J. Biosci., Vol. 1, Number 3, September 1979, pp. 295–304. © Printed in India.

Characterisation of lactic dehydrogenase from *Lactobacillus casei*

VANITA A. PADGAONKAR, S. F. D'SOUZA and G. B. NADKARNI
Biochemistry and Food Technology Division, Bhabha Atomic Research Centre, Bombay 400 085

MS received 7 June 1978; revised 26 April 1979

Abstract. Lactic dehydrogenase from *Lactobacillus casei* ATCC 7469 has been purified to homogeneity by a two-step affinity chromatography procedure which gave an yield of 35%. The enzyme specifically catalysed the conversion of pyruvate to lactate. The enzyme was maximally active at pH 4·6, which was shifted to 5·4 in the presence of fructose 1,6-biphosphate. The enzyme had a molecular weight of 70,800 with two identical subunits, unlike the lactic dehydrogenase from other sources. Histidine and primary amino groups appeared to be involved in catalysis.

Keywords. Lactic dehydrogenase; *Lactobacillus casei*; structure-function; regulation.

Introduction

Lactobacillus casei, a facultative anaerobe derives energy and metabolites for growth and multiplication exclusively through, fermentation of glucose to L-lactic acid (Gunsalus and Schuster, 1961; Naik and Nadkarni, 1968). Lactic dehydrogenase, (EC 1.1.1.27), the terminal enzyme in the pathway for glucose utilisation, is also involved in the regeneration of NAD^+ for the continuous operation of the fermentative process (DeMoss *et al*., 1953). Several studies on the comparative aspects of lactic dehydrogenase from different species of lactic acid bacteria have been reported (Mizushima and Kitahara, 1962; Gasser *et al*., 1970; Dennis and Kaplan, 1960; Holland and Pritchard, 1975; Gordon and Doelle, 1976; Hensel *et al*., 1977a, b). However, differences have been observed in the properties of the enzyme from each of these strains of Lactobacillus (Mizushima and Kitahara, 1962; Gasser, 1970; Doelle, 1971). Thus, L-lactic dehydrogenase was not initially detected in *L. casei*, while flavin-linked D- and L-lactic dehydrogenases and NAD-specific D-lactic dehydrogenase were isolated (Mizushima and Kitahara, 1962). Subsequent attempts established the presence of NAD-linked L-lactic dehydrogenase in *L. casei* (Dennis and Kaplan, 1960; Doelle, 1971) which was apparently unidirectional for conversion of pyruvate to lactate, thus eluding detection on electrophoresis (Gasser, 1970). Recent studies on *L. casei* ATCC 393 have indicated the regulatory characteristics of this enzyme (Holland and Pritchard, 1975; Gordon and Doelle,

1976; Hensel *et al*., 1977a). Thus fructose-1,6-diphosphate (FDP) was shown to be an activator of lactic dehydrogenase.

Earlier work from this laboratory on *L. casei* ATCC 7469 has brought out various aspects of regulation of the fermentative pathway (Naik and Nadkarni, 1968), including gluconeogenesis in this organism (Pradhan and Nadkarni, 1975) and the functional involvement of multiple forms of enzymes in different adaptive states (Kaklij and Nadkarni, 1970; 1974a, b). The present report describes some additional observations on the structure and function of L-lactic dehydrogenase from this bacterium.

Materials and methods

Materials

All the chemicals used in these studies were of the highest purity. The components of the bacterial growth medium were Difco Products, the substrates and reagents were purchased from Sigma Chemical Company, St. Louis, MO, USA and Sepharose 4B and Sephadex G 200 from Pharmacia, Uppsala, Sweden. Blue Sepharose 6B was kindly provided by Dr. B. K. Bachhawat.

Culture conditions

The cultures of *L. casei* ATCC 7469 were maintained on a medium containing per litre: glucose, 10 g; bactotryptone (Difco), 10 g; yeast extract (Difco), 10 g; K_2HPO_4 5 g; and salt solution, 20 ml (which contained per 100 ml, $MgSO_4 \cdot 7H_2O$, 800 mg; $MnSO_4 \cdot 7H_2O$, 162 mg; NaCl, 40mg; $FeSO_4$, 40mg and 12N HCl, 2·0 ml). The organism was maintained by biweekly transfers on agar stabs having the same composition. The cells were used after two successive transfers in the liquid medium (10 ml). The organism grown in 150 ml of the medium was harvested at 0 to 4° C in a Sorvall RC–2B centrifuge at 8000 g for 20 min and the cells washed with cold isotonic saline (0·95%).

Preparation of cell-free extract

The cells were suspended in 0·1 M phosphate buffer pH 7·0 containing 5 mM 2-mercaptoethanol and were extracted using an Aminco–French pressure cell at 4000–6000 psi. The unbroken cells and cell debris were removed by centrifugation at 12,000 g for 60 min and discarded. The supernatant was used as the cell-free extract for the enzyme assays. The protein was estimated by Lowry's Folin-phenol reagent (Lowry *et al*., 1951) using bovine serum albumin as the standard.

Assay of lactic dehydrogenase

The reaction mixture in a total volume of 3 ml contained 0·1 M acetate buffer (pH 4·6) containing 0·3 mM NADH, 20 mM pyruvate and 0·005 ml of the extract. Reaction was started with addition of NADH and decrease in the absorbance at 340 nm was measured. The unit of lactic dehydrogenase activity has been expressed as μmol of NADH oxidised per min at 30° C.

Purification of lactic dehydrogenase from L. casei

The cell extract was brought to 40% saturation by adding solid ammonium sulphate with constant stirring. The precipitate was allowed to stand for 2 h, separated by centrifugation at 12,000 g for 45 min, and discarded. The supernatant solution was brought to 70% saturation of the salt and the precipitate thus obtained was allowed to stand for 16–20 h, collected by centrifugation, and was dissolved in 0·01 M sodium phosphate buffer, pH 6·0 containing 5 mM 2-mercaptoethanol. This was dialysed against repeated changes of the buffer to remove traces of ammonium sulphate.

Dialysed preparation of the enzyme (700 mg) was loaded on a column (15 × 1·7 cm) of Sepharose 4B containing an oxamate group (Holland and Pritchard, 1975). This was prepared by using hexamethylene diamine as a spacer molecule which was covalently bound to CNBr-activated Sepharose 4B and the oxalate was linked, using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodimide metho-*p*-toluene sulphonate, to the spacer molecule (Hensel *et al.*, 1977b). The column was washed with 0·01 M phosphate buffer pH 6·0 till the washings were free of protein. Lactic dehydrogenase was eluted by increasing the buffer concentration from 0·01 M to 0·05 M. The enzymatically active fractions were then pooled, concentrated by precipitating with solid ammonium sulphate (70% saturation) and dialysed as above.

The dialysed sample (105 mg) was then loaded on a column (9 × 0·9 cm) of Blue Sepharose 6B previously equilibrated with 0·01 M phosphate buffer pH 6·4· The column was washed with the same buffer till the washings were free of protein. Lactio dehydrogenase was eluted with 5 mM NADH (figure 1). The results of the purification show a 40-fold increase in sp. act. with 35% recovery (table 1).

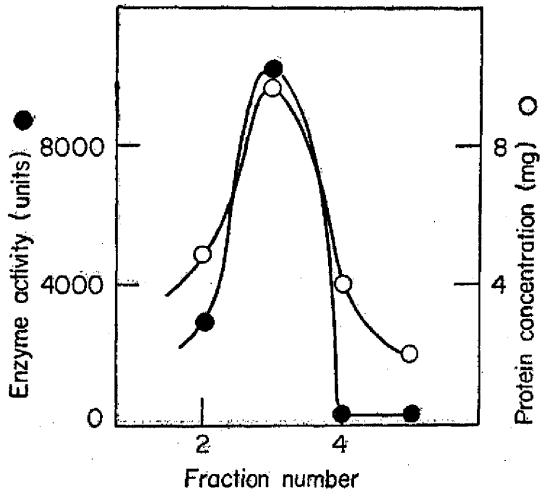

Figure 1. Affinity chromatography of lactic dehydrogenase from *L. casei* on Blue Sepharose 6B. Details are stated in the text. Elution was carried out with 5 mM NADH and 2 ml fractions were collected.

Table 1. Purification of lactic dehydroganese from *L. casei.*

Fraction	Total units[a] (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg of protein)	Purification (fold)	Recovery (%)
Crude extract	28,989	1200	24·2	1	100
$(NH_4)_2SO_4$ fractionation (40–70% saturation)	27,849	706	39·5	1·6	96
Oxamate affinity column chromatography	18,576	105	176·9	7·3	64
Blue Sepharose 6B affinity chromatography	10,217	11	954·8	39·2	35

[a] The unit of activity has been expressed as μmol of NADH oxidised.

Polyacrylamide disc gel electrophoresis

The protein samples obtained were subjected to polyacrylamide disc gel electrophoresis according to the procedure of Davis (1966), in a Canalco Model–12 apparatus.

Results

The enzyme preparation eluted from blue sepharose column also showed a single protein band on disc gel electrophoresis pointing to the homogeneity as shown in figure 2. The enzyme thus obtained was stable at 4° C for at least 20 days.

Molecular weight of *L. casei* lactic dehydrogenase determined by Sephadex G–200 gel filtration (Andrews, 1964) was observed to be 70,000. The following markers were used: myoglobin, ovalbumin, bovine serum albumin and aldolase. After treatment with 0·1% sodium dodecyl sulphate (SDS) the molecular weight was found to be 36,300 (figure 3). Mobility of lactic dehydrogenase in Sod. dod. SO_4-polyacrylamide gel electrophoresis also indicated the value of 35,480, suggesting presence of two identical subunits.

Properties of the enzyme

The equilibrium of the reaction was essentially towards the conversion of pyruvate to lactate. The enzyme did not show any activity with D- or L-lactate, while it was weakly active with glyoxylate as substrate. The activity with varying concentrations of pyruvate, keeping NADH concentration at 0·3 mM gave a sigmoidal response and the apparent K_m ($S_{0·5}$) value for pyruvate at pH 4·6 was calculated to be 5 mM.

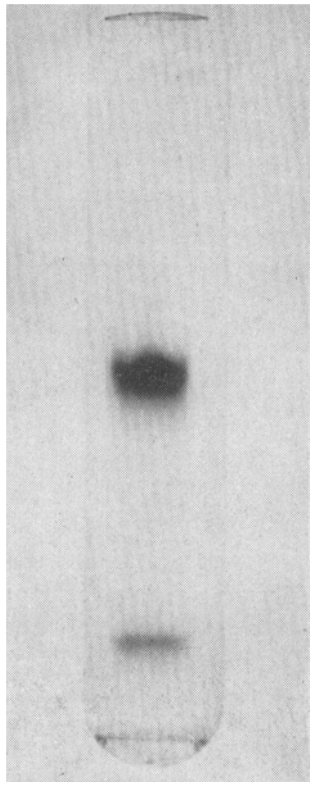

Figure 2. Polyacrylamide gel electrophoresis of the purified lactic dehydrogenase from *L. casei*. The preparation (20 μg) was in Tris-glycine buffer pH 8·3 for running in 7·5% gel. The electrophoresis was carried out for 90 min at 4–6° C, with constant potential of 240 volts and 5 mA of current per tube. The staining was performed with Amido black. The lower band represents marker dye bromophenol blue.

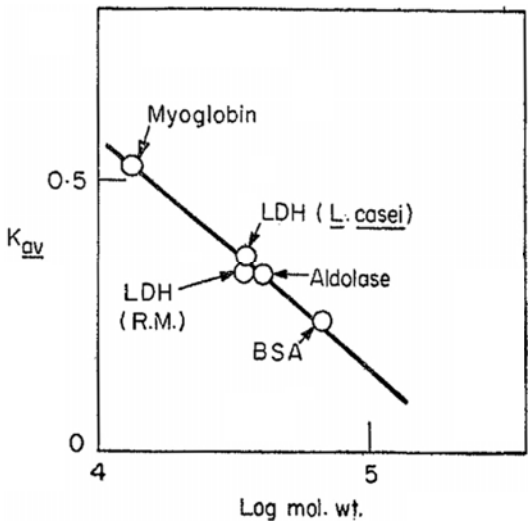

Figure 3. The proteins (5 mg) were incubated with 1% Sod. dod. SO_4 in 0·1 M phosphate buffer (pH 7·0) for 3 h at 37° C and then chromatographed on (64 × 2·1 cm). Sephadex G 200 column previously equilibrated with 1% Sod. dod. SO_4. R.M.—Rabbit muscle lactic dehydrogenase.

Effect of pH on activity

The pH activity curves of lactic dehydrogenase pointed to two pH optima of 4·2 and 4·6 at 2 mM and 20 mM pyruvate respectively, as shown in figure 4. The shape of the activity curve as a function of pyruvate concentration was found to be dependent on pH of the assay system. At lower pH values (3·8–4·2), the activity

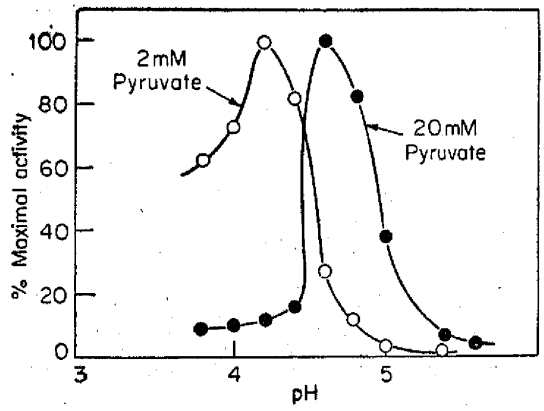

Figure 4. pH activity curves of *L. casei* lactic dehydrogenase at two concentrations of pyruvate. The activities obtained were 570 units/mg protein at pH 4·2 and 950 units/mg at pH. 4·6. The per cent maximal activities are plotted to compare the relative responses.

curves were hyperbolic, while the increase in pH (4·4–5·4) caused sigmoidal responses, suggesting co-operative binding of the substrate on the enzyme at higher pH values.

Activation of lactic dehydrogenase by FDP

The effect of glycolytic intermediates including glucose 6-phosphate, fructose 6-phosphate (F6P), fructose-1,6-biphosphate (FDP), glyceraldehyde 3-phosphate and phosphoenolpyruvate added to the assay system at 0·1 mM concentration indicated that only FDP could activate the enzyme while the other glycolytic intermediates were without any effect. In the presence of FDP, the pH optimum was shifted from 4·4 to 5·4. The sigmoidal responses caused by pyruvate at higher pH values were also abolished in the presence of FDP as shown in table 2. Analysis of the

Table 2. Kinetic properties of lactic dehydrogenase of *L. casei*.

pH	Lineweaver—Burk plot		K_m (mM)		Hill Coeff.	
	No FDP	With FDP*	No FDP	With FDP	No FDP	With FDP
3·8	Linear	..	1·1	..	0·9	..
4·2	Linear	..	1·3	..	1·3	..
4·4	Nonlinear	Linear	2·5	1·7	1·9	1·2
4·6	Nonlinear	Linear	5	1·1	2·1	1·4
5·4	Nonlinear	Linear	20	1·9	2·4	1·6

* The concentration of FDP added was 0·1 mM. K_m was obtained from Lineweaver-Burke plot or from half maximal activation depending upon the shape of the curve.

kinetic data using Hill equation gave approximate values of 2·0 and 1·0 for the interaction coefficients for pyruvate in the absence and presence of FDP, respectively. These results indicated that FDP caused alterations in the enzyme with removal of the regulatory constraints. At lower pH values (3·8–4·2), FDP did not exert any effect on the enzyme activity. The K_m value for pyruvate was also lowered in the presence of FDP. The varying concentration of FDP caused activation of the enzyme in a sigmoid fashion. However, it is not clear if this sigmoid behaviour could also be attributed to the shifting of pH optimum in the presence of FDP. The elution pattern of the enzyme on Sephadex G 200 in presence and absence of FDP, as shown in figure 5, indicated that the activator caused alterations in the protein.

Possible functional groups

The functional amino acids of *L. casei* lactic dehydrogenase were ascertained by chemically modifying certain residues as described earlier (Kaklij and Nadkarni,

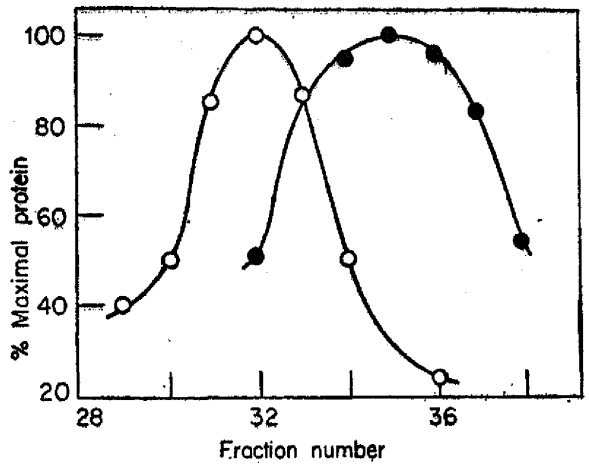

Figure 5. Sephadex G 200 gel filtration profile of *L. casei* lactic dehydrogenase in presence and absence of FDP (0·02 mM). The enzyme preparation (4mg) was loaded on the column (64 × 2·1 cm) previously equilibrated appropriately. The elution was carried out with 0·05 M acetate buffer pH 5·4 and 3·5 ml fractions were collected for protein estimation, The plots are made with reference to maximal protein eluted.

1974a; Kelkar and Nadkarni, 1977). The inactivation of *L. casei* lactic dehydrogenase by photo-oxidation in the presence of Rose Bengal and methylene blue as described earlier (Hoffee *et al*., 1965) suggests the importance of histidine residues. The addition of pyruvate to the enzyme did not afford any protection against this inactivation as shown in table 3.

There was progressive loss of lactic dehydrogenase activity when acetic anhydride was added to the system in increasing concentrations (Riordan and Vallee, 1963) with complete inactivation at 20 mM. Sodium sulphite (0.1 mM) a reagent known to protect –SH groups from acetylation did not afford any protection. An estimation of thiol groups of lactic dehydrogenase in presence and absence, of pyruvate or FDP indicated that only three thiol groups were available to react with5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (Todd and Gronow, 1969). In the presence of 4 M guanidineHCl, however, 22–23 thiol groups could be exposed for reaction with DTNB. Iodoacetamide and N-ethylmaleimide (1 mM) showed 65 and 72% inhibition respectively, after 60 min of preincubation, *p*-Chloromercuribenzoate (0.5 mM) was comparatively less effective showing only 38% inhibition after 60 min preincubation.

The inactivation of the enzyme caused by incubation with 3% 2,4,6-trimtrobenzene sulphonate for 120 min, suggested the essentiality of primary amino groups for catalysis (Freedman and Radda, 1968), However, the enzyme was not affected by sodium borohydride reduction (Hoffee *et al*., 1965) in the presence of pyruvate.

Incubation of lactic dehydrogenase with two different concentrations (0·42 and 0·85 mM) of tetranitromethane (TNM) (Lane and Dekker, 1969) did not influence the activity at either alkaline or acidic pH. It has been Indicated that TNM causes

Table 3. Photoinactivation of *L. casei* lactic dehydrogenase.
The reaction mixture (1 ml) contained lactic dehydrogenase preparation (65 μg) in 0·1 M phosphate buffer pH 7·4 with and without the additions as indicated below. The mixture was exposed to 500 watt projector lamp for 20 min and aliquots (0·005 ml) were tested for the enzyme activity.

Additions	Per cent activity retained
Enzyme	100
Enzyme + light	100
Enzyme + methylene blue (0·01%)	73·7
Enzyme + methylene blue + light	2·6
Enzyme + pyruvate (20 mM) + methylene blue + light	5·3
Enzyme + Rose Bengal (0·0007%)	94·7
Enzyme + Rose Bengal + Light	6·6
Enzyme + pyruvate (20 mM) + Rose Bengal + light	6·6

nitration of tyrosine residues at alkaline pH and oxidation of thiol groups under acidic environment (Lane and Dekker, 1969).

Discussion

The foregoing results indicate that the preparation of lactic dehydrogenase from *L. casei* ATCC 7469 is composed of two identical subunits each having molecular weight of 35–36000. These results are at variance with the observations reported with *L. casei* ATCC 393 (Hensel *et al.* 1977a, b) and some other species of lactic acid bacteria (Gasser *et al.* 1970). Most of these earlier studies have shown that the molecular weight of lactic dehydrogenase was in the range of 140000, with four subunits. The present preparation therefore differs from the general pattern of tetrameric arrangement of lactic dehydrogenase from most other sources (Everse and Kaplan, 1973). The molecular weight 70,000 of our enzyme resembled that of *L. leichmanii* (Gasser *et al.* 1970). Electrophoretic characterisation of lactic dehydrogenases from 146 strains of different species of lactobacilli indicated that the NAD-dependent enzyme from *L. casei* and *L. fermenti* alone were not catalyzing the reverse reaction and hence could not be stained for activity by usual methods employed for dehydrogenases (Gasser, 1970). The results presented above also indicate the specificity of the enzyme for pyruvate as substrate.

The enzyme could be modulated by substrate concentration, pH of the system and FDP as observed for some bacterial lactic dehydrogenases (Jago *et al.*, 1971; Holland and Pritchard, 1975; Gordon and Doelle, 1976; Hensel *et al.*, 1977a). The differential pH optima of the present preparation observed at two concentrations of pyruvate, may suggest variations in molecular conformations, compatible with the observed changes in affinity for the substrate. Non-linear reciprocal plots and Hill coefficient of 2 at higher pH, indicate positive co-operativity for pyruvate (Hensel *et al.*, 1977). The sigmoid responses in relation to pyruvate concentrations were abolished in the presence of FDP. However, the enzyme does not seem to be entirely dependent upon FDP for activity (Holland and Pritchard, 1975). FDP did not seem to have an activating efect on lactic dehydrogenase from some other species of Lactobacilli (Gasser *et al.*, 1970). The present data also show that the enhanced activity caused by FDP could also be attributed to structural alterations in the enzyme.

References

Andrews, P. (1964) *Biochem. J.*, **91,** 222.

Davis, B. J. (1966) *Ann. N.Y. Acad. Sci.*, **121,** 404.

DeMoss, R. D., Gunsalus, I. C. and Bard, R. C. (1953) *J. Bacteriol.*, **66,** 10.

Dennis, D. and Kaplan, N. O. (1960) *J. Biol. Chem.*, **235,** 810.

Doelle, H. W. (1971) *J. Bacteriol.*, **108,** 1283.

Everse, J. and Kaplan, N. O. (1973) *Adv. Emymol.*, **37,** 61.

Freedman, R. B. and Radda, G. K. (1968) *Biochem. J.*, **108,** 383.

Gasser, F. (1970) *J. Gen. Microbiol.*, **62,** 223.

Gasser, F., Duodoroff, M. and Contopoulos, R. (1970) *J. Gen. Microbiol.*, **62,** 241.

Gordon, G. L. and Doelle, H. W. (1976) *Eur. J. Biochem.*, **67,** 543.

Gunsalus, I. C. and Schuster, C. W. (1961) in *Bacteria* (New York: Academic Press), **2, 1.**

Hensel, R., Mayr, U., Stetter, K. O. and Kandler, O. (1977a) *Arch. Microbiol.*, **112,** 81.

Hensel, R., Mayr, U., Fujiki, H. and Kandier, O. (1977b) *Eur. J. Biochem.*, **80,** 83.

Hoffee, P., Lai, C. Y. and Horecker, B. L. (1965) *J. Biol. Chem.*, **240,** 1512.

Holland, R. and Pritchard, G. G. (1975) *J. Bacteriol.*, **121,** 777.

Jago, G. R., Nichol, L. W., O'dea, K. and Sawyer, W. H. (1971) *Biochem. Biophys. Acta*, **250,** 271.

Kaklij, G. S. and Nadkarni, G. B. (1970) *Arch. Biochem. Biophys.*, **140,** 334.

Kaklij, G. S. and Nadkarni, G. B. (1974a) *Arch. Biochem. Biophys.*, **160,** 47.

Kaklij, G. S. and Nadkarni, G. B. (1974b) *Arch. Biochem. Biophys.*, **160,** 52.

Kelkar, S. M. and Nadkarni, G. B. (1977) *Indian J. Biochem. Biophys.*, **14,** 305.

Lane, R. S. and Dekker, E. E. (1969) *Biochem. Biophys. Res. Commun.*, **36,** 973.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193,** 265.

Millar, B. S. and Schwert, G. W. (1963) *J. Biol. Chem.*, **238,** 3249.

Mizushima, S. and Kitahara, K. (1962) *Jpn. J. Gen. Appl. Microbiol.*, **8,** 130.

Naik, V. R. and Nadkarni, G. B. (1968) *Arch. Biochem. Biophys.*, **123,** 431.

Pradhan, P. G. and Nadkarni, G. B. (1975) *Arch. Biochem. Biophys.*, **171,** 65.

Riordan, J. F. and Valee, B. L. (1963) *Biochemistry*, **2,** 1460.

Todd, P. and Gronow, M. (1969) *Anal Biochem.*, **28,** 369.