Biochimica et Biophysica Acta, 370 (1974) 441–449
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

BBA 67353

THE DEXTRANSUCRASE ISOENZYMES OF *LEUCONOSTOC MESENTEROIDES* NRRL B-1299

MIKIHIKO KOBAYASHI and KAZUO MATSUDA

Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai (Japan)

(Received May 13th, 1974)

SUMMARY

Dextransucrase (EC 2 4 1 5) activity of *Leuconostoc mesenteroides* NRRL B-1299 strain was shown to be inducible by sucrose, and detected both in the culture supernatant and centrifuged residue fractions The total enzyme activity reached a maximum in 18–24 h after inoculation, with the extracellular and intracellular enzymes in the ratio of 2 3 From the sediment, 41 3% of the total intracellular activity was solubilized by successive treatments with disruption, lysozyme digestion, and deoxycholate extraction The extracellular enzyme gave a number of active bands on acrylamide-disc electrophoresis, whereas the intracellular enzyme gave only two bands Furthermore, the multiple forms of the extracellular enzyme, with the monomer having a molecular weight of 42 000, acted as the oligomeric isoenzymes by gel electrophoresis according to Hedrick, J L and Smith, A J ((1968) Arch Biochem Biophys 126, 155–164) On the other hand, the intracellular enzymes with a similar molecular weight of 74 000, were characterized as the charge isomer proteins These isoenzymes also differed from each other in their enzymatic characteristics (i e optimum pH, temperature, and K_m)

INTRODUCTION

The biosynthesis of the dextran has been extensively studied mainly with the extracellular dextransucrase (sucrose 1,6-α-D-glucan 6-α-glucosyltransferase, EC 2 4 1 5) from NRRL B-512 and B-512F strains of *Leuconostoc mesenteroides* [1, 2], and some properties of the partially purified enzyme have been clarified [2] Recently, however, it has been shown that the dextrans elaborated by *L. mesenteroides* NRRL B-1299 have a highly branched structure [3], with different solubility and heterogeneous molecular weight distributions among the five fractions [4], in contrast to the B-512 dextran thus far studied Little is known about the formation of branching point and non-α-1,6-linkages in the highly branched dextrans

As an approach to the study of dextran synthesis in the B-1299 strain, the dextransucrase of this strain was studied The present paper shows that the enzyme occurs in the extracellular and intracellular forms in this strain and that both exhibit the respective, characteristic isoenzyme patterns

MATERIALS AND METHODS

Cultivation

L mesenteroides NRRL B-1299 was grown on the following medium for 18–24 h at 27 °C The medium was composed of 2% sucrose, 1 5% K_2HPO_4, 0 5% peptone, 0 5% yeast extracts, 0 001% $MgSO_4$ $7H_2O$, 0 001% $MnCl_2$ $4H_2O$ and 0 001% NaCl The pH was adjusted to 7 4

Isolation of the extracellular enzyme

Cells in the late log phase were harvested by centrifugation at 8000 × *g* for 20 min at 0 °C The clear supernatant was subjected to $(NH_4)_2SO_4$ fractionation, and the fraction of 30–70% satn was collected, redissolved in 0 005 M sodium phosphate buffer (pH 5 2), dialyzed against the same buffer at 4 °C, and used for assaying the extracellular dextransucrase The sedimented cells were washed twice with 0 2 M sodium acetate buffer (pH 5 2), and stored as a freeze-dried powder, which was stable for several months

Solubilization of the intracellular enzyme

The washed cells were disrupted by grinding with an equal amount of quartz sand in 0 2 M sodium acetate buffer (pH 5 4) at 0 °C, the cell debris were removed by centrifugation at 8000 × *g* for 20 min, and the sediment was submitted to the same procedure two more times The supernatant was dialyzed overnight against 0 005 M sodium phosphate buffer (pH 6 0) The sediment was suspended in the acetate buffer, and digested with lysozyme (1 0 mg/ml) for 60 min at 30 °C The digest was then centrifuged at 8000 × *g* for 20 min, and the resulting residue was washed with the same buffer, and extracted with 0 04% (w/v) sodium deoxycholate with mechanical stirring for 60 min at 0 °C The solubilized protein fraction thus obtained was combined with the supernatant fractions from disruption and lysozyme digestion, and dialyzed overnight against 0 005 M sodium phosphate buffer (pH 6 0) The activity of this solubilized fraction was designated as the "intracellular" enzyme activity The final sediment fraction, designated as the cell-bound enzyme fraction, contained cell debris and water-insoluble dextran

Enzyme assay

Dextransucrase activity was determined by measuring the release of reducing sugar by the Nelson–Somogyi method [5, 6] The crude extracellular enzyme activity was assayed by incubating a suitable amount of the enzyme at 35 °C for 60 min in a mixture containing sucrose (125 mg/ml) and McIlvaine's buffer (pH 5 2) The assay of the crude intracellular enzyme activity was carried out at pH 6 0 (McIlvaine's buffer) and 40 °C One unit of the enzyme activity was defined as the quantity that produced reducing sugars equivalent to 1 0 mg of fructose per min under the above conditions [7]

Enzyme reaction with [^{14}C]sucrose

[U-^{14}C]Sucrose (10 0 Ci/mole) was purchased from Daiichi Pure Chemicals Co , Tokyo The reaction mixture contained in a final volume of 50 μl 5 μl of [U-^{14}C]-sucrose (855 μg, 4 10^4 cpm), 10 μl of McIlvaine's buffer, and 30 μl of enzyme solution

(40 units/ml) After incubation at 35 or 40 °C for a suitable period of time, the reaction was stopped by heating for 3 min at 100 °C, and applied to a Toyo-Roshi No 51A filter paper Paper chromatography was carried out by the descending method using water-saturated *n*-butanol as the solvent The chromatogram was cut into segments (1 cm × 2 cm) and each was counted for radioactivity in a scintillation solution containing 4 g diphenyloxazole and 0 1 mg *p*-bis[2-(5-phenyloxazolyl)]-benzene per l of toluene, using a liquid-scintillation spectrometer (Horiba Model LS-500)

Protein

Protein was determined by the absorbance at 280 nm or by the method of Lowry et al [8] with crystalline bovine serum albumin as a standard

Polyacrylamide-gel electrophoresis

Gel electrophoresis was performed according to Maurer [9], using the gel system No 6 with 5 0–11 0% acrylamide Veronal buffer (pH 7 5) was used as the electrode buffer Samples were applied to the spacer gels in 50% (v/v) glycerol solution The columns were electrophoresed at 4 °C, applying 5 mA to each column Duplicate columns were run for each sample, one column being stained with 0 25% Coomassie brilliant blue solution in a mixed solvent of water–methanol–acetic acid (5 5 1, by vol) to disclose the protein bands, and the other cut into 2-mm segments to assay for the enzyme activity by incubating with the usual assay components under optimum conditions with gentle stirring The following proteins were used as markers, ovalbumin (4 6 10^4), bovine albumin (6 7 10^4, dimer 1 34 10^5, trimer 2 01 10^5), γ-globulin (1 60 10^5), and horse apoferritin (4 5 10^5)

RESULTS

Enzyme production

The production of dextransucrase in *L mesenteroides* NRRL B-1299 usually occurred logarithmically 2–4 h behind the growth of the cells and reached a maximum in 18–24 h after inoculation As shown in Fig 1, while the amount of reducing sugar (fructose) in the medium reached a maximum in 9 h and decreased rapidly [10], the dextransucrase activity continued to increase, reaching a maximum in 18–24 h At this stationary state, the ratio of the total activity in the cell-free medium to that in

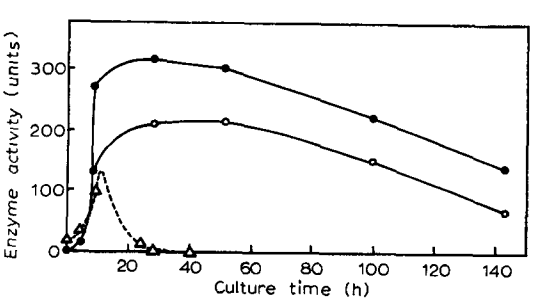

Fig 1 Production of dextransucrase in *L mesenteroides* NRRL B-1299 Cells were grown on 2% sucrose as a sole sourse of sugar, and the extracellular enzyme (○—○), the intracellular enzyme (●—●), and fructose (△---△) were measured

the sediment was 2 3 and gradually decreased to about 1 2 with the prolonged incubation

Inducibility of the dextransucrase activity

The culture of *L mesenteroides* grown on 2% sucrose, having the enzyme activity of about 40 units/ml, was inoculated into a fresh medium containing 2% glucose as the sole carbon source After a 24-h incubation and successive re-inoculations, the enzyme activity diminished to less than 1/10 of the initial activity (Fig 2) However, the enzyme activity was restored to the initial level when the culture on 2% glucose was transferred back to a 2% sucrose medium Therefore, the dextransucrase of this strain seems to be inducible by sucrose, as that in other strains of *L mesenteroides* [10]

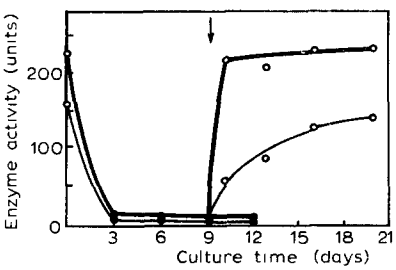

Fig 2 Induction of dextransucrase by sucrose The activity of the extracellular (———) and intracellular (▬▬▬) enzymes, grown on 2% glucose (●) or 2% sucrose (○), was assayed The cells grown on 2% sucrose were inoculated into the medium containing 2% glucose After several re inoculations into 2% glucose medium, the cells were again transferred, as indicated by an arrow, to the medium containing 2% sucrose

Solubilization of the intracellular enzyme

The freeze-dried sediment, containing the cells and water-insoluble dextran, was disrupted by grinding with quartz sand The precipitate was collected by centrifugation, redissolved in 0 1 M sodium acetate buffer (pH 5 4) and digested with lysozyme at 30 °C The digest was then centrifuged and the residue was treated with deoxycholate, and the extract was separated from the residue by centrifugation The combined supernatants from the above three extraction procedures were dialyzed against 0 005 M sodium phosphate buffer (pH 6 0) This cell-free extract, designated as the intracellular enzyme fraction, was examined for activity against sucrose and its enzymatic properties The total enzyme activity solubilized by these procedures amounted to approx 41 3% of the whole activity of the sediment (Table I) After these treatments, however, 42 3% of the initial enzyme activity was still retained in the residual cell-bound fraction

Dextransucrase action demonstrated with labeled sucrose

The synthesis of dextran from sucrose by dextransucrase was studied with ^{14}C-labeled substrate As shown in Fig 3a, the extracellular dextransucrase actually converted the glucose residue of [^{14}C]sucrose into the polymer fraction, concomitant with the liberation of fructose Both the incorporation and liberation increased with reaction time The intracellular enzyme also showed essentially the same incorpora-

TABLE I

SOLUBILIZATION OF THE INTRACELLULAR ENZYME BY VARIOUS TREATMENTS FROM THE CELLS OF *L MESENTEROIDES* NRRL B-1299

500 mg of freeze-dried cells (1 1 10^4 units) was employed

Fraction	Enzyme activity (%)		
	Supernatant*	Precipitate*	Recovered
Extraction procedure			
(1) disruption	16 0	84 0	100
(2) lysozyme digestion	10 4	89 6	98 7
(3) deoxycholate extraction	28 7	71 3	89 9
Total**	41 3	42 3	83 6

* After centrifugation at 8000 × g for 20 min

** These values were corrected for the recovery

tion pattern as that of the extracellular enzyme (Fig 3b) It can be noted that glucose was formed in the course of the reaction only in a small amount in both cases Thus, the action of the sucrose-hydrolyzing enzyme such as invertase would be excluded, and the production of glucose should be explained on the basis of the intermediate formation of glycosyl radicals from sucrose [11]

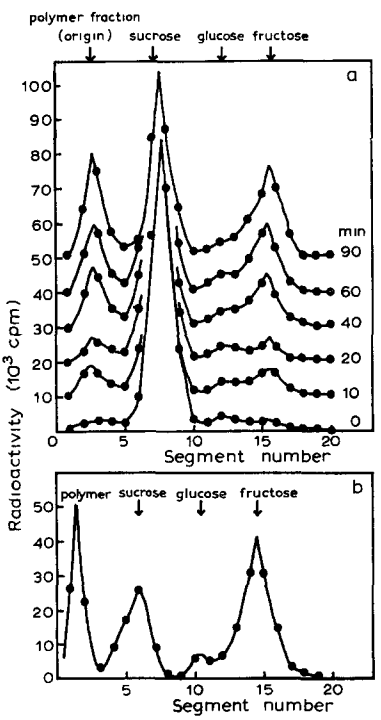

Fig 3 Dextran synthesis from [U-^{14}C]sucrose by dextransucrase The assay conditions were described in the text (a) Incorporation of [^{14}C]glucose into the polymer fraction by the extracellular dextransucrase as a function of the incubation time (b) Incorporatoin of [^{14}C]glucose into the polymer fraction by the intracellular dextransucrase after 180 min of reaction time

The isoenzyme pattern of the extracellular dextransucrase

The heterogeneity of the extracellular dextransucrase was examined with polyacrylamide-gel electrophoresis The enzyme fraction showed the presence of two major bands and at least six minor bands with enzyme activity The two main components had the R_m (relative mobility) values of 0 15 and 0 93, respectively, at a gel concentration of 7 5% Investigation of the relationship of these multiple forms was made by the method of Hedrick and Smith [12] When the logarithm of the R_m of a protein was plotted as a function of the gel concentration, a linear relationship was observed Plots of log R_m versus gel concentration for each component of the extracellular enzyme yielded several nonparallel lines (Fig 4a) Extrapolation of each line showed that the lines are intersected in the vicinity of a 0% gel concentration A nearly proportional decrease in the slope of each oligomeric form is the characteristic of the size of the isomer proteins [12]

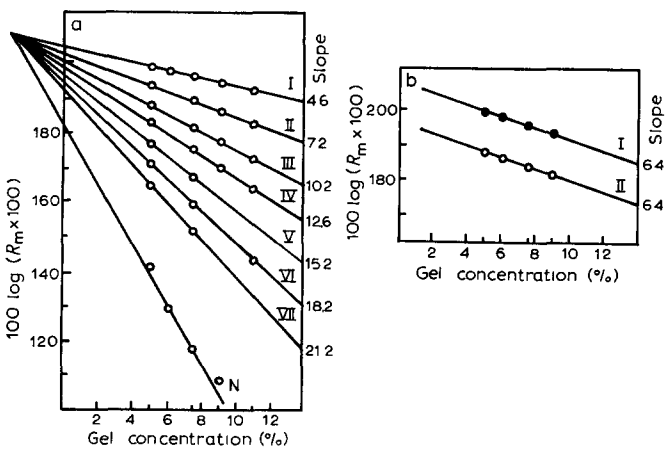

Fig 4 The effect of gel concentrations on the mobility of dextransucrase isoenzymes The negative slopes of each line are noted on the right of the figure (a) The extracellular isoenzymes (b) The intracellular isoenzymes

The molecular weight of these isoenzymes were estimated from a calibration curve Fig 5 illustrates the relationship between the slope of the plotted lines and the molecular weight of the various protein markers The molecular weights of the extracellular isoenzymes were calculated as follows, 42 000 for isoenzyme I (monomer), 83 000 for isoenzyme II (dimer), 127 000, 169 000, 212 000 and so on Isoenzyme N had a value of over 450 000 Among these extracellular isoenzymes, forms I and N occurred consistently but other isoenzymes with molecular weights up to 212 000 were detected with varying frequency Isoenzyme N seemed to be a highly aggregated form and it was impossible to decide its exact molecular weight by this gel system alone These results demonstrated that the extracellular isoenzymes of dextransucrase may be classified into a size isomer family as described by Hedrick and Smith

The extracellular dextransucrase was eluted in five peaks from a DEAE-cellulose column (Fig 6a) The first peak, eluted with 0 1 M NaCl, corresponding to isoenzyme N as evidenced by polyacrylamide-gel electrophoresis, was the aggregated form of isoenzyme I The following three peaks, eluted with 0 1–0 2 M NaCl, probably

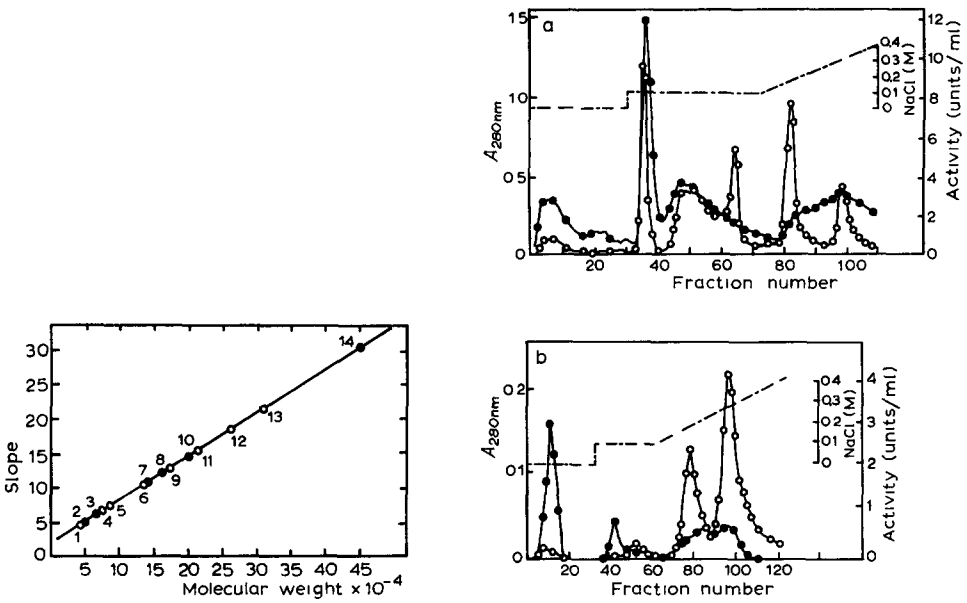

Fig 5 The molecular weight determination of the isoenzymes by the slope–molecular weight relationship The procedures were essentially the same as described by Hedrick and Smith [12] The slopes of the isoenzymes were calculated from the data shown in Fig 4 Enzymes plotted (○) were as follows 1, extracellular isoenzyme I (monomer), 4, intracellular isoenzyme I and II, 5, extracellular isoenzyme II (dimer), 6, III (trimer), 9, IV (tetramer), 11, V (pentamer), 12, VI (hexamer), 13, VII (heptamer) Marker proteins (●) used were as follows 2, ovalbumin, 3, bovine albumin (monomer), 7, bovine albumin (dimer), 8, γ-globulin, 10, bovine albumin (trimer), 14, horse apoferritin

Fig 6 Elution pattern of dextransucrase from a DEAE-cellulose column ○—○, dextransucrase activity, ●—●, protein, — —, concentration of NaCl (a) The extracellular dextransucrase The column (2 8 cm × 18 cm) was equilibrated with 0 005 M sodium phosphate buffer at pH 5 4 Fractions of 15 ml were collected and assayed for the enzyme activity at 35 °C as described in the text (b) The intracellular dextransucrase The column (2 2 cm × 18 cm) was equilibrated with 0 005 M sodium phosphate buffer at pH 6 0 Fractions of 10 ml were collected and assayed at 40 °C

corresponded to mixtures of isoenzymes II–VI The last peak, eluted with 0 3 M NaCl, corresponded to isoenzyme I, the monomeric subunit

The isoenzyme pattern of the intracellular dextransucrase

The intracellular enzyme was also analyzed by gel electrophoresis and two distinct bands with enzyme activity were observed And, in this case, two parallel lines were obtained by plotting log R_m against the gel concentration (Fig 4b) This showed that the intracellular enzymes differed in net charges but were the same in the molecular size Thus, they should be classified into the charge isomer family. Their molecular weights were estimated as approx 74 000 from the calibration curve (Fig 5) Furthermore, Fig 6b shows a clear separation of the isoenzymes by the DEAE-cellulose column The first peak, eluted with 0 15 M NaCl, corresponded to the slow moving isoenzyme II, shown in Fig 4b The second peak, eluted with 0 3 M NaCl, was identical to the fast moving isoenzyme I Compared with the monomeric form of the extracellular isoenzymes, the molecular weight of the intracellular iso-

enzymes were found to be somewhat high These results suggest that a marked difference exists between the extracellular and intracellular isoenzymes

DISCUSSION

The present study has shown that the dextransucrase activity of *L mesenteroides* NRRL B-1299 was induced by sucrose but not by glucose, as in the case of other strains [10]

Hehre et al [13] and Suzuki [14] described the intracellular dextransucrase activity from the cells of this strain In contrast to these findings, Smith [15] reported that the major part of the dextransucrase activity was retained in the particulate fraction and could not be solubilized by various procedures

The present study has indicated that the dextransucrase activity occurred both in the supernatant and in the sediment of the culture in a ratio of 2 3 in terms of total enzyme activity

Various methods of solubilizing the enzyme from the cells were attempted, and 41 3% of the activity of the cells was recovered by three steps of solubilization procedures However, 42 3% of the enzyme activity was still retained in the residual fraction, containing the cell-debris and water-insoluble dextran Whether the residual activity was bound to the cell-membrane or to the water-insoluble dextran remains to be clarified

Evidence for the dextransucrase reaction was obtained by experiments with [^{14}C]sucrose, the enzymes of both intra- and extracellular origin transferred glucosyl residues of the labeled sucrose into the polymer fraction On the other hand, there are few reports on the presence of a second enzyme with activity on sucrose in some dextran-producing bacteria, *L mesenteroides* NRRL B-512, *Streptococcus bovis* (strain I) [16] and *Lactobacillus* strain RWM-13 [17] In the present experiment, however, the amounts of glucose produced in the course of reaction was very small, as compared with fructose (Figs 3a, b) There is no work available concerning the isolation and characterization of invertase in the *L mesenteroides* B-1299 strain These results indicate that the invertase activity of this strain may not play a significant, if any, role in the extracellular and intracellular enzyme reactions

The ioenzyme pattern of the extracellular and the intracellular dextransucrase was demonstrated by the electrophoresis in polyacrylamide gel The method proposed by Hedrick and Smith [12] proved convenient for the rapid and accurate determination of the molecular weight of these enzymes, since the molecular weights of impure proteins can be determined by this method providing that a specific chemical or biological test for the identification of the protein is available An application of this technique has been reported for the demonstration of multiple forms of phosphorylase in sweet corn [18]

The extracellular isoenzymes differed each other in their properties, such as pH optima and Michaelis constants (data not shown) The elution pattern of the extracellular dextransucrase from a DEAE-cellulose column also clearly demonstrated the multiplicity of this enzyme Thus, the monomeric isoenzyme I and its highly aggregated form, isoenzyme N, were well separated by DEAE-cellulose column chromatography, whereas the intermediate oligomeric isoenzymes appeared between them in three peaks as a mixture of these oligomers

Two intracellular enzymes, shown to belong to the charge isomer family, were eluted in two peaks from a DEAE-cellulose column at pH 6 0 by a linear NaCl gradient These enzymes also differed in their pH optima and Michaelis constants (data not shown) Purification of these enzymes and the structure of their reaction products will be reported elsewhere

The occurrence of different transglucosylases in the *L mesenteroides* species has not yet been observed But in the case of predominant streptococcal groups isolated from human dental plaque, such as *S sanguis* [19] and *S mutans* [20], several distinct transglucosylases were isolated Although these enzymes were characterized by their isoelectric points, pH optima, K_m values and electrophoretic mobilities in polyacrylamide gel, their role in the polysaccharide synthesis has not yet been clarified sufficiently

Dextran elaborated by *L mesenteroides* NRRL B-1299 is heterogeneous in its molecular weight and solubility [4] Methylation analysis of five fractions of this dextran has shown the occurrence of α-1,3- and α-1,2-linkages in the dextran [3] Thus it is important to elucidate the functional roles of each isoenzyme in the dextransucrase reaction, correlating them with the structural characteristics of the product dextran

ACKNOWLEDGEMENTS

The authors would like to thank Dr K Tamari for stimulating discussions and Dr H Nagayama for helpful suggestions during the preparation of this manuscript

REFERENCES

1 Hehre, E J (1951) Adv Enzymol 11, 297–337
2 Ebert, K H and Schenk, G (1968) Adv Enzymol 30, 179–221
3 Kobayashi, M , Shishido, K , Kikuchi, T and Matsuda, K (1973) Agric Biol Chem 37, 2763–2769
4 Kobayashi, M , Shishido, K , Kikuchi, T and Matsuda, K (1973) Agric Biol Chem 37, 357–365
5 Nelson, N (1944) J Biol Chem 153, 375–380
6 Somogyi, M (1945) J Biol Chem 160, 69–73
7 Koepsell, H J and Tsuchiya, H M (1952) J Bacteriol 63, 293–295
8 Lowry, O H , Rosebrough, N J , Farr, A L and Randall, R J (1951) J Biol Chem 193, 265–275
9 Maurer, H R (1968) Disk Elektrophorese, pp 32–110, Walter de Gruyter, Berlin
10 Neely, W B and Nott, J (1962) Biochemistry 1, 1136–1140
11 Goodman, A , Weil, R M and Stern, K G (1955) J Biol Chem 217, 977–985
12 Hedrick, J L and Smith, A J (1968) Arch Biochem Biophys 126, 155–164
13 Hehre, E J and Suzuki, H (1966) Arch Biochem Biophys 113, 675–683
14 Suzuki, Y (1965) J Vitaminol 11, 320–326
15 Smith, E E (1970) FEBS Lett 12, 33–37
16 Bailey, R W and Bourne, E J (1961) Nature 191, 277–278
17 Dunican, L K and Seeley, Jr, H W (1963) J Bacteriol 86, 1079–1083
18 Lee, E Y C and Braun, J J (1973) Ann N Y Acad Sci 210, 115–128
19 Newbrun, E (1971) Caries Res 5, 124–134
20 Guggenheim, B and Newbrun, E (1969) Helv Odontol Acta 13, 84–97