Biochem. J. (1968) **110**, 395
Printed in Great Britain

The Effect of Temperature on Catalytic and Regulatory Functions of Pyruvate Kinases of the Rainbow Trout and the Antarctic Fish *Trematomus bernacchii*

BY G. N. SOMERO AND P. W. HOCHACHKA
Department of Zoology, University of British Columbia, Vancouver 8, B.C., Canada

(*Received* 27 *May* 1968)

1. The effects of temperature on the catalytic and regulatory properties of pyruvate kinases from the temperate-zone rainbow trout and the Antarctic fish *Trematomus bernacchii* were examined. 2. The K_m value of pyruvate kinase for one of its two substrates, phosphoenolpyruvate, is temperature-dependent, and is lowest at temperatures that closely coincide with the habitat temperatures of the two fishes. 3. Two regulatory functions of pyruvate kinase, feedforward activation by fructose diphosphate and feedback inhibition by ATP, are temperature-independent. Enzyme–ADP interaction is also temperature-independent. 4. It is concluded that enzyme–substrate and enzyme–modulator interactions are important factors in short-term and in evolutionary adaptations by poikilotherms to changes in temperature. Though the K_m for substrate may vary in apparently adaptive manners, the regulatory functions of an enzyme appear to be unchanged over the range of temperatures experienced by the organism in Nature.

The effect of temperature on the catalytic and regulatory properties of enzymes of poikilotherms is poorly understood. In studies of mammalian and bacterial enzymes, sharp and frequently differential effects of temperature on these properties have been observed. Often these effects are of such magnitude as to appear incompatible with poikilothermic existence. For example, the interaction of rat liver fructose 1,6-diphosphatase with the negative modulator AMP is so highly temperature-sensitive that the K_i for AMP at 40° is nearly 100 times that at 2° (Taketa & Pogell, 1965). An even more striking example of temperature sensitivity of enzyme–modulator interactions is found with *Escherichia coli* thymidine kinase (Iwatsuki & Okazaki, 1967). At 37°, thymidine 5′-triphosphate inhibits enzymic activity by approx. 75%; at all temperatures below 30°, thymidine 5′-triphosphate activates the enzyme. Enzyme–substrate interactions may also exhibit marked temperature-dependences. The affinity of *E. coli* thymidine kinase for substrate at 20° is about 10 times that at 37° (Iwatsuki & Okazaki, 1967). Comparable changes in enzyme–substrate affinity, as measured by K_m changes, have been observed with other enzymes (Cowey, 1967; Helmreich & Cori, 1964; Himes & Wilder, 1968; Hochachka & Somero, 1968). An important consequence of these K_m changes is that at low, and usually physiological, concentrations of substrate, enzymic activity may be greater at low temperatures than at high temperatures.

To examine the action of temperature on the catalytic and regulatory functions of poikilothermic enzymes, we have initiated studies of several key enzymes involved in glycolysis and gluconeogenesis in fishes. This paper presents the results of a study of PyK* of the temperate-zone rainbow trout (*Salmo gairdnerii*) and of the Antarctic fish *Trematomus bernacchii*.

MATERIALS AND METHODS

Experimental animals. Adult rainbow trout weighing approx. 175g. were kept at temperatures of 4–8°. *Trematomus bernacchii* were captured in McMurdo Sound, Antarctica, by Mr Paul K. Dayton of the University of Washington, and were frozen until used. This species experiences an average habitat temperature of −1·9° (Littlepage, 1965) and has an upper lethal temperature of 6° (Somero & DeVries, 1967). Its metabolic rate is highly cold-adapted (Somero, Giese & Wohlschlag, 1968; Wohlschlag, 1964). Rats were of the Wistar strain.

Preparation of PyK. Fish white dorsal muscle and rat skeletal muscle were homogenized with 4–5 vol. of 0·01 M-tris–HCl buffer, pH 7·4, containing 2mM-EDTA. The crude homogenate was centrifuged at 12000*g* for 20min., and the pellet was discarded. The supernatant was brought to 40%

*Abbreviations: PyK, pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40); FDP, fructose 1,6-diphosphate; PEP, phosphoenolpyruvate.

saturation with solid $(NH_4)_2SO_4$ and stirred for 1 hr. at room temperature. The suspension was then centrifuged as above, the pellet discarded, and the supernatant brought to 75% saturation with solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in a minimal volume of 0·01 M-tris–HCl buffer, pH 7·4. The enzyme was stable at 0–4° for several days, and if frozen was stable for several weeks. Portions of the stock enzyme solution were exhaustively dialysed against 0·01 M-tris–HCl buffer, pH 7·4, before use in the assays.

Assay of PyK activity. The spectrophotometric assay of Bücher & Pfleiderer (1955) was employed. Lactate dehydrogenase activity was coupled to pyruvate formation, and the rate of PyK activity was measured as the decrease in E_{340} due to NADH. Tris–HCl buffers were used in all experiments. Because of the sharp temperature-dependence of the pK of tris, care was taken to adjust the buffers to pH 7·4 at all assay temperatures. The specific compositions of the assay media are given in the Figure legends. Biochemicals were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., with the exception of PEP, which was obtained from Calbiochem, Los Angeles, Calif, U.S.A.

Electrophoresis of PyK. Muscle, liver and brain homogenates were analysed electrophoretically to determine whether tissue-specific isoenzymes of PyK occur in the rainbow trout. These assays were kindly performed by Dr Walter Susor of the University of Washington, by the procedure given by Susor & Rutter (1968).

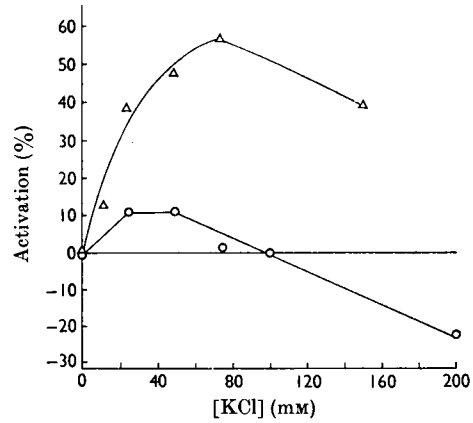

Fig. 1. Effect of K^+ concentration on the reaction velocity of rainbow-trout M-PyK (○) and rat muscle PyK (△). The assay mixture contained, in a total volume of 2·0 ml.: 0·05 M-tris–HCl buffer, pH 7·4; 0·15 mM-NADH; 0·23 mM-ADP; 1 mM-PEP; 8 mM-$MgSO_4$; lactate dehydrogenase (diluted); PyK (added last); and various concentrations of KCl. The assay temperature was 25°.

RESULTS AND DISCUSSION

Isoenzyme forms of rainbow-trout PyK. PyK functions at an important branch point in the glycolytic and gluconeogenic pathways. In mammalian tissues such as muscle, which depend highly on glycolysis, PyK occurs in a form distinguishable from the PyK of gluconeogenic tissues such as liver (Tanaka, Harano, Morimura & Mori, 1965; Tanaka, Harano, Sue & Morimura, 1967*a*; Susor & Rutter, 1968). Similarly, PyK in rainbow trout occurs in at least two forms. White muscle possesses an isoenzyme that exhibits a relatively slow anodal migration, approx. 1·5 cm./hr. on cellulose acetate at 250 v and pH 7·0. This isoenzyme, which we term M-PyK, is activated by FDP. M-PyK was used in all kinetic studies described below. A second isoenzyme, which is found in liver and brain, shows a more rapid anodal migration, approx. 3·1 cm./hr. at 250 v. This isoenzyme, which we term L-PyK, is insensitive to FDP.

The FDP effects on trout M-PyK and L-PyK are opposite to those observed with the mammalian isoenzymes. In the latter system, FDP activates PyK in liver, whereas the muscle isoenzyme is FDP-insensitive (Tanaka *et al.* 1967*a*). Mammalian brain PyK, like trout brain PyK, is FDP-insensitive.

Ion requirements of rainbow-trout M-PyK. PyK of rat and that of the rainbow trout have similar ion requirements. In common with most PyK enzymes, the trout enzyme is activated by K^+, albeit to a far smaller extent than the rat enzyme (Fig. 1). This low K^+-dependence may have adaptive significance, for intracellular K^+ concentrations in trout muscle decrease after exposure to low temperatures (Hickman, McNabb & Nelson, 1964). Wide variation in K^+ activation among species was reported by Boyer (1953).

Trout M-PyK has an absolute Mg^{2+} requirement. The K_a for Mg^{2+} exhibits a definite, albeit irregular, variation with temperature. Minimal K_a (1·5 mM) occurs at 15°; maximal K_a (3·2 mM) is observed at 20°. These K_a values fall within the range reported for mammalian PyK enzymes.

The effect of Ca^{2+} on trout PyK is complex. High concentrations of Ca^{2+} are inhibitory (Fig. 2), a common attribute of PyK enzymes. However, under conditions of low Ca^{2+} and optimum Mg^{2+} concentrations, Ca^{2+} activates the enzyme (Fig. 2). The percentage activation increases as the temperature is lowered, an effect of possible regulatory significance for the organism. The basis of this complex interaction with Ca^{2+} is unclear. However, the data do suggest that Ca^{2+} may bind to sites remote from Mg^{2+}-binding sites.

Effect of temperature on enzyme–substrate interactions and maximal velocities. The PEP-saturation curves for all of the PyK enzymes of this study are hyperbolic at all temperatures and pH values examined. With both fish enzymes, the K_m for PEP varies with temperature in apparently adaptive manners (Fig. 3).

First, in the upper thermal range for the trout

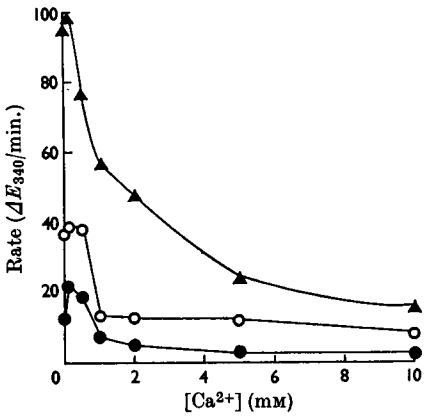

Fig. 2. Effect of Ca^{2+} on the activity of rainbow-trout M-PyK at a series of temperatures: ●, 5°; ○, 12°; ▲, 18°. Assay conditions were the same as described in Fig. 1, except that the concentration of K^+ was 75mM, the concentration of PEP was 2mM, and Ca^{2+} concentrations were varied as indicated.

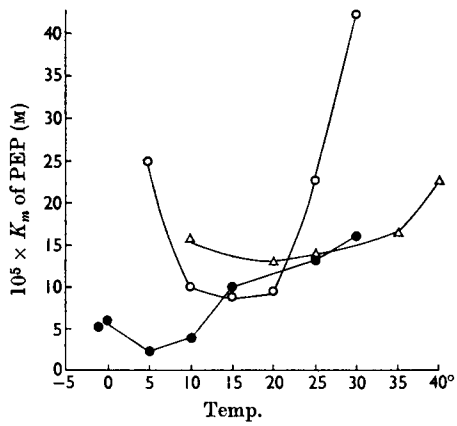

Fig. 3. Effect of temperature on the K_m of PEP of rainbow-trout M-PyK (○), *Trematomus* PyK (●) and rat muscle PyK (△). Assay conditions were as described in Fig. 1, except that the concentration of K^+ was 75mM, and the concentration of PEP was varied over the range 0·01–10mM. The K_m values were determined from double-reciprocal plots ($1/v$ versus 1/[PEP]).

enzyme (10–25°), the direct relationship between temperature and K_m for PEP may act as an important mechanism for stabilizing rates of enzymic activity when the organism experiences rapid changes in habitat temperature (Table 1). Thus, at low substrate concentrations, when the K_m for substrate is important in determining the rate of catalysis, the decrease in K_m that occurs with a

Table 1. *Temperature coefficients, Q_{10}, of the rainbow-trout PyK reaction at high and low concentrations of substrate (PEP)*

Methods were as described in the Materials and Methods section. The rate values on which the temperature coefficients are based are given in Fig. 4.

	Q_{10}	
Temp. range	0·1mM-PEP	1mM-PEP
10–20°	4·2	5·3
15–25	3·3	4·6

fall in temperature decreases the Q_{10} of the reaction by approx. 30%. These results are consistent with observations made on other enzyme systems (Cowey, 1967; Hochachka & Somero, 1968; Zydowo, Makarewicz, Umiastowski & Purzycka, 1965).

Secondly, temperature-dependent K_m changes appear to play an important role in evolutionary adaptation to temperature. The temperature at which the K_m for substrate is lowest, which we define as the 'thermal optimum' of the enzyme, closely coincides with the habitat temperatures of the two fishes. PyK from the rainbow trout has a minimal K_m for PEP near 15°; the *Trematomus* enzyme has a minimal K_m near 0–5° (Fig. 3). The minimal K_m for PEP of trout M-PyK occurs at temperatures that closely approximate to the temperature of optimum performance for this species, 15° (Fry, 1957).

In addition to having a lower temperature optimum, the absolute value of the K_m of PEP of the *Trematomus* enzyme is lower than that of the trout enzyme. Evolutionary adaptation of enzymes to low temperatures may therefore lead to decreases in K_m for substrate as well as to lower thermal optima.

In contrast with the two fish enzymes, rat PyK does not exhibit a minimal K_m near the rat's body temperature (Fig. 3).

A seemingly paradoxical property of the plots of K_m versus temperature in Fig. 3 is the sharp increase in K_m at temperatures several degrees above the minimal temperature encountered by the two fish species (−1·8° for *Trematomus*; 2–4° for the trout). If the temperature-dependent K_m changes discussed above are involved only in promoting stability of the rate of catalytic activity (Table 1), then one would predict that the K_m would continue to decrease with decreasing temperatures down to temperatures near the lower lethal limits for the organisms. Clearly, this is not the case for either PyK (Fig. 3) or for several lactate dehydrogenase isoenzymes we have studied (Hochachka & Somero, 1968).

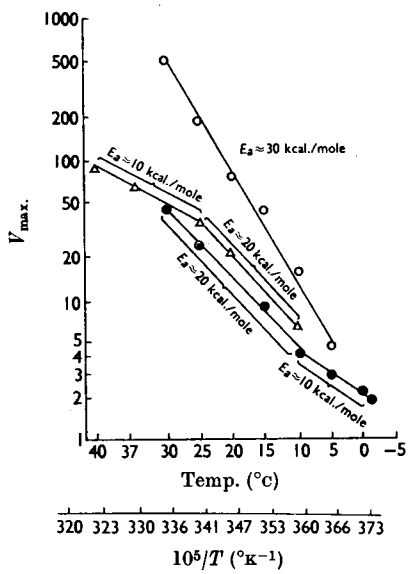

Fig. 4. Arrhenius plots of PyK activity [log $V_{max.}$ versus $1/T$ (°K)] for rainbow-trout PyK (○), *Trematomus* PyK (●) and rat muscle PyK (△). Assay conditions were as described in Fig. 1, except that the concentration of K^+ was held constant at 75mM. $V_{max.}$ values were obtained from plots of $1/v$ versus 1/[PEP].

A possible resolution of this paradox may be related to alterations in metabolic pathway participation at different temperatures. Acclimatization of fishes to different temperatures is known to promote changes in the relative activities of certain metabolic pathways (Ekberg, 1958; Hochachka & Hayes, 1962; Somero *et al.* 1968). At low temperatures the relative participation of glycolysis is decreased, and pentose-shunt activity assumes a quantitatively more important role in carbohydrate metabolism. It is conceivable that these acclimatization phenomena could involve immediate effects of temperature on enzyme–substrate interactions, as well as changes in quantities of enzymes due to altered rates of enzyme synthesis (Smith, 1967). Indeed, the competitive abilities of different metabolic pathways for common intermediates are known to vary with temperature (Hochachka, 1968), and these changes may be related to differential effects of temperature on the K_m values of the enzymes of different pathways.

Arrhenius plots of log $V_{max.}$ versus $1/T$ are presented in Fig. 4. With both fish enzymes, the temperature at which maximum activity occurs lies well beyond the upper lethal temperature of the species, 6° in *Trematomus bernacchii* (Somero & DeVries, 1967) and 25–27° for the trout (Fry & Gibson, 1953).

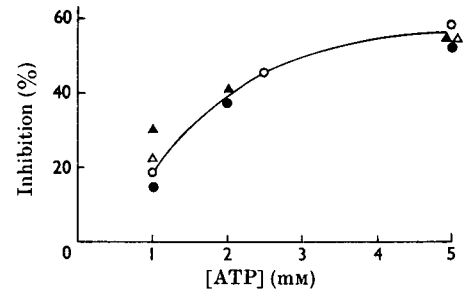

Fig. 5. Inhibition of rainbow-trout M-PyK by various concentrations of ATP at different temperatures: ●, 10°; ○, 15°; ▲, 20°; △, 25°. Assay conditions were as described in Fig. 1, except that the concentration of K^+ was 75mM and the concentration of PEP was 2mM.

Table 2. *FDP activation of rainbow-trout M-PyK at a series of temperatures*

Assay conditions were as described in Fig. 1, except that K^+ concentration was 75mM, and 0·1mM-FDP was added to the assay system. The same enzyme preparation was used in all determinations.

Temp.	Activation (%) 0·5mM-PEP	2mM-PEP
7°	190	—
10	200	180
13	230	—
15	240	195
16	240	210
17	240	—
20	240	190
25	210	200

Activation energies of the PyK reactions are also given in Fig. 4. The significance of activation energies in temperature adaptation has been discussed by several authors (Read, 1964*a*,*b*; Somero *et al.* 1968; Vroman & Brown, 1963). It has been proposed that enzymes from cold-adapted species possess low activation-energy characteristics, and therefore function as highly efficient catalysts. Though a positive correlation between habitat temperature and activation energy does not hold for all enzymes (Read, 1964*a*,*b*; Hochachka & Somero, 1968), a correlation is observed for the two fish PyK enzymes. The activation energy of the pyruvate kinase reaction of *Trematomus* is about one-third that of the trout reaction.

The breaks in the linearity of the Arrhenius plots of the rat and *Trematomus* PyK enzymes exhibit an interesting relationship. At temperatures near the two organisms' body temperatures, the acti-

vation energies of the PyK reactions are approx. 10kcal./mole. At temperatures removed from body temperature, the enzymes are markedly less efficient catalytically, as the activation energies rise to approx. 20kcal./mole. Other workers have reported similar breaks in the Arrhenius plots of rat PyK activity (Kayne & Suelter, 1968).

Effect of temperature on regulatory properties of trout M-PyK. In sharp contrast with the temperature-dependence of PyK–PEP interaction, the K_m for ADP varies by less than 10% (average K_m 0·26mM) over the range 5–25°. The basis for this difference between the two substrates may lie in the fact that the adenylates are common substrates for many enzymes and, in addition, play major roles in regulating enzymic activity (Atkinson & Walton, 1967*a*,*b*). If the activities of many enzymes involved in ATP metabolism are affected by the relative concentrations of the adenylates, then proper metabolic regulation over the range of temperatures encountered by a poikilotherm may require that enzyme–adenylate interactions be temperature-independent.

That such is also the case for PyK–ATP interaction is evident in Fig. 5. The effect of ATP, a competitive inhibitor of PyK, is unchanged over the range of temperatures examined.

A second major regulating mechanism characteristic of a number of PyK enzymes is feedforward activation by FDP (Hess, Haeckel & Brand, 1966; Maeba & Sanwal, 1968; Milman & Yurovitski, 1967; Susor & Rutter, 1968; Taylor & Bailey, 1967). For trout M-PyK, the K_a for FDP at 15° is approx. 2μM. FDP lowers the K_m for PEP and also increases the $V_{max.}$ of the reaction. The relative effect on K_m is greater; 0·1mM-FDP can produce up to 16-fold activation of the enzyme at low (0·01mM) concentrations of PEP. FDP also completely reverses ATP inhibition. Similar findings have been reported for the PyK enzymes of loach embryos (Milman & Yurovitski, 1967) and rat liver (Tanaka, Sue & Morimura, 1967*b*).

FDP-sensitivity of the enzyme varied among preparations, an observation also reported by Susor & Rutter (1968). We have no information on the basis of this variability.

FDP activation of trout M-PyK shows no pH-dependence over the range pH 6·5–7·5, and is affected little by temperature between 7° and 25° (Table 2). Although activation increases by approx. 20% between 7–10° and 20°, this change is small compared with the large temperature effects on regulatory properties observed with other enzymes (Ingraham & Maaløe, 1967; Iwatsuki & Okazaki, 1967; Taketa & Pogell, 1965; Worcel, 1966). The temperature-independence of the regulatory properties of trout M-PyK suggest that feedforward and feedback controls of enzymic activity are equally effective at all temperatures that this species is likely to encounter in Nature.

Stability of enzyme activity in poikilotherms therefore would appear to be promoted by two distinct thermal properties: (1) temperature-dependent enzyme–substrate interactions, and (2) temperature-independent enzyme–modulator interactions, particularly enzyme–adenylate interactions.

The authors are indebted to Mrs Myrna Bosomworth for her excellent technical assistance. These studies were supported by the National Research Council of Canada. G. N. S. is a National Science Foundation Post-Doctoral Fellow.

REFERENCES

Atkinson, D. E. & Walton, G. M. (1967*a*). *J. biol. Chem.* **242**, 3239.
Atkinson, D. E. & Walton, G. M. (1967*b*). *J. biol. Chem.* **242**, 3241.
Boyer, P. D. (1953). *J. cell. comp. Physiol.* **42**, 71.
Bücher, T. & Pfleiderer, G. (1955). In *Methods in Enzymology*, vol. 1, p. 435. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Cowey, C. B. (1967). *Comp. Biochem. Physiol.* **23**, 969.
Ekberg, D. R. (1958). *Biol. Bull., Wood's Hole*, **114**, 308.
Fry, F. E. J. (1957). In *Physiology of Fishes*, vol. 1, p. 1. Ed. by Brown, M. W. New York: Academic Press Inc.
Fry, F. E. J. & Gibson, M. B. (1953). *J. Hered.* **44**, 56.
Helmreich, E. & Cori, C. F. (1964). *Proc. nat. Acad. Sci., Wash.*, **52**, 647.
Hess, B., Haeckel, R. & Brand, K. (1966). *Biochem. biophys. Res. Commun.* **24**, 824.
Hickman, C. P., jun., McNabb, R. A.& Nelson, J. S. (1964). *Canad. J. Zool.* **42**, 577.
Himes, R. H. & Wilder, T. (1968). *Arch. Biochem. Biophys.* **124**, 230.
Hochachka, P. W. (1968). *Comp. Biochem. Physiol.* **25**, 107.
Hochachka, P. W. & Hayes, F. R. (1962). *Canad. J. Zool.* **40**, 261.
Hochachka, P. W. & Somero, G. N. (1968). *Comp. Biochem. Physiol.* (in the Press).
Ingraham, J. L. & Maaløe, O. (1967). In *Amer. Ass. Advanc. Sci. Publ. no.* 84: *Molecular Mechanisms of Temperature Adaptation*, p. 77. Ed. by Prosser, C. L. Baltimore: Horn–Shafer.
Iwatsuki, N. & Okazaki, R. (1967). *J. molec. Biol.* **29**, 155.
Kayne, F. J. & Suelter, C. H. (1968). *Biochemistry*, **7**, 1968.
Littlepage, J. L. (1965). In *Biolcgy of the Antarctic Seas*, vol. 2, p. 1. Ed. by Lee, M. O. Washington: American Geophysical Union.
Maeba, P. & Sanwal, B. D. (1968). *J. biol. Chem.* **243**, 448.
Milman, L. S. & Yurovitzki, Yu. G. (1967). *Biochim. biophys. Acta*, **146**, 298.
Read, K. R. (1964*a*). *Biol. Bull., Wood's Hole*, **127**, 489.
Read, K. R. (1964*b*). *Grad. Sch. Oceanogr. Univ. R.I. Publ. no.* 2: *Proc. Symp. exp. Marine Ecol.* p. 39.
Smith, M. W. (1967). *Biochem. J.* **105**, 65.
Somero, G. N. & DeVries, A. L. (1967). *Science*, **156**, 257.
Somero, G. N., Giese, A. C. & Wohlschlag, D. E. (1968). *Comp. Biochem. Physiol.* **26** 223.

Susor, W. A. & Rutter, W. J. (1968). *Biochem. biophys. Res. Commun.* **30**, 14.
Taketa, K. & Pogell, B. M. (1965). *J. biol. Chem.* **240**, 651.
Tanaka, T., Harano, Y., Morimura, H. & Mori, R. (1965). *Biochem. biophys. Res. Commun.* **21**, 55.
Tanaka, T., Harano, Y., Sue, F. & Morimura, H. (1967*a*). *J. Biochem., Tokyo*, **62**, 71.
Tanaka, T., Sue, F. & Morimura, H. (1967*b*). *Biochem. biophys. Res. Commun.* **29**, 444.
Taylor, C. B. & Bailey, E. (1967). *Biochem. J.* **102**, 32c.
Vroman, H. E. & Brown, J. R. C. (1963). *J. cell. comp. Physiol.* **61**, 129.
Wohlschlag, D. E. (1964). In *Biology of the Antarctic Seas*, vol. 1, p. 33. Ed. by Lee, M. O. Washington: American Geophysical Union.
Worcel, A. (1966). *Biochim. biophys. Acta*, **113**, 178.
Zydowo, M., Makarewicz, W., Umiastowski, J. & Purzycka, J. (1965). *Acta biochim. pol.* **12**, 319.