BBA 66675

RAT THYMOCYTE PHOSPHOFRUCTOKINASE: SOME KINETIC PROPERTIES COMPARED WITH THOSE OF MUSCLE PHOSPHOFRUCTOKINASE*

TAKESHI YAMADA[a] AND HARUMI OHYAMA[b]

[a]*Division of Biology,* [b]*Division of Radiation Health, National Institute of Radiological Sciences, Chiba (Japan)*

(Received March 15th, 1972)

SUMMARY

Phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) was extracted from rat thymocytes and partially purified by the following steps: ammonium sulfate fractionation, heat treatment, TEAE-cellulose column chromatography and affinity chromatography on blue dextran polyacrylamide gel. Some properties of the enzyme thus obtained were compared with these of muscle phosphofructokinase. Thymocyte phosphofructokinase showed a higher affinity for TEAE cellulose, higher susceptibility to ATP inhibition, and greater sensitivity to allosteric effectors than the muscle enzyme. The concentration of fructose 6-phosphate required to give half-maximal velocity was higher for the thymocyte enzyme.

INTRODUCTION

It is well established that phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) plays an important role in the regulation of carbohydrate metabolism in different mammalian tissues[1–3]. We have recently demonstrated that the enzyme is also one of the most important enzymes in the regulation of glycolysis in thymocytes, and its regulatory properties are profoundly affected by X-ray irradiation, that is ,aerobic glycolysis is markedly stimulated by low doses of X-rays as a result of the enhancement of the phosphofructokinase reaction[4–6]. Chapman *et al.*[7] have shown that the radiation-induced decrease in allosteric activity is due to a reduced enzyme sensitivity to ATP inhibition in the irradiated enzyme prepared from rabbit muscle. Recently, Chumachenko *et al.*[8] have reported increased specific activity of phosphofructokinase in muscle homogenate prepared from an irradiated rabbit.

Abbreviation: TEAE, *O*-(triethylaminoethyl)-.

* Contribution No. 230 from the Division of Biology, National Institute of Radiological Sciences, Chiba, Japan.

In the course of investigation of the effect of X-rays on thymocyte phosphofructokinase, it became necessary to obtain purified thymocyte phosphofructokinase. This paper describes the partial purification of this enzyme and results of TEAE-cellulose column chromatography and kinetic studies as compared with those obtained from the muscle phosphofructokinase.

MATERIALS AND METHODS

Animals

Male albino rats of Wistar strain aged 2 to 3 months from our breeding colony were used for the experiments.

Chemicals and enzymes

Rabbit muscle aldolase (EC 4.2.1.13), triosephosphate isomerase (EC 5.3.1.1) and α-glycerophosphate dehydrogenase (EC 1.1.1.8), cyclic AMP were obtained from Boehringer, Mannheim. NADH was purchased from Oriental Yeast Co. Ltd., Tokyo. The potassium salt of fructose 6-phosphate (fructose 1,6-diphosphate free), AMP, ADP and dithiothreithol were from Sigma. ATP was purchased from Kojin Co. Ltd., Tokyo, and purified by DEAE cellulose column chromatography as follows. A solution of ATP (200 mg in 10 ml distilled water)was applied to the DEAE cellulose (Serva, $-Cl^-$ type) column (1 cm × 6 cm). After removal of ADP and AMP by elution of 0.005 M HCl, ATP was eluted by 0.1 M HCl containing 0.1 M NaCl, and then immediately neutralized by NaOH. AMP and ADP remaining in the purified ATP fraction were less than 0.01% of ATP.

Measurement of enzyme activity

Phosphofructokinase was assayed by coupling with excess aldolase, triosephosphate isomerase and α-glycerophosphate dehydrogenase and following the rate of oxidation of NADH at 340 nm at 25 °C, using a Gilford model 240 multiple sample absorbance recording spectrophotometer. Units of activity were defined as the number of μmoles of fructose 1,6-diphosphate produced per min under the standard conditions. The standard assay mixture contained in a total volume of 1.0 ml, 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 1 mM EDTA, 0.05 mM NADH, 1 mM fructose 6-phosphate, 1 mM ATP, 10 mM mercaptoethanol, 0.01 mg α-glycerophosphate dehydrogenase, 0.01 mg triosephosphate isomerase, 0.05 mg aldolase and the phosphofructokinase preparation. For kinetic studies, the assay medium was modified to contain 50 mM imidazole–HCl buffer, pH 6.9 or 7.2, or 50 mM Tris–HCl buffer, pH 8.0, 0.05 mM NADH, 1 mM dithiothreithol, 5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, the same amount of the auxillary enzymes as the standard system (freed from $(NH_4)_2SO_4$ by a high speed centrifugation at 12 000 × rev./min), ATP and fructose 6-phosphate in final concentrations as indicated at figures and tables. By a preliminary experiment, it was found that $(NH_4)_2SO_4$ had a marked stimulative effect on the enzyme activity under the conditions used. Therefore, 5 mM $(NH_4)_2SO_4$ was added to the assay medium after the removal of the salt from the auxillary enzymes as mentioned above in order to maintain a constant concentration of $(NH_4)_2SO_4$. All reactions were started by the addition of fructose 6-phosphate after allowing 3 min for temperature equili-

bration at 25 °C. All experiments comparing the kinetics of muscle and thymocyte phosphofructokinase were carried out with approx. 0.01 unit of enzyme in each assay.

Preparation of blue dextran polyacrylamide gel column for affinity chromatography

Blue dextran immobilized by fixation in cross-linked polyacrylamide gel was prepared according to the slightly modified method of Kopperschläger *et al.*[9]. Polymerization was performed in 100 ml 50 mM phosphate buffer (pH 7.3) containing 9.5 g acrylamide, 0.5 g *N,N'*-methylene-bis-acrylamide, 1.3 g blue dextran (Pharmacia), 1 g *N,N,N',N'* tetramethylethylendiamine, 0.3 g $(NH_4)_2S_2O_8$. After polymerization the gel was pulverized by passing through a stainless steel mesh (100 mesh), washed with 50 mM phosphate buffer and then packed to a column (1.5 cm × 6 cm).

Protein determination

Protein determination in the purified steps was performed by the method of Lowry *et al.*[10] with bovine serum albumin as a standard.

RESULTS

Preparation of thymocyte phosphofructokinase

Step 1. Extraction. Thymus glands were removed from about 50 rats and pooled in ice-cold Krebs–Ringer phosphate buffer (pH 7.4). All further operations were performed at 0–4 °C unless otherwise stated. The pooled tissues were then minced with scissors and filtered through a platinum mesh. The thymocyte pellet obtained by a low speed centrifugation was resuspended in a 5-fold volume of the extraction medium containing 0.03 M Tris phosphate, pH 8.0, 1 mM EDTA, and was then homogenized with a Waring blender. The resulting homogenate was centrifuged at 60 000 × *g* for 40 min after adjusting pH to 8.0.

Step 2. Ammonium sulfate fractionation. The supernatant fluid was then fractionated with $(NH_4)_2SO_4$. The precipitate obtained between 35–50% saturation was dissolved in the dilution medium containing 25 mM Tris–HCl, pH 8.0, 25 mM $(NH_4)_2SO_4$, 1 mM EDTA, 0.1 mM mercaptoethanol, 0.1 mM ATP and 0.01 mM fructose 1,6-diphosphate (Solution A).

Step 3. Heat treatment. The enzyme solution was then immersed in a water bath and kept at 55 °C for 3 min.

Step 4. TEAE-cellulose chromatography. After removal of the precipitate, the heat-treated supernatant was applied to a Sephadex G-25 column previously equilibrated with Solution A to remove the remaining $(NH_4)_2SO_4$. The fractions containing phosphofructokinase activity eluted by the same medium were combined and applied to a TEAE-cellulose column previously equilibrated with the same buffer. Elution was carried out with a solution containing linear gradients of both $(NH_4)_2SO_4$ (0.025 to 0.2 M) and Tris–HCl buffer (0.025 to 0.05 M, pH 8.0). There were two peaks (see Fig. 1, thymocyte) the first, a small sharp peak, was eluted in fractions 29 to 34, followed by the major broad peak of the activity of the fractions 38 to 60. The latter main portion of the activity peak of thymocyte phosphofructokinase, which accounted for about 70% of the total activity, was pooled and concentrated, and dialyzed against 50 mM phosphate buffer, pH 7.1, containing 0.5 mM EDTA and 5 mM mercaptoethanol.

Step 5. Blue dextran polyacrylamide gel chromatography. The dialyzed TEAE-chromatographed fraction was applied to the blue dextran polyacrylamide gel column. The column was then washed with the phosphate buffer. The enzyme eluted with a gradient of 0.025 M to 0.5 M $(NH_4)_2SO_4$ in a buffer containing 25 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.1 mM ATP, 0.01 mM fructose 1,6-diphosphate. The fractions containing the enzyme were combined, concentrated and dialyzed against Solution A again. The enzyme that was carried through the above steps is referred to as the purified thymocyte phosphofructokinase and was used for the kinetic studies described below.

A summary of the purification data obtained for a typical preparation is given in Table I.

TABLE I

PURIFICATION OF RAT THYMOCYTE PHOSPHOFRUCTOKINASE

About 50 thymus glands were used for the extraction. The standard assay system was used for the assay of all fractions.

Fraction	*Vol. (ml)*	*Activity (units/ml)*	*Protein (mg/ml)*	*Spec. act. (units/mg)*
Crude extract	88	0.42	3.1	0.13
$(NH_4)_2SO_4$ fraction	15	1.75	6.4	0.27
Heat treatment	15	1.67	2.1	0.79
TEAE-cellulose	1.8	4.14	0.7	5.9
Blue dextran column	2.0	2.2	0.048	46

Preparation of muscle phosphofructokinase

Rat muscle phosphofructokinase was prepared by the method of Tanaka *et al.*[11] including TEAE-cellulose chromatography, and then further purified by the blue dextran polyacrylamide gel affinity chromatography in a similar manner, described in the preparation of thymocyte enzyme to give a final activity of 176 units per mg.

Affinity for TEAE-cellulose

For comparison, in the upper part of Fig. 1 is shown the behaviour of muscle phosphofructokinase chromatographed under identical conditions. Muscle phosphofructokinase was eluted earlier in a relatively narrow peak, while thymocyte enzyme was eluted later in two peaks mentioned already, which began in the falling portion of the muscle peak. Rechromatography of each enzyme gave an elution profile identical to its original, as shown in Fig. 1. Therefore, thymocyte phosphofructokinase seemed to possess a higher affinity for TEAE-cellulose than the muscle enzyme. The existence of chromatographically distinguishable forms of phosphofructokinase has also recently been reported for rat[12], rabbit[13], and human tissues[14].

Sensitivity to ATP inhibition

Fig. 2 shows the response of both phosphofructokinases to increasing concentrations of ATP at pH 6.9, 7.2, and 8.0 in the presence of 0.1 mM fructose 6-phosphate. The difference in sensitivity to the inhibition by ATP between both enzymes is appa-

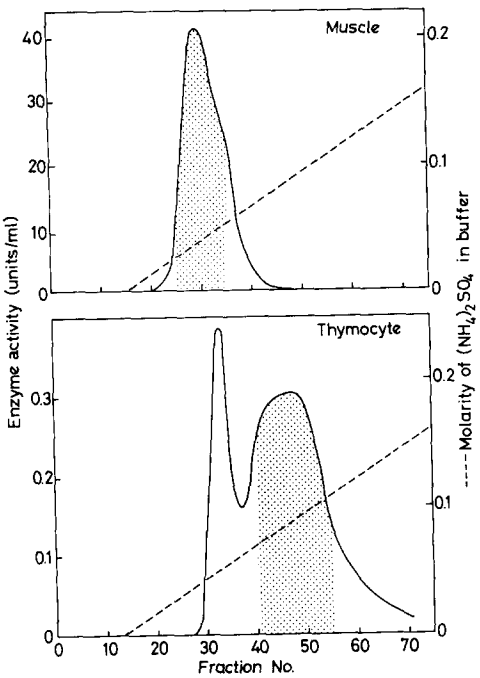

Fig. 1. TEAE-cellulose column chromatography of rat thymocyte and muscle phosphofructokinase. The curves represent separate runs of muscle (upper) and thymocyte (lower) phosphofructokinase on a column (1.5 cm × 15 cm) of TEAE-cellulose. The detailed procedures are described in the text. The flow rate was 20 ml/h. 4-ml fractions were collected. Activity was expressed as units per ml. The fractions shadowed with dots were collected for further purification procedures.

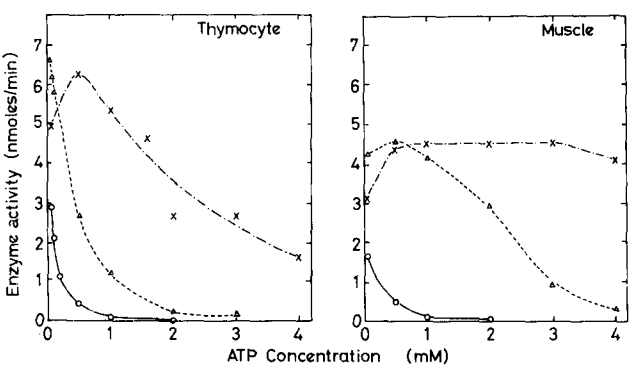

Fig. 2. ATP inhibition of rat thymocyte (left) and muscle (right) phosphofructokinase. The assay mixture for kinetic studies (see text) was used. Enzyme activity is expressed as nmoles of fructose 1,6-diphosphate produced per min under the conditions used. ○—○, pH 6.9; △---△, pH 7.2; ×–·–×, pH 8.0.

rent in the figure. The thymocyte phosphofructokinase was inhibited by ATP in lower concentrations than that required to inhibit the muscle enzyme to a same extent at pH 7.2. For example, the concentrations required to reduce the velocity to half that at optimum ATP concentration were 2 mM for muscle enzyme and

0.4 mM for thymocyte phosphofructokinase at pH 7.2. Whereas the enzyme from muscle was not inhibited by ATP at pH 8.0, the thymocyte enzyme was inhibited even at that pH.

The effect of 0.1 mM cyclic AMP on both enzymes was tested, since adenine nucleotides as such are known to relieve the ATP inhibition[15] (Fig. 3). Although cyclic AMP increased the activity of both enzymes, remarkable stimulation was observed with the thymocyte enzyme, especially at pH 7.2, where the activity of the enzyme could be almost completely relieved from ATP inhibition at any concentration of ATP used. It can been seen also from Fig. 3 that the maximal activity achieved by cyclic AMP stimulation with the thymoctye phosphofructokinase is higher than that observed with the same amount (0.01 unit) of the muscle enzyme at any pH used.

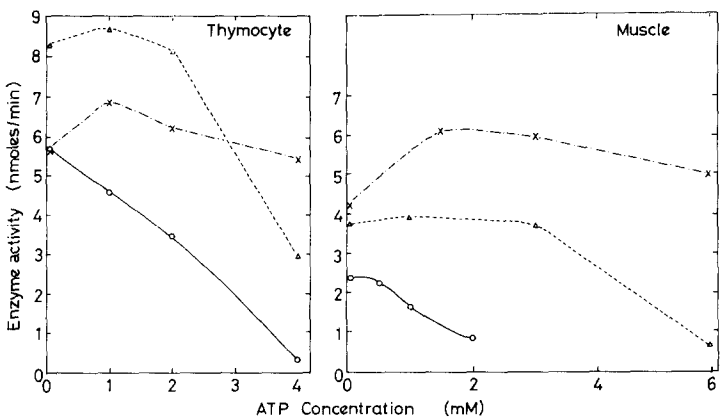

Fig. 3. Release from ATP inhibition by cyclic AMP. Left: rat thymocyte phosphofructokinase, right: muscle phosphofructokinase. Assay conditions were identical with those described in the legend to Fig. 2, except that 0.1 mM cyclic AMP was added. Enzyme activity was expressed as nmoles of fructose 1,6-diphosphate produced per min under the conditions used. ○—○, pH 6.9; △---△, pH 7.2; ×-·-×, pH 8.0.

The effects of varying concentrations of ADP, AMP, and cyclic AMP on thymocyte and muscle phosphofructokinase are shown in Fig. 4. Muscle phosphofructokinase required about 8 to 10 times higher concentrations of these activators to achieve rate increases comparable to those elicited by the nucleotides with thymocyte enzyme. With both enzymes, cyclic AMP at saturation increased the activity to a higher level than that achieved with saturating levels of AMP and ADP. The maximal activity of the thymocyte enzyme achieved by saturating concentrations of AMP and ADP as well as cyclic-3',5'-AMP was higher than that of the muscle enzyme of an equal amount (0.01 unit) by more than a factor of two. From the above results it seems likely that the thymocyte phosphofructokinase is more susceptible to ATP inhibition and more sensitive to nucleotide activation.

Kinetics for fructose 6-phosphate

When the activity was determined at a given level of ATP with varying levels of fructose 6-phosphate, a sigmoid response curve typical of allosteric enzymes was

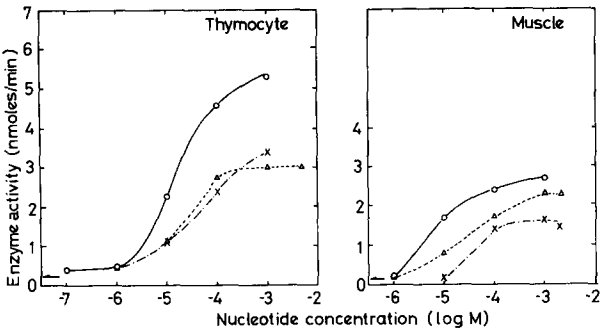

Fig. 4. Activation of phosphofructokinase by adenine nucleotides. Left: rat thymocyte phosphofructokinase, right: muscle enzyme. Assay conditions were identical with those described in the legend to Fig. 2, except that ATP concentration was 1 mM, pH 6.9, and AMP, ADP or cyclic AMP was added to give a final concentration indicated on the abscissa. The points on ordinates indicated by an arrow (←) represent the activity levels assayed without any addition of the nucleotide activator. Enzyme activity was expressed as nmoles of fructose 1,6-diphosphate produced per min under the conditions used. ○—○, cyclic AMP; △---△, AMP; ×-·-×, ADP.

TABLE II

KINETIC DATA FOR FRUCTOSE 6-PHOSPHATE

Assays were carried out at pH 6.9, 7.2 and 8.0 with varying concentrations of fructose 6-phosphate and with ATP as indicated. Other conditions were the same as described in the legend to Fig. 2. The data obtained were plotted as log fructose 6-phosphate concn *versus* log $v/V-v$. The interaction coefficient (n) was obtained from the slope of the plotted data. K' is the concentration (in mM) of fructose 6-phosphate required to achieve half-maximal velocity.

ATP concn (mM)	*Enzyme*	*pH*					
		6.9		*7.2*		*8.0*	
		K'	n	K'	n	K'	n
0.05	Thymocyte	0.12	1.7	0.063	1.5	0.036	1.2
	Muscle	0.063	0.7	0.040	1.0	0.015	1.2
1	Thymocyte	0.75	1.8	0.28	1.8	0.05	1.5
	Muscle	0.27	1.2	0.047	0.9	0.015	1.2

observed with both the thymocyte and the muscle enzyme. A Hill plot[16] of such data gave straight lines from which the concentration of substrate required to give half-maximal velocity (fructose 6-phosphate$_{0.5}$) and the interaction coefficient, which is presumed to be determined by the number of interacting sites for fructose 6-phosphate and the strength of interaction among them could be calculated. These data are given in Table II. With both enzymes the fructose 6-phosphate$_{0.5}$ and the interaction coefficient increased by increasing ATP concentration from 0.05 to 1 mM. At both levels of ATP, the concentration of fructose 6-phosphate required to give half-maximal velocity was higher for the thymocyte phosphofructokinase by a factor of two or more. While little difference in interaction coefficients could be observed at 0.05 mM ATP between the two enzymes, there appeared to be considerable difference between the coefficients of the two enzymes at a higher ATP concentration of 1 mM. The coefficient is approx. 2 for the thymocyte phosphofructokinase, whereas the

value for the musle enzyme is about 1. It is possible from these results that the affinity of muscle enzyme for fructose 6-phosphate is higher than that of the thymocyte phosphofructokinase, and the cooperative interaction among the fructose 6-phosphate binding sites is stronger for the thymocyte phosphofructokinase. The value of fructose 6-phosphate$_{0.5}$ decreased with increasing pH for both enzymes. The enzymes, when assayed at pH 8.0 with 0.05 mM ATP, exhibited approximate Michaelis–Menten kinetics.

DISCUSSION

Tanaka *et al.*[11] have recently demonstrated that at least four types of phosphofructokinase from various tissues of the rat can be distinguished from each other on the basis of immunological properties and by TEAE-cellulose chromatography under the identical conditions employed in this experiment. These enzymes were named type I, II, III, and IV in order of their elution from the cellulose column. In agreement with the results of Tanaka *et al.*[11], a distinct difference between thymocyte and muscle phosphofructokinase was observed in the elution profile on TEAE-cellulose column chromatography (Fig. 1). Muscle enzyme could be eluted earlier from the column, at the identical fractions reported by Tanaka *et al.*[11] who named the muscle phosphofructokinase "Type I" enzyme. The first, minor peak of thymocyte enzyme corresponds to the Type II enzyme and the second, major peak can be identified as Type III by comparison with the results of Tanaka *et al.*[11]. Accordingly, rat thymocytes mainly contain the Type III isozyme of phosphofructokinase and the type II enzyme as a minor fraction.

The most interesting results which were obtained in this experiment are the higher susceptibility of thymocyte Type III enzyme to ATP inhibition and the greater sensitivity to allosteric activators such as adenine nucleotides (Figs 2–4). These results are in agreement with the observation of Kemp[13], who also found that muscle phosphofructokinase from rabbit was less sensitive to ATP inhibition than the liver enzyme. The greater sensitivity of thymocyte enzyme to ATP inhibition should be related to the observations that lower levels of ATP[4,6] have been reported for thymocytes than muscle[17] and heart[18]. Thymocyte phosphofructokinase, therefore, may be very sensitive to the allosteric regulation by the ATP level at the physiological concentration in its tissue (2 to 3 mM)[4,6].

Kinetic studies with fructose 6-phosphate revealed several interesting properties of the thymocyte phosphofructokinase. It is of particular interest to note that the half-maximal concentration of fructose 6-phosphate (0.28 mM) at pH 7.2 and 1 mM ATP was almost equal to its intracellular concentration of 0.27 mM (ref. 4). This means that the activity of thymocyte phosphofructokinase might be well regulated by a minor change in fructose 6-phosphate within the cell.

Data on nucleotide activation suggest that the intracellular levels of ADP (0.2 mM)[4,6] and AMP (1.9 mM)[4,6] are high enough to affect thymocyte phosphofructokinase. Thus, it is feasible to assume that the thymocyte phosphofructokinase is well regulated by ATP and fructose 6-phosphate and readily responds to a minor fluctuation of adenine nucleotide activators under physiological conditions.

Investigations of X-ray effects on phosphofructokinase so far performed with the muscle enzyme have demonstrated a marked action of X-ray irradiation against

the allosteric properties of the enzyme[7,8]. The results of the present investigation, which showed the stronger allosteric properties of thymocyte phosphofructokinase, suggest that the effect of X-rays on thymocyte phosphofructokinase might be more profound than observed with the muscle enzyme, and therefore deserves a further investigation.

ACKNOWLEDGEMENT

The authors wish to thank professor T. Tanaka and Dr T. An, Osaka University Medical School, for their generous help and encouragement.

REFERENCES

1 M. C. Scrutton and M. F. Utter, *Annu. Rev. Biochem.*, 37 (1968) 249.
2 J. V. Passonneau and O. H. Lowry, *Adv. Enzyme Regul.*, 2 (1964) 265.
3 T. E. Mansour, *Adv. Enzyme Regul.*, 8 (1969) 37.
4 H. Ohyama, T. Yamada, T. Kumatori and S. Minakami, *Int. J. Radiat. Biol.*, 13 (1967) 457.
5 T. Yamada and H. Ohyama, *Int. J. Radiat. Biol.*, 14 (1968) 169.
6 T. Yamada, H. Ohyama, T. Kumatori and S. Minakami, *Int. J. Radiat. Biol.*, 15 (1969) 497.
7 A. Chapman, T. Sanner and A. Pihl, *Biochim. Biophys. Acta*, 178 (1969) 74.
8 Y. V. Chumachenko, P. D. Dvornikova, L. S. Lototskaya, S. P. Matsui, T. T. Volodina and M. F. Gulyi, *Radiobiologyia (U.S.S.R.)*, 11 (1971) 846.
9 G. Kopperschläger, W. Diezel, R. Freyer, S. Liebe and E. Hofmann, *Eur. J. Biochem.*, 22 (1971) 40.
10 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
11 T. Tanaka, T. An and Y. Sakaue, *J. Biochem. Tokyo*, 69 (1971) 609.
12 C. B. Taylor and M. Bew, *Biochem. J.*, 119 (1970) 797.
13 R. G. Kemp, *J. Biol. Chem.*, 246 (1971) 245.
14 R. B. Layzer, L. P. Rowland and W. J. Band, *J. Biol. Chem.*, 244 (1969) 3823.
15 K. Uyeda and E. Racker, *J. Biol. Chem.*, 240 (1965) 4682.
16 A. V. Hill, *J. Physiol.*, 40 (1910) 4 p.
17 C. J. Threlfall and H. B. Stoner, *Br. J. Exp. Path.*, 38 (1957) 339.
18 J. R. Williamson, *J. Biol. Chem.*, 246 (1971) 245.