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CHARACTERIZATION AND PARTIAL PURIFICATION OF DIHYDROXYACETONE KINASE IN *DUNALIELLA SALINA*

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Summary

Dihydroxyacetone kinase from *Dunaliella salina* is stabilized against inactivation by maintainance in the presence of 2 M glycerol. In the stabilized form a two-step purification procedure resulted in an enzyme preparation of about 440-fold purity which gave three bands (78 000—100 000 daltons) in the absence of denaturing agents on a polyacrylamide gel.

The enzyme is specific for dihydroxyacetone and Mg^{2+}-ATP complex as its substrates. It has a sharp pH activity curve with a pH optimum around 7.5 and little activity below 6.

It is suggested that dihydroxyacetone kinase plays a central role in the mechanism of osmoregulation via glycerol in *Dunaliella*.

Introduction

The unicellular green algae of the genus *Dunaliella* are among the few living organisms with the capacity for growth in a broad range of NaCl concentrations. By varying its internal concentration of glycerol this alga can endure drastic changes in the osmotic pressure of its external environment [1,2]. It is therefore required that *Dunaliella* provide a metabolic pathway by which glycerol can be rapidly accumulated and removed.

Two novel enzymes, which are most likely involved in glycerol metabolism [3], have been described in *Dunaliella*. The first is dihydroxyacetone reductase (glycerol:$NADP^+$ 2-oxidoreductase (dihydroxyacetone-forming), EC 1.1.1.156)

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Abbreviation: Tricine, *N*-Tris(hydroxymethyl)methylglycine.

[4—6] which mediates the interconversion of glycerol and dihydroxyacetone via the reduction and oxidation of $NADP^+$ and NADPH, respectively. This enzyme is distinct from the enzyme glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate:(acceptor) oxidoreductase, EC 1.1.99.5), which has also been briefly reported to be present in *Dunaliella* [3]. The second novel enzyme of this algal genus is dihydroxyacetone kinase [7]. This enzyme catalyzes the phosphorylation of dihydroxyacetone by ATP. Since dihydroxyacetone kinase was found to be rather unstable, only partial purification was possible due to the rapid loss of activity.

In this study we report on the stabilization of dihydroxyacetone kinase, its purification and its further characterization.

Materials and Methods

All chemicals were of reagent grade quality. The following chemicals were obtained from Sigma Chemical Company:$NADP^+$; NADPH; ATP (grade I); GTP (Type I); dithiothreitol; dihydroxyacetone; DL-glyceraldehyde-3-phosphoric acid; α-glycerophosphate dehydrogenase (Type I); triose phosphate isomerase (Type I). Glyceraldehyde was purchased from Calbiochem. DEAE-cellulose (DE52) and Sephadex G-200 (superfine) were obtained from Whatman and Pharmacia, respectively.

Dunaliella salina or *parva* were grown as previously described [2]. The cells were harvested and the enzyme purified as summarized in Table I: 8 l *D. salina* culture, autotrophically grown in 3 M salt were concentrated by centrifugation at 2000 × *g* for 30 min at room temperature and washed twice in 3 M NaCl/10 mM Tricine (pH 7.5) by centrifugation at 6000 × *g* for 10 min. After cooling on ice for 10 min, the cells were subjected to osmotic shock with 9 vols. ice-cold 5 mM Tricine (pH 7.5), the mixture kept on ice and mixed for 10 min ('cell fragments'). From this point on all operations were carried out at 4°C. Glycerol was added to a final concentration of 2 M, and the solution was centrifuged at 48 000 × *g* for 15 min to obtain the 'cell-free extract'. The pellet of broken cells was washed once with 9 vols. 2 M glycerol/5 mM Tricine (pH 7.5), and the wash added to the extract.

The extract was diluted with 3 vols. 0.5 M glycerol/5 mM Tricine (pH 7.5) and applied to a 2.1 × 29 cm column of DEAE-cellulose (Whatman DE52) pre-equilibrated with 0.5 M glycerol/50 mM NaCl/5 mM Tricine (pH 7.5).

The column was washed with the equilibration buffer until no protein appeared in the eluted buffer followed by a linear gradient of 50—150 mM NaCl in 0.5 M glycerol/5 mM Tricine (pH 7.5). The activities of dihydroxyacetone reductase and dihydroxyacetone kinase and the protein concentrations were determined (Fig. 1) for the collected fractions. The former was completely eluted during the application of the extract and the washing of the column. The elution peak of dihydroxyacetone kinase specific activity were pooled, diluted with 2 vols. 0.5 M glycerol/5 mM Tricine (pH 7.5) and applied to a 1 ml DEAE-cellulose (Whatman DE52) column equilibrated with the same buffer in order to concentrate the protein. The protein was eluted in 1-ml fractions with the above buffer containing 0.3 M NaCl. Glycerol was immediately added to the eluted fractions to a final concentration of 2 M. The active frac-

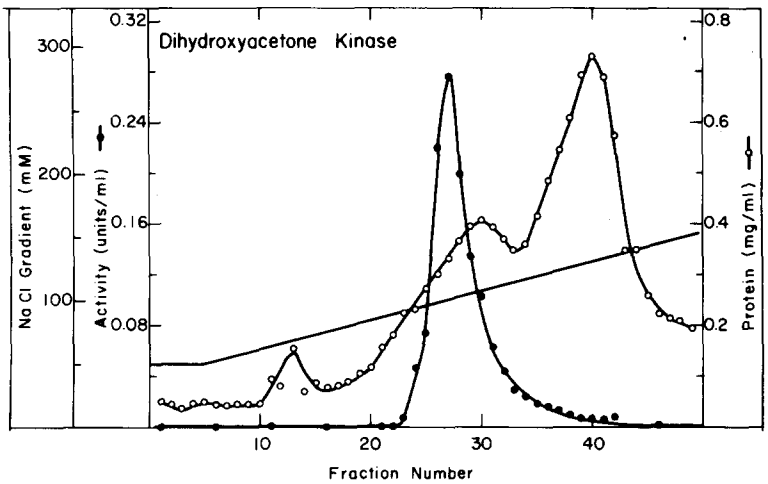


Fig. 1. Elution profile of dihydroxyacetone kinase from a DEAE-cellulose column. Elution volume measurements began with the application of the NaCl gradient. Volume per fraction was 11 ml. The fractions were collected at an elution rate of 1.4 ml/min.

tions were pooled. Using the method of Sachs and Painter [10], the concentrated sample was applied to a 2.5 × 60 cm Sephadex G-200 superfine column equilibrated with 0.5 M glycerol/5 mM Tricine (pH 7.5) with a void volume of 50 ml as determined by bromophenol blue elution. A flow rate of 0.6 ml/min was maintained. The elution profile of the enzyme from the Sephadex is presented in Fig. 2. Again, the enzyme fractions with the highest specific activity were pooled and applied to a 1 ml DEAE-cellulose column for concentration as described above.

Dihydroxyacetone kinase was also purified from *D. parva* cells with similar results.

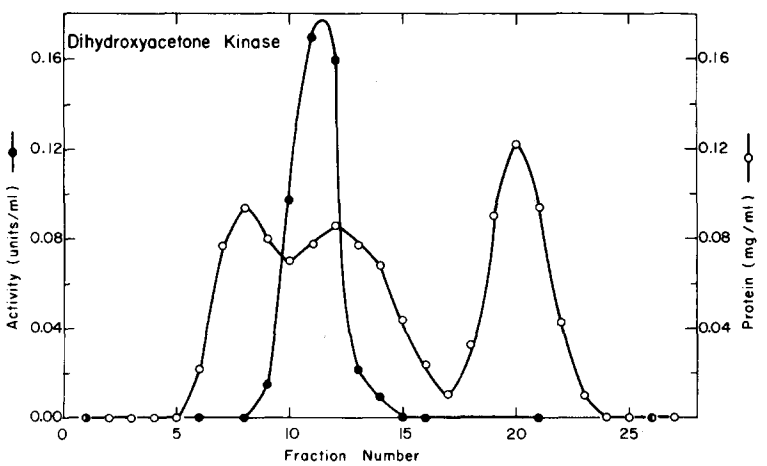


Fig. 2. Elution profile of dihydroxyacetone kinase from a Sephadex G-200 superfine column. Elution volume measurements began with the application of protein. Volume per fraction was 7.4 ml. The fractions were collected at an elution rate of 0.6 ml/min.

The activities of dihydroxyacetone reductase and dihydroxyacetone kinase were followed during the purification stages using a Cary 16 recording spectrophotometer at 340 nm. The former activity was determined by following dihydroxyacetone reduction as described by Ben Amotz and Avron [5]; and dihydroxyacetone kinase activity was determined by the method of Lerner and Avron [7] with the addition of ATP initiating the reaction. In both cases a unit of enzyme activity is defined as the amount of enzyme which oxidizes or reduces 1 μmol of NADPH or $NADP^+$ per min at 25°C under the specified conditions. For higher sensitivity, we occasionally used for assay a Cary 118 recording spectrophotometer, an Eppendorf 1100 fluorimeter connected to a recorder with exciting light through the 313 + 366 filter and a 420 nm cutoff protecting the photomultiplier [7], or an Aminco-Chance dual wavelength spectrophotometer set at 355—380 nm. Protein concentration was determined by absorbance reading at 280 nm and 260 nm [8].

Vertical acrylamide-slab gel electrophoresis was performed with the lower, separating gel (10 cm high) composed of a gradient of 5—15% bisacrylamide solution (30% acrylamide/0.8% bisacrylamide) in a Tris buffer (pH 8.8), and the upper or stacking gel (2.5 cm high) composed of 3% bisacrylamide in a Tris buffer (pH 6.8). The running buffer was 25 mM Tris-glycine (pH 8.9). Electrophoresis was carried out at room temperature at 80 mV for 45 min followed by 150 mV for about 100 min or until the dye front of bromophenol blue began to be eluted from the gel. Since the enzyme preparation was stored in 2 M glycerol only bromophenol blue was added to the sample buffer.

Staining of the gels was carried out by the method of Diezel et al. [9]. The intensity of the stain was determined on a Gilford 240 spectrophotometer from an enlarged photographic negative of the gel.

Results

Enzyme stability. Cell-free extract was incubated in the presence of various concentrations of NaCl and glycerol at 4°C over a period of 9 days in order to determine the best conditions for stabilization of dihydroxyacetone kinase activity (Fig. 3). In the presence of glycerol at a concentration of 2 M, the enzyme was maintained with essentially constant activity at about 85% of the original value. The reasons for the initial increase (Fig. 3) in activity and the subsequent decrease are not clear. 20 mM Tricine and 1 mM dithiothreitol had little effect. The addition of 0.2 M NaCl to the crude extract caused a rapid loss of dihydroxyacetone kinase activity; in the presence of 0.5 M glycerol the decay was retarded and in 2 M glycerol, NaCl had little effect. A faster loss of dihydroxyacetone kinase activity occurred when the crude extract was subjected to higher temperatures. Thus, incubation of the enyzme at 50°C for 20 min completely inactivated the enzyme in a polyol-less medium, while 85% of the activity was maintained when either 2 M glycerol or 1 M sucrose were present. Since glycerol and sucrose were similarly effective, a general protection by polyols against inactivation seems to be operating.

When the purified enzyme was stored at −20°C in the presence of 2 M glycerol/5 mM Tricine (pH 7.5)/0.3 M NaCl, activity was maintained with no loss over a period of at least 2 months despite repeated thawing and freezing.

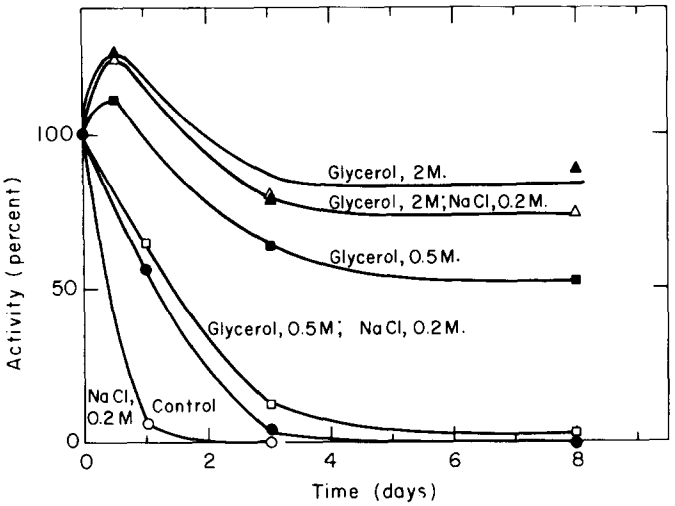


Fig. 3. The effect of glycerol on enzyme stability. The following were added to a cell-free extract maintained at 4°C and containing 5 mM Tricine (●———●); 0.2 M NaCl (○———○); 0.5 M glycerol (■———■); 0.5 M glycerol + 0.2 M NaCl (□———□); 2 M glycerol (▲———▲); 2 M glycerol + 0.2 M NaCl (△———△). Samples were removed at the times indicated and assayed.

Purity. The purity of the enzyme preparation was followed during the different phases of purification by both the specific activity and gel electrophoresis. The final enzyme preparation was about 440-fold purer than the cell fragments (Table I). When subjected to electrophoresis on a 5—15% gradient of polyacrylamide gel three major bands were observed (78 000, 88 000 and 100 000 daltons, Fig. 4). It is not clear at present which of the bands correspond to dihydroxyacetone kinase.

No dihydroxyacetone reductase activity was detected in the purified enzyme preparation, some triose phosphate isomerase activity was detected amounting to about 1% of the activity of dihydroxyacetone kinase.

Molecular weight. The partition coefficient [11] K_{av}, of the purified *D. parva* dihydroxyacetone kinase was determined on Sephadex G-200 by the rapid flow method of Sachs and Painter [10]. Dihydroxyacetone kinase eluted at K_{av} 0.31 indicating a Stokes' radius of 3.5 nm which is equivalent to a globular protein of a molecular weight of about 65 000.

Affinity for substrates and substrate specificity. The apparent K_m values for dihydroxyacetone and ATP were calculated from Lineweaver-Burk plots for the pure enzyme. Addition of 2 M glycerol to the assay buffer had no effect on either the apparent K_m or V in either case. As previously reported [7] the apparent K_m toward dihydroxyacetone was around 10 μM but in the purified enzyme preparation no substrate inhibition (see Ref. 7) was found at concentrations up to 1 mM (data not shown here). The apparent K_m toward ATP in presence of excess Mg^{2+} was around 80 μM (Fig. 5). The double reciprocal plot of the purified enzyme did not curve downwards as was described for the crude extract [7].

There was no activity detected when 130 μM glyceraldehyde was substituted for dihydroxyacetone, nor was the enzyme active when 1 mM GTP was substituted for ATP.

TABLE I

PURIFICATION PROCEDURE FOR DIHYDROXYACETONE KINASE FROM *DUNALIELLA SALINA*

Fraction	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)	Yield (%)
D. salina fragments	72.5	5075	29.0	0.0006	1	100
Cell-free extract	152.5	455	24.6	0.054	9	88
DEAE-cellulose gradient (concentrated pooled fractions)	5.5	5.6	5.3	0.94	158	18
Sephadex G-200 (pooled fractions)	22.2	1.34	3.6	2.65	442	12

Mg^{2+} dependence. As is evident from Fig. 5, dihydroxyacetone kinase is inactive when Mg^{2+} is not present. Half-maximal activity occurs in the presence of around 1 mM Mg^{2+}. Beyond 14 mM, Mg^{2+} becomes slightly inhibitory, possibly due to a general salt effect.

Since there is no dihydroxyacetone kinase activity in the absence of Mg^{2+}, it can be assumed that it is a Mg^{2+}-ATP complex with which the enzyme reacts. The data of Fig. 6 indicate that excess free Mg^{2+} is not, or is only slightly, inhibitory. However, as can be seen in Table II, excess free ATP is strongly inhibitory. Thus, with 0.4 mM Mg^{2+}, increasing the ATP concentration from 0.2 to 0.6 mM inhibited the reaction by more than 60%, while with 1 mM Mg^{2+}

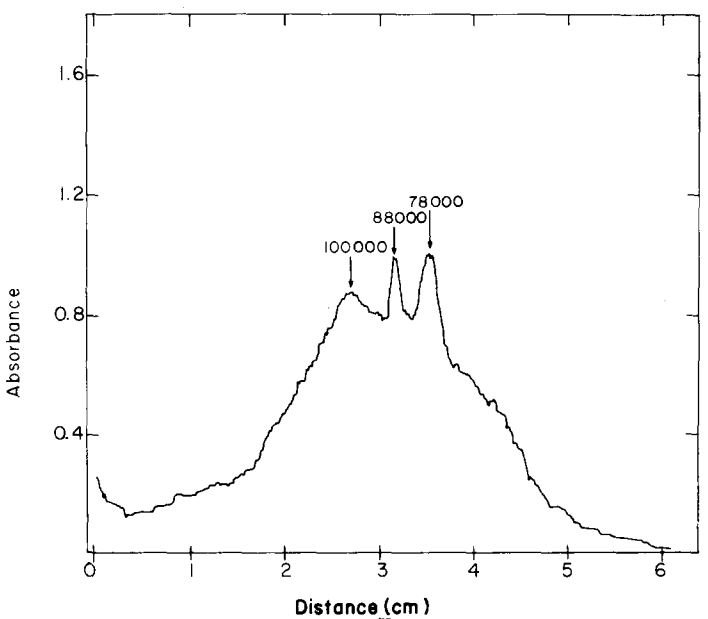
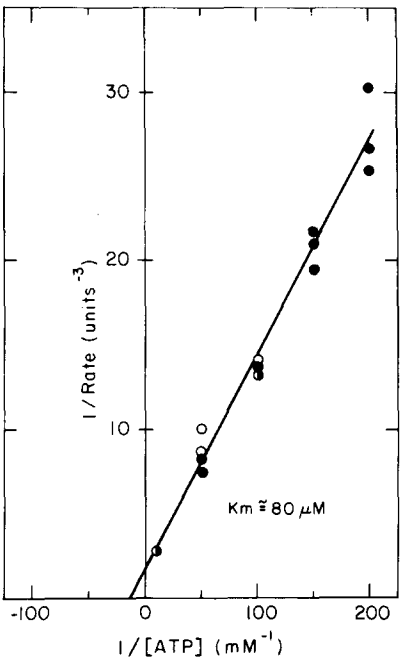


Fig. 4. Profile of polyacrylamide gel chromatography of the purest enzyme preparation. Electrophoresis was performed without the presence of denaturing agents with 22 μg protein applied to gel. Molecular weights were determined by comparison to molecular weight standards.



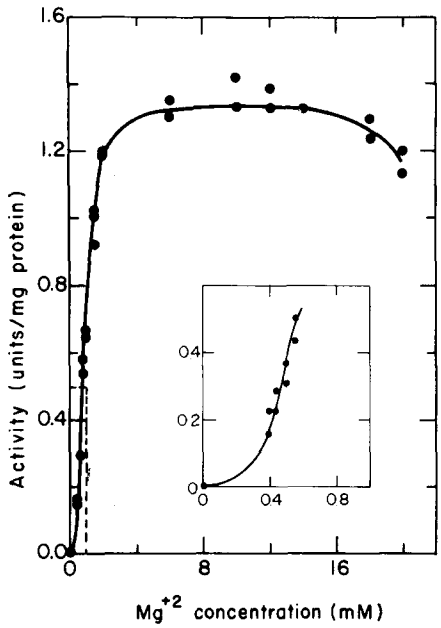


Fig. 5. The apparent K_m of purified dihydroxyacetone kinase for ATP. Activity was measured both in the absence (●———●) and presence of 2 M glycerol (○———○) in the reaction mixture. 2.1 μg of purified enzyme were added to the reaction mixture.

Fig. 6. The dependence of the rate of the reaction on the concentration of Mg^{2+}. 2.4 μg of purified enzyme preparation were used. The reaction mixture contained 0.6 mM ATP and $MgCl_2$ as indicated. The insert shows the data enclosed by the dotted line in more detail and on an expanded scale.

the same change stimulated the rate by over 30%. This inhibitory effect causes an apparent lag in the activity vs. Mg^{2+} concentration curve (Fig. 6), the magnitude of which depends upon the ATP concentration at which the curve is run.

pH activity curve. The dependence of activity on pH of the purified enzyme is shown in Fig. 7. There is a sharp increase in activity between pH 5.5 to pH 6.5 from essentially no activity to about 75% of maximal activity. The pH optimum is around 7.5.

TABLE II

INHIBITION OF DIHYDROXYACETONE KINASE BY EXCESS FREE ATP

1 μg of purified enzyme per reaction mixture

Mg^{2+} Concentration (mM)	Activity with (units/mg protein)	
	0.2 mM ATP	0.6 mM ATP
0.40	0.52	0.20
0.66	0.76	0.80
1.00	1.14	1.50

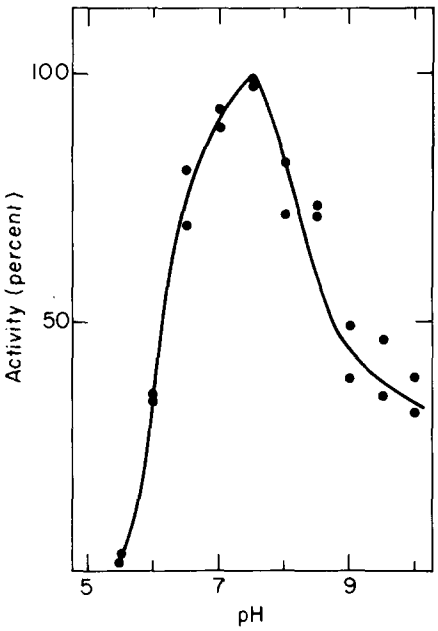


Fig. 7. pH profile of purified dihydroxyacetone kinase. A 50 mM Tris-maleic acid buffer was used over the entire pH range. Control experiments were performed to insure that the small amount of NaOH required to bring the buffer to the indicated pH did not affect the enzymic activity, and that the glycerophosphate dehydrogenase added was sufficient to ensure that the phosphorylation of dihydroxyacetone was the rate-limiting step at all the pH values tested.

Absorption spectrum. The absorption spectrum of the most purified preparation had a single peak at 280 nm, and no absorption in the visible region.

Discussion

The presence of two novel enzymes in algal cells of the genus *Dunaliella* has previously been reported [3—7]. Both enzymes, dihydroxyacetone reductase and dihydroxyacetone kinase, have been suggested to play a central role in the metabolism of glycerol which serves as the major osmoregulatory solute in these algae [3].

Dihydroxyacetone kinase was proposed to be the key enzyme involved in the conversion of glycerol to insoluble polysaccharide which is suggested to occur when the algae face a decrease in the salt concentration of the medium. Dihydroxyacetone, which is maintained at equilibrium with glycerol by dihydroxyacetone reductase, is thought to be phosphorylated by dihydroxyacetone kinase, and the phosphorylated product converted to the osmotically inactive polysaccharide via the glycolytic pathway.

Prior studies of dihydroxyacetone kinase found the enzyme too unstable to enable significant purification. By the addition of 2 M glycerol to the enzyme solution the enzyme is stabilized. Activity is maintained high presumably by preventing detrimental conformational changes [12]. It is noteworthy that the natural environment of the enzyme within these algal cells contains glycerol at similar concentrations [2]. Stabilization of dihydroxyacetone kinase has

enabled the development of the purification procedures and the further characterization of the enzyme described herein.

The rather sharp pH activity profile of the dihydroxyacetone kinase indicates a possible regulating role of H^+. Thus, small changes in intracellular pH in the pH 6—7 region will be accompanied by large changes in enzymic activity.

References

1 Craigie, J.S. and Mclachlan, J. (1964) Can. J. Bot. 42, 777—778
2 Ben Amotz, A. and Avron, M. (1973) Plant Physiol. 51, 875—878
3 Ben Amotz, A. and Avron, M. (1978) in Energetics and Structure of Halophilic Microorganisms (Caplan, S.R. and Ginzburg, M., eds.), pp. 529—541, Elsevier, Amsterdam
4 Ben Amotz, A. and Avron, M. (1973) FEBS Lett. 29, 153—155
5 Ben Amotz, A. and Avron, M. (1974) Plant Physiol. 53, 628—631
6 Borowitzka, L.J. and Brown, A.D. (1974) Arch. Microbiol. 96, 37—52
7 Lerner, H.R. and Avron, M. (1977) Plant Physiol. 59, 15—17
8 Warburg, O. and Christian, W. (1941) Biochem. Z. 310, 384—421
9 Diezel, W., Kopperschlager, G. and Hofmann, E. (1972) Anal. Biochem. 48, 617—620
10 Sachs, D.H. and Painter, E. (1972) Science 175, 781—782
11 Laurent, T.C. and Killander, J. (1964) J. Chromatogr. 14, 317—330
12 Citri, N. (1973) in Advances in Enzymology (Meister, A., ed.), Vol. 37, pp. 397—648, Interscience Publishers, New York