BBA 66467

IMMOBILISATION OF α-AMYLASE BY ATTACHMENT TO SOLUBLE SUPPORT MATERIALS

J. R. WYKES, P. DUNNILL AND M. D. LILLY

Biochemical Engineering Section, Department of Chemical Engineering, University College London, Torrington Place, London W.C.1 (Great Britain)

(Received July 8th, 1971)

SUMMARY

1. α-Amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) has been immobilised by attachment to soluble amino-*s*-triazinyl derivatives of dextran 2000 DEAE-dextran 2000 and to CM-cellulose (Edifas B10).

2. These amylase derivatives show up to 67% of the specific activity of the free enzyme.

3. The pH optimum shows 0.5 unit shifts depending on the charge of the support.

4. Attachment of amylase to CM-cellulose and DEAE-dextran increases the stability of the enzyme at neutral pH. Attachment to CM-cellulose increases the heat stability of the enzyme.

5. The increased stability of the CM-cellulose–amylase compared with free amylase was demonstrated in the continuous hydrolysis of starch in an ultrafilter reactor.

INTRODUCTION

Recent work on immobilised enzymes has concentrated on attachment of enzymes to water-insoluble supports such as cellulose[1] and dextran derivatives[2], polyacrylamide[3], and porous glass[4]. Using such immobilised enzymes in a reactor it is apparent that the rate of diffusion of substrate to the enzyme active sites and steric factors may limit the conversion of substrate to products[5,6]. Such effects are likely to be more pronounced with substrates of high molecular weight. To retain some of the advantages of immobilisation, but at the same time, retain good accessibility with high molecular weight substrates, the attachment of enzymes to soluble supports has been investigated. Recently we reported on the properties of soluble immobilised chymotrypsin for casein hydrolysis in an ultrafilter reactor[7]. As a second representative enzyme acting on high molecular weight substrates α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) was chosen because of its economic use in starch liquification and as studies have been made on its attachment to insoluble supports[8-11].

The effects on the activity, optimum pH for activity and stability at various pH values and temperatures of attachment of α-amylase to dextran, DEAE-dextran and CM-cellulose have been investigated.

MATERIALS

A purified α-amylase (Maxamylase) was a gift from Royal Netherlands Fermentation Industries, Delft, Holland. Edifas, grade B10 (soluble CM-cellulose), was a gift from ICI Ltd., Stevenson, Ayrshire, Scotland. Dextran-2000 and DEAE-dextran (both mean mol. wt. $2 \cdot 10^6$) were obtained from Pharmacia Ltd. Soluble starch from maize was obtained from BDH, Poole, Dorset. Membranes used for ultrafiltration were obtained from Amicon Corporation, Lexington, Massachusetts, U.S.A.

METHODS

s-Triazine derivatives of dextran, DEAE-dextran and CM-cellulose were obtained by reaction with 2-amino-4,6-dichloro-*s*-triazine prepared from cyanuric chloride as described previously[12]. 10 g of soluble polymer were dissolved in 100 ml of water (200 ml in the case of CM-cellulose) and adjusted to pH 11.0. 0.5 g *s*-triazine in 20 ml of 50% acetone:water (v/v) were also adjusted to pH 11.0 and the two solutions mixed and stirred vigorously. The pH was maintained at 11.0 by addition of alkali in a pH-stat until 0.2 mequiv of alkali per g dry weight of polymer had been added. The reaction was stopped by reducing the pH to between 3 and 4 with HCl. Unreacted *s*-triazine was removed by precipitation of the polymer with 2 vol. of acetone. The precipitate was collected by centrifugation, redissolved in 100 ml of water and the washing process repeated twice. The *s*-triazine activated polymer was finally dried in a vacuum desiccator and stored at —10°.

Immobilised α-amylase preparations were made by reacting α-amylase with *s*-triazinyl derivatives (0.5 g) of dextran, DEAE-dextran and CM-cellulose in borate buffer, pH 8.8, for 16 h at room temperature. The reaction volumes, weights of α-amylase added and borate concentrations for each experiment are given in Table I. The *s*-triazinyl derivatives contained 0.2 mequiv of *s*-triazine per g dry weight of polymer, except for the preparation of CM-cellulose–amylase (3) where the derivative contained 1.0 mequiv per g dry weight of polymer.

The attachment reaction was stopped by adjusting the pH to 6.0 and the unbound enzyme was removed by ultrafiltration using an Amicon XM100 membrane.

TABLE I

REACTION CONDITIONS FOR IMMOBILISATION OF α-AMYLASE

α-Amylase derivative	*Reaction vol. (ml)*	*Buffer concn. (M)*	*Amylase added (mg)*
Dextran (1)	20	0.125	50
Dextran (2)	20	0.20	60
DEAE-dextran	50	0.02	50
CM-cellulose (1)	20	0.125	50
CM-cellulose (2)	17.5	0.20	75
CM-cellulose (3)	20	0.125	75

The buffer used for washing contained 0.05 M glycerophosphate, 0.1 M NaCl, 0.001 M $CaCl_2$, pH 6.0.

Bound protein was estimated using the method of LOWRY *et al.*[14]. In the case of DEAE-dextran–amylase no result could be obtained as a precipitate formed even though none formed with *s*-triazinyl DEAE-dextran alone. Bound protein in this case was therefore calculated from the $A_{280\ nm}$ of the added α-amylase and the $A_{280\ nm}$ of the ultrafiltrate which contained unbound α-amylase.

Stock solutions of α-amylase (5.0 mg/ml) were prepared in 0.05 M glycerophosphate (pH 6.0) containing 0.1 M NaCl and 0.001 M $CaCl_2$.

α-Amylase was assayed using soluble starch as substrate in buffers that did not precipitate Ca^{2+}. The standard reaction mixture was made up of 1.0 ml of 2% starch solution in 0.20 M NaCl + 0.002 M $CaCl_2$, 0.90 ml of 0.10 M glycerophosphate buffer (pH 6.0) and 0.10 ml of enzyme solution containing 5–30 μg protein. The reducing groups formed were estimated using 2,5-dinitrosalicylic acid[13] after incubation at 50°, usually for 3 min. Since maltose was used as a standard in the assay, results are expressed in terms of μmoles of maltose formed.

Where the pH optimum of α-amylase derivatives was investigated the initial rate of the hydrolysis was obtained using reaction mixtures containing 1 ml of 2% starch solution in 0.02 M NaCl + 0.002 M $CaCl_2$, 0.90 ml of a mixed buffer 0.02 M in acetate, glycerophosphate and Tris of appropriate pH, 0.10 ml of enzyme solution containing 5–30 μg protein diluted in the same buffer immediately prior to use. In experiments to measure the stability of the α-amylase preparation at different pH values and temperatures, the enzyme was diluted in the appropriate 0.10 M buffer either with or without Ca^{2+} and 0.1 ml was assayed in a standard reaction mixture at pH 6.0. In cases where the enzyme preparation was incubated in the absence of Ca^{2+} and the dilution of the enzyme solution was less than 50 times, the preparation first was dialysed against a Ca^{2+} free buffer overnight at 5°.

The hydrolysis of starch at 70° by amylase or CM-cellulose–amylase in a continuous-flow ultrafiltration reactor was examined. The reactor was fitted with an Amicon PM 10 membrane to retain the enzyme or immobilised enzyme, and contained 50 ml of substrate to which 50 μg of α-amylase or 50 μg of CM-cellulose–amylase (Preparation 3) was added. 0.4% starch solution in 0.1 M NaCl, 0.001 M $CaCl_2$ and 0.02 M Tris·HCl buffer (pH 6.5) was fed in under a pressure of 18 lb/inch² gauge. The filtrate volume collected per hour was measured and 1- or 2-ml samples were used for the estimation of reducing groups. The output was calculated as μmoles of maltose per h.

RESULTS

α-Amylase has been attached to uncharged dextran, DEAE-dextran and soluble CM-cellulose. α-Amylase has an isoelectric point pH 6.0 (ref. 15) approximately. Since attachment to supports was done at pH 8.8 the protein was negatively charged at this pH and attachment to positively charged supports was favoured. Conversely attachment to negatively charged supports may be expected to be low. In the latter case mutual repulsion of the enzyme and support can be partially overcome by raising the ionic strength of the reaction mixture used for attachment.

Table II compares the specific activity of various dextran-amylase and CM-cellulose–amylase preparations. The dextrans showed good uptake of protein with

TABLE II

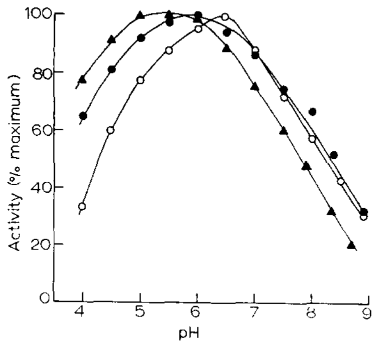
ACTIVITY OF AMYLASE PREPARATIONS

Preparation	*Protein added (mg)*	*Uptake (%)*	*Support (mg/g)*	*Specific activity**
α-Amylase	—	—	—	420
Dextran–amylase (1)	50	61	61	250
Dextran–amylase (2)	60	65.5	80	283
DEAE-dextran–amylase	50	90	90	140
CM-cellulose–amylase (1)	50	6.4	6.2	250
CM-cellulose–amylase (2)	75	17.1	25.5	106
CM-cellulose–amylase (3)	75	19.2	28.8	44

* μmoles · min^{-1} · mg^{-1}protein.

25–67% of the specific activity of the free amylase. With CM-cellulose uptake of protein was very much reduced but the specific activity remained high. Increasing the ionic strength increased the protein attached to CM-cellulose but the specific activity was adversely affected. There was no advantage in using a CM-cellulose more highly substituted with *s*-triazinyl residues as again the specific activity was reduced possibly due to multiple attachment of each enzyme molecule to the support.

The pH optimum of each α-amylase preparation was determined in a mixed buffer system of low ionic strength (Figs. 1 and 2). Attachment to CM-cellulose shifted the optimum from pH 6.0 to 6.5 and reduced the activity on the acid side of the optimum. However, little increase in activity occurred on the alkaline side compared with the free enzyme (Fig. 1). Attachment to DEAE-dextran gave a derivative with maximum activity at about pH 5.5 and the whole profile was shifted 0.5 pH units to the acid side compared with the free enzyme (Fig. 1). The dextran-amylase had a broad optimum activity between pH 5.5 and 6.5 compared with the sharper optimum for the free enzyme at pH 6.0 but without a significant shift in profile (Fig. 2).



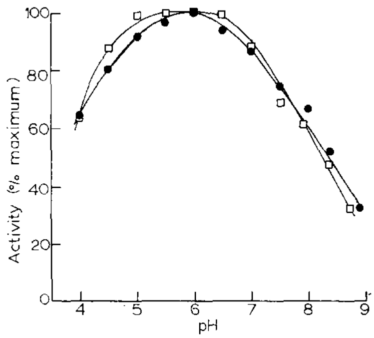


Fig. 1. The effect of attachment of α-amylase to soluble DEAE-dextran and CM-cellulose on the pH profile of starch hydrolysis. Enzyme activities were assayed at 50° with 1.0% starch solution. The pH was controlled using a mixed buffer system 0.01 M in acetate, glycerophosphate and Tris. ●—●, amylase; ▲—▲, DEAE-dextran–amylase; ○—○, CM-cellulose–amylase.

Fig. 2. The effect of attachment of amylase to dextran on the pH profile of starch hydrolysis. The reaction conditions were the same as described in Fig. 1. ●—●, amylase; □—□, dextran–amylase.

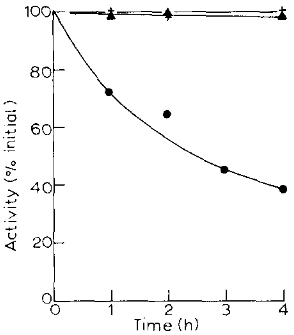


Fig. 3. Decay of α-amylase in the presence of Ca^{2+} incubated at 50° in buffers of different pH. α-Amylase was diluted in the buffers indicated, incubated at 50° and 0.1 ml assayed in the standard reaction mixture at pH 6.0 after various time intervals. ●—●, 0.10 M glycerophosphate buffer pH 5.0; +—+, 0.10 M glycerophosphate buffer (pH 7.0); ▲—▲, 0.10 M Tris·HCl buffer (pH 9.0). All buffers contained 0.001 M $CaCl_2$.

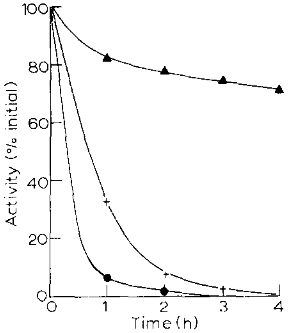


Fig. 4. Decay of α-amylase incubated at 50° in buffers of different pH in the absence of Ca^{2+}. α-Amylase was diluted in the buffers indicated, incubated at 50° and 0.1 ml assayed in the standard reaction mixture at pH 6.0 after various time intervals. ●—●, 0.10 M glycerophosphate buffer (pH 5.0); +—+, 0.10 M glycerophosphate buffer (pH 7.0); ▲—▲, 0.10 M Tris·HCl (pH 9.0).

The effect of immobilisation on the stability of α-amylase was investigated. α-Amylase is stabilised by starch and Ca^{2+} (refs. 16,17). Fig. 3 shows that the stability of α-amylase in the presence of Ca^{2+} decreased below pH 7. There was no loss of activity over the period of the assay. In the absence of Ca^{2+} (Fig. 4) stability at the comparable pH was decreased and maximum stability occurred at pH 9.0. Since α-amylase was less stable in the absence of Ca^{2+}, effects of attachment to soluble supports were more likely to be apparent by incubating the enzyme preparation in the absence of

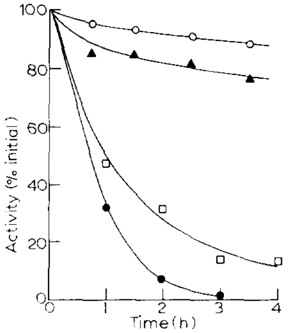


Fig. 5. Decay of soluble immobilised derivatives of amylase at pH 7.0 and 50° in the absence of Ca^{2+}. After dialysis, overnight at 5° the soluble immobilised derivatives of amylase were diluted in 0.10 M glycerophosphate buffer (pH 7.0) and incubated at 50°. The stock solution of α-amylase was diluted 50 × in the same buffer. 0.1-ml samples were assayed in the standard reaction mixture at pH 6.0 after various time intervals. ●—●, amylase; □—□, dextran–amylase (Preparation 2); ▲—▲, DEAE-dextran–amylase; ○—○, CM-cellulose–amylase (Preparation 2).

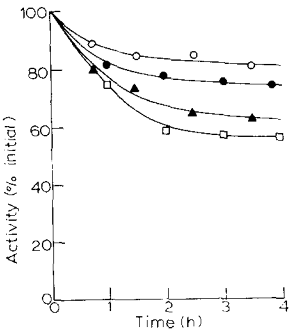


Fig. 6. Decay of soluble immobilised derivatives of amylase at pH 9.0 and 50° in the absence of Ca^{2+}. The same procedure as given in Fig. 5 was followed except that 0.1 M Tris·HCl buffer pH 9.0 was used. ●—●, amylase; □—□, dextran–amylase (Preparation 2); ▲—▲, DEAE-dextran–amylase; ○—○, CM-cellulose–amylase (Preparation 2).

Ca^{2+}. The stability of each α-amylase derivative was investigated at pHs 5.0, 7.0 and 9.0. There was no increase in stability at pH 5.0, but at pH 7.0 both DEAE-dextran–amylase and CM-cellulose–amylase were more stable than the free enzyme (Fig. 5). These differences were not so marked at pH 9.0, only CM-cellulose–amylase having marginally increased stability compared to the free enzyme (Fig. 6).

In view of the increased stability obtained on attachment to DEAE-dextran and CM-cellulose apparent at pH 7.0 it was of interest to see if such derivatives could be used for the hydrolysis of starch on a continuous basis. This can be done in a stirred reactor from which the products are removed by ultrafiltration through a suitable membrane and simultaneously replaced by feeding in fresh starch solution[18]. Since both free and immobilised amylase were relatively stable in the presence of Ca^{2+}, these experiments were done at 70° to increase the rate of denaturation and to take advantage of the maximum rates of hydrolysis of starch.

An initial experiment was done to examine the stability of α-amylase, DEAE-dextran–amylase and CM-cellulose–amylase (Preparation 3 was used) at 70° in the presence of 0.001 M Ca^{2+} at pH 7.0. Under these conditions there is a marked stabilisation of the amylase on attachment to CM-cellulose compared with attachment to DEAE dextran and free α-amylase (Fig. 7). CM-cellulose was therefore chosen for use in a reactor experiment for comparison with free α-amylase. Equal amounts of protein were introduced into the reactor in each case. The specific activity of the CM-cellulose–amylase (Preparation 3) was 10% of that of the free enzyme. On reaching a steady state the CM-cellulose–amylase was producing half the amount of hydrolysed starch compared with the maximum amount produced by the free enzyme. The free enzyme lost 82% of its activity after 70 h compared with the CM-cellulose–amylase which lost 32% of its activity in the same time (Fig. 8).

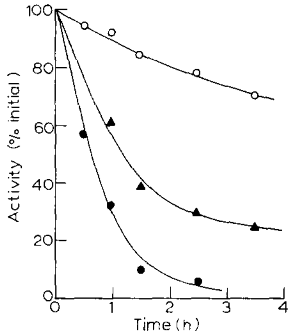


Fig. 7. Decay of soluble immobilised derivatives of amylase at pH 7.0 and 70° in the presence of Ca^{2+}. The same procedure as given in Fig. 6 was followed except that the buffer contained 0.001 M $CaCl_2$ and the incubation was at 70°. ●—●, amylase; ▲—▲, DEAE-dextran–amylase; ○—○, CM-cellulose–amylase (Preparation 3).

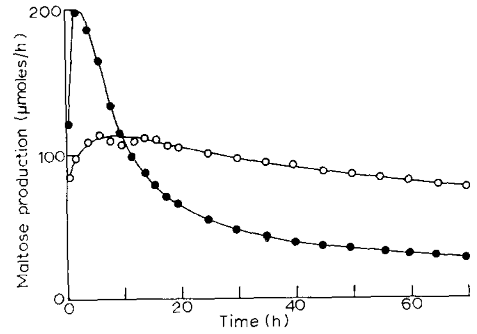


Fig. 8. The continuous hydrolysis of starch in a reactor using amylase (●) and CM-cellulose–amylase (○) as catalysts.

DISCUSSION

There have been few reports of enzymes attached to soluble supports. Chymotrypsin has been attached to soluble CM-cellulose using an acid azide method[19]. Chymotrypsin has also been attached to polycarboxylic acid polymers using *N*-ethyl-5-phenylisoxazolium 3-sulphonate as coupling agent[20]. In general these soluble enzymic derivatives show good uptake of added protein and good retention of activity. No further information is available on their properties. Attachment of chymotrypsin to dextran compared with attachment to insoluble DEAE-cellulose showed the advantages of using a soluble support for an enzyme attacking a high molecular weight substrate and the use of such a derivative in an ultrafilter reactor[7].

α-Amylase has been chemically attached to several insoluble supports. MANECKE[8] has employed nitrated copolymers of methylacrylic acid, methylacrylic acid–*m*-fluoroanilide crosslinked with divinylbenzene and nitrated copolymers of methylacrylic acid, 4- or 3-fluorostyrene and divinylbenzene. Enzyme activities did not exceed 3% of those of the free enzyme and enzyme stability was not enhanced. BARKER *et al.*[9] have attached α-amylase to micro-crystalline cellulose *via* diazotised 3-(*p*-aminophenoxy)-2-hydroxypropyl ethers and 2-hydroxy-3-(*p*-isothiocyanatophenoxy) propyl ethers of cellulose. Derivatives exhibited up to 6% of the free enzyme activity and enhanced stability, 20% remaining after 7 days at 45°. BARKER *et al.*[10] have also attached α-amylase *via* diazo and isothiocyanato coupling to cross-linked copolymers of acrylamide incorporating arylamino and acid hydrazide groups. Derivatives exhibited up to 16% of the free enzyme activity and enhanced stability: up to 25% remaining after 4 days at 45°. LEDINGHAM AND HORNBY[11] attached α-amylase to CM-cellulose, *p*-aminobenzyl-cellulose and polystyrene *via* azide and diazo coupling techniques. Specific activities were respectively 4.3, 4.9 and 9.2% of the free enzyme. The specific activities of the soluble immobilised α-amylase described here which are as high as 67% of the free enzyme compare very favourably with insoluble derivatives.

Attachment to the charged supports, DEAE-dextran and soluble CM-cellulose, causes shifts in the pH optimum compared with the free amylase. Surrounding an enzyme with negatively charged carboxyl groups might be expected to cause a localised lowering of pH with respect to the buffer. Hence there will be an apparent alkaline shift in the pH optimum. This appears to be true for the acid side of the pH activity curve of soluble CM-cellulose-amylase but the alkaline side is only slightly affected. DEAE-dextran would be expected to have the opposite effect and this was shown by a shift to a lower pH on the alkaline side of the optimum (Fig. 3). However the effect on the pH optimum is evidently distorted as attachment to dextran causes a broadening of the pH activity profile. Shifts in pH optimum have been observed for various enzymes attached to charged insoluble supports[21,22] but the effect has not previously been reported for soluble immobilised enzymes.

It is known that α-amylase is stabilised in the presence of starch[15] a polymer of glucose α-1.4 linked. Attachment to dextran had little effect on the stability of α-amylase incubated at 50° in the absence of Ca^{2+}. This may be because dextran is a α-1.6 linked polymer of glucose and is not of the correct stereochemical configuration to induce stabilisation. Attachment to DEAE-dextran does however stabilise the α-amylase. This may be because positively charged DEAE groups can partially substitute for Ca^{2+}. Stabilisation is however most marked on attachment to CM-cellulose. Even though negatively charged groups are present the cellulose (β-1.4 linked glucose) may be sufficiently similar to starch to induce stabilisation.

The stability of the soluble CM-cellulose-amylase was also demonstrated under operational conditions. BUTTERWORTH *et al.*[18] have demonstrated the use of free α-amylase in an ultrafilter reactor for the continuous hydrolysis of starch at 40°. Under their conditions 34% of the enzyme was apparently lost through the membrane in 4 days. We have repeated this experiment at 70° and shown an advantage using an immobilised enzyme. Enhanced stability is observed for the soluble derivative: 68% of the activity is retained at 70° over a period of 70 h whereas the free amylase retains 18% of its activity over the same period.

ACKNOWLEDGEMENTS

The authors are pleased to acknowledge the skilled technical assistance of Y. Saloojee and the support of the Science Research Council.

REFERENCES

1 B. P. SURINOV AND S. E. MANOILOV, *Biokhimiya*, 31 (1966) 387.
2 R. AXEN, J. PORATH AND S. ERNBACH, *Nature*, 214 (1967) 1302.
3 K. MOSBACH, *Acta Chem. Scand.*, 24 (1970) 2093.
4 H. H. WEETALL, *Science*, 166 (1969) 615.
5 M. D. LILLY, W. E. HORNBY AND E. M. CROOK, *Biochem. J.*, 100 (1966) 718.
6 R. AXEN, P. A. MYRIN AND J. C. JANSON, *Biopolymers*, 9 (1970) 401.
7 S. P. O'NEILL, J. R. WYKES, P. DUNNILL AND M. D. LILLY, *Biotech. Bioeng.*, 13 (1971) 319.
8 G. MANECKE, *Pure Appl. Chem.*, 4 (1962) 507.
9 S. A. BARKER, P. J. SOMERS AND R. E. EPTON, *Carbohyd. Res.*, 8 (1968) 491.
10 S. A. BARKER, P. J. SOMERS, R. E. EPTON AND J. V. MCLAREN, *Carbohyd. Res.*, 14 (1970) 287.
11 W. M. LEDINGHAM AND W. E. HORNBY, *FEBS Lett.*, 5 (1969) 118.
12 G. KAY AND M. D. LILLY, *Biochim. Biophys. Acta*, 198 (1970) 276.
13 W. S. WHELAN, in R. L. WHISTLER, *Methods in Carbohydrate Chemistry*, Vol. 4, Academic Press, New York, 1964, p. 252.
14 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
15 E. H. FISCHER AND E. A. STEIN, in P. D. BOYER, H. LARDY AND K. MYRBACK, *The Enzymes*, Vol. 4, Academic Press, New York, 2nd ed., 1961, p. 313.
16 *Bacterial Amylase*, Royal Dutch Fermentation Industries, Ltd., Delft, Technical Information Bulletin, 1969.
17 M. H. MOSELEY AND L. KEAY, *Biotech. Bioeng.*, 12 (1970) 251.
18 T. A. BUTTERWORTH, D. I. C. WANG AND A. J. SINSKEY, *Biotech. Bioeng.*, 12 (1970) 615.
19 M. A. MITZ AND L. J. SUMMARIA, *Nature*, 189 (1961) 576.
20 R. P. PATEL, D. V. LOPIEKES, S. P. BROWN AND S. PRICE, *Biopolymers*, 5 (1967) 577.
21 L. GOLDSTEIN, Y. LEVIN AND E. KATCHALSKI, *Biochemistry*, 3 (1964) 1913.
22 L. GOLDSTEIN, in D. PERLMAN, *Fermentation Advances*, Academic Press, New York, 1969.