero, G. N. & Hochachka, P. W. (1968). *Biochem. J.* **110**, 395.
- Taketa, K. & Pogell, B. M. (1963). *Biochem. biophys. Res. Commun.* **12**, 229.
- Taketa, K. & Pogell, B. M. (1965). *J. biol. Chem.* **240**, 651.
- Trivedi, B. & Danforth, W. H. (1967). *J. biol. Chem.* **241**, 4110.
- Underwood, A. & Newsholme, E. A. (1965). *Biochem. J.* **95**, 767.
- Wallace, J. C. & Newsholme, E. A. (1967). *Biochem. J.* **104**, 378.
- Weber, G., Singhal, R. L. & Srivastava, S. K. (1965). In *Advances in Enzyme Regulation*, vol. 3, p. 43. Ed. by Weber, G. Oxford: Pergamon Press Ltd.
- Williamson, J. R., Cheung, W. Y., Coles, H. S. & Herczeg, B. E. (1967). *J. biol. Chem.* **242**, 5112.