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CALORIMETRIC ANALYSIS OF SUGARS AND SUGAR DERIVATIVES WITH AID OF AN ENZYME THERMISTOR

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ABSTRACT

Enzymic determination of L-ascorbic acid, D-galactose, D-glucose, cellobiose, lactose, and sucrose with an enzyme thermistor is described. The enzyme thermistor is a simple flow-calorimeter designed for routine analysis and measures metabolites by the heat produced in a small column with immobilized enzyme. This instrument may be used for determination of discrete samples as well as for continuous monitoring. As the change of enthalpy produced by the primary reaction is usually large enough to make the measurement sufficiently accurate, simple assay procedures can be designed, an advantage of great importance when the samples are crude and complex.

INTRODUCTION

Direct analysis of various sugars in separate samples, and also continuous monitoring of such sugars, is needed in such fields as medicine, food technology, and fermentation. Current methods are usually operated for analysis of discrete samples. Separation is achieved via such chromatographic techniques, as ion exchange chromatography[1], high-performance liquid chromatography (l.c.)[2], and gas chromatography[3]. Enzymic analysis of carbohydrates has thus far mainly been based on spectrophotometry[4].

Sometimes a specific enzymic step may be monitored directly[5], but as a rule one or more auxiliary enzyme(s) must be used to obtain a measurable product[6]. Especially when analyzing complex samples, it would be advantageous if a given sugar component could be measured directly in a one-step procedure by using a specific enzyme, as the products formed in subsequent steps might already be present in the sample and thereby necessitate a blank determination. Generally, the change in enthalpy in association with an enzymic reaction is large enough to be useful for such direct determination, which eliminates the need of further steps. This paper describes some methods using enthalpimetry combined with immobilized enzymes in sugar analyses.

MATERIALS AND METHODS

D-Galactose oxidase (E.C. 1.1.3.9) 200 units/mg, from *Dactylium dendroides*, D-glucose oxidase (E.C. 1.1.3.4) 200 units/mg, from *Aspergillus niger*, and invertase (E.C. 3.2.1.26) 400 units/mg, from bakers yeast were purchased from Sigma, St. Louis, Mo., U.S.A. L-Ascorbate oxidase (E.C. 1.10.3.3) from *Cucurbita* and β-D-glucosidase (E.C. 3.2.1.21) from almonds, were obtained from Boehringer Mannheim, West Germany. Lactase, 66 units/mg, from *Aspergillus niger* was obtained from Société Rapidase, Séclin, France.

D-Galactose, cellobiose, and L-ascorbic acid were obtained from Merck, Darmstadt, West Germany. Controlled-pore glass (CPG-10, mean pore diameter 73 nm, 80–120 mesh) was purchased from BDH, Poole, England, and concanavalin A–Sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of analytical grade.

Enzyme immobilization. — The enzymes were immobilized on controlled-pore glass by following procedures described elsewhere[6,7].

The biospecific, reversible immobilization of glycoproteins was conducted on concanavalin A–Sepharose (column size 0.5 mL) by introducing the enzyme as a short pulse into the buffer flow that was continuously pumped through the thermistor (flow rate 0.85 mL/min). On passage of the lectin-column binding took place. Washing with salt (1 mol/L of sodium chloride) eliminated unspecifically bound enzyme. At this point, the thermistor was ready for enzymic analyses. When it was time to recharge the column, the old enzyme was first washed out by a pulse of 0.1 mol/L of glycine · HCl, pH 2.2 and, after reconditioning to the perfusion buffer, fresh enzyme could be introduced[8].

Assay procedure. — The sample to be analyzed was introduced as a pulse into the continuous flow by means of a three-way valve. In all experiments involving enzymes bound to controlled-pore glass, a flow of 1.0 mL/min was used. On passage of the sample through the enzyme column, a reaction takes place and heat is liberated. The temperature at the outlet of the enzyme column is registered by a thermistor[7].

Assay of sucrose: The reaction studied was:

$$\text{sucrose} + H_2O \xrightarrow{\text{invertase}} \text{D-glucose} + \text{D-fructose}.$$

Invertase was immobilized either on controlled-pore glass or on concanavalin A–Sepharose. Perfusing buffer was 0.1M sodium citrate–phosphate, pH 5.5. Samples of different concentrations of sucrose dissolved in the same buffer were introduced as already described.

Assay of lactose: The reaction(s) studied was:

$$\text{lactose} + H_2O \xrightarrow{\text{lactase}} \text{D-glucose} + \text{D-galactose}$$

$$\text{D-glucose} + O_2 \xrightarrow{\text{D-glucose oxidase}} \text{D-glucono-1,5-lactone} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2} O_2.$$

Lactase immobilized on controlled-pore glass was used. As the heat of hydrolysis proved too little to give sufficient sensitivity to an assay based on the heat of the primary reaction, the glass-bound lactase preparation was placed in a precolumn situated outside the apparatus and a preparation of D-glucose oxidase and catalase bound to controlled-pore glass was placed inside the thermistor unit. The perfusing buffer was 0.1M sodium acetate, pH 5.0. An alternative arrangement with a mixture of the two preparations of immobilized enzymes within the thermistor bed was also used.

Assay of galactose: The reaction studied was:

$$\text{D-galactose} + O_2 \xrightarrow{\text{D-galactose oxidase}} \text{D-}\textit{galacto}\text{-hexodialdose} + H_2O_2.$$

D-Galactose oxidase was bound to controlled-pore glass and packed in the thermistor column. The perfusing buffer used was 0.1M Tris·HCl, pH 7.4. When not in use, the system was equilibrated with Tris buffer containing mM copper sulfate, Cu^{2+} ions reportedly increasing the stability of the D-galactose oxidase column[9].

Assay of L-ascorbic acid: The reaction studied was:

$$2\ \text{L-ascorbate} + O_2 \xrightarrow{\text{L-ascorbate oxidase}} 2\ \text{dehydroascorbate} + 2\ H_2O$$

The enzyme was immobilized by biospecific reversible immobilization on concanavalin A–Sepharose packed in the thermistor column. The column used contained 0.5 mL of gel to which was added 4.5 U of enzyme. The perfusing buffer used was 0.1M sodium acetate, pH 5.5.

RESULTS AND DISCUSSION

A problem in using conventional methods for analysis of disaccharides has been the need of several auxiliary enzymic steps and additional substrates to obtain colored products. In the experiments reported here, we applied several different disaccharidases for hydrolysis of their respective disaccharide into monosaccharides in the hope that the heat of the primary reaction would be sufficient to permit quantitative analysis. The various reactions differed widely in the heat they evolved, and thus in their sensitivities (Table I). The heat of hydrolysis of sucrose was much greater than that of hydrolysis of the other disaccharides. Thus, determination of

TABLE I

Compound	*Enzyme(s)*	*Concentration range (mM)*
Sucrose	Invertase	1–100
Lactose	β-D-Galactosidase + D-glucose oxidase + catalase	0.25–5
Cellobiose	β-D-Glucosidase + D-glucose oxidase + catalase	0.1–10
D-Galactose	D-Galactose oxidase	0.5–10
D-Glucose	D-Glucose oxidase + catalase	0.05–0.7
L-Ascorbic acid	L-Ascorbate oxidase	0.05–0.6

lactose required a subsequent enzyme-catalyzed step involving D-glucose oxidase to obtain sufficiently high signals. This result implies that calorimetric determination of disaccharides also requires auxiliary enzymic step(s), but only with such enzymes as D-glucose oxidase or D-galactose oxidase, preferably combined with catalase. However, when analyzing samples containing both the disaccharide and the monosaccharide, a correction for the content of monosaccharide must be made. This may be performed by the use of a split-flow enzyme thermistor[10,11] where enzymes for the splitting of disaccharides and the auxilliary enzymes are in one flow line, and in the other line is an identical amount of the auxiliary enzymes, but no enzyme to split the disaccharide. In those instances, the temperature difference between the two columns gives a measure on the content of disaccharide present in the sample. Another alternative, which has been used, for instance, in the analysis of cellobiose[11] in the presence of D-glucose, is to keep the disaccharide-splitting enzyme in a pre-column to the D-glucose oxidase thermistor. By switching the flow through or around the precolumn, a difference in heat evolved is observed. This difference may readily be correlated to the amount of cellobiose present in the sample. At high concentrations of monosaccharides in comparison with that of the disaccharide to be converted, pretreatment of the sample to decrease the amount of monosaccharide is a possibility. In practice, this does not make the method more complicated. Furthermore, the stability of such a system as D-glucose oxidase is high, and the sum of the enthalpies of the reactions is substantial[11].

The fact that sucrose can be analyzed directly makes it possible to obtain a linear relationship between temperature response and concentration over extended ranges as, unlike methods using oxidases as auxiliary enzymes, there is no need for oxygen.

Fig. 1 gives standard curves for sucrose within two different ranges, 0–10 and 0–100mM, indicating both a high sensitivity and a wide range of linearity. The temperature peaks reproduced in Fig. 2 give an impression of the precision of the method, which is usually within ±2% r.s.d., as well as the rapidity of analysis using moderate flow rates and rather small sample-volumes. With larger sample-volumes,

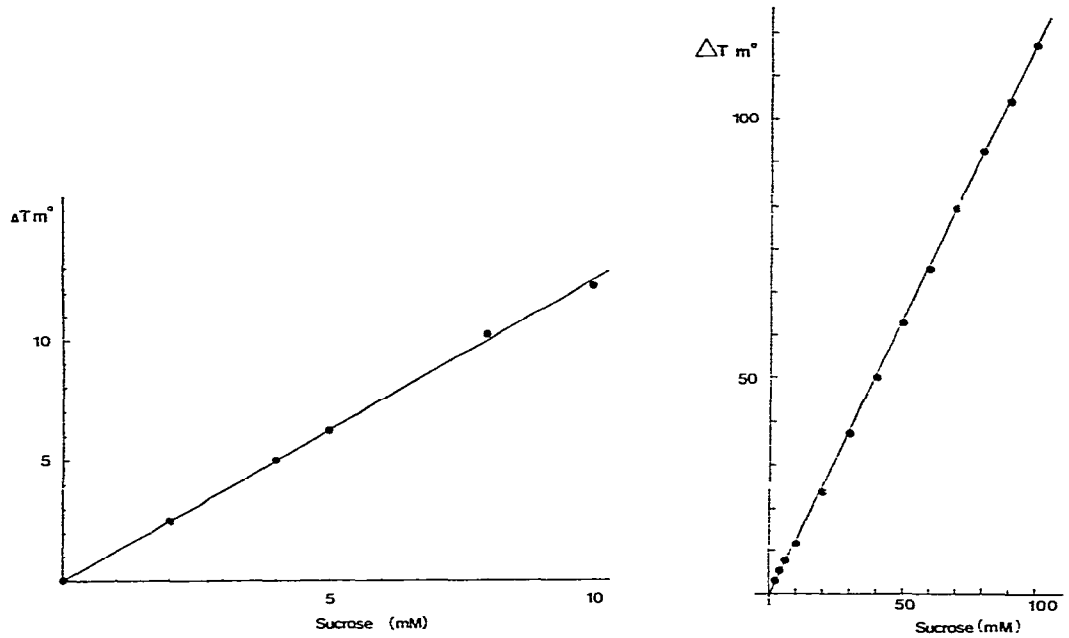

Fig. 1. Calibration curves showing the heat response ΔT (mdegree), measured as peak heights, for enzyme-thermistor assay of sucrose using invertase immobilized on controlled porous glass. Further experimental details are given in the text.

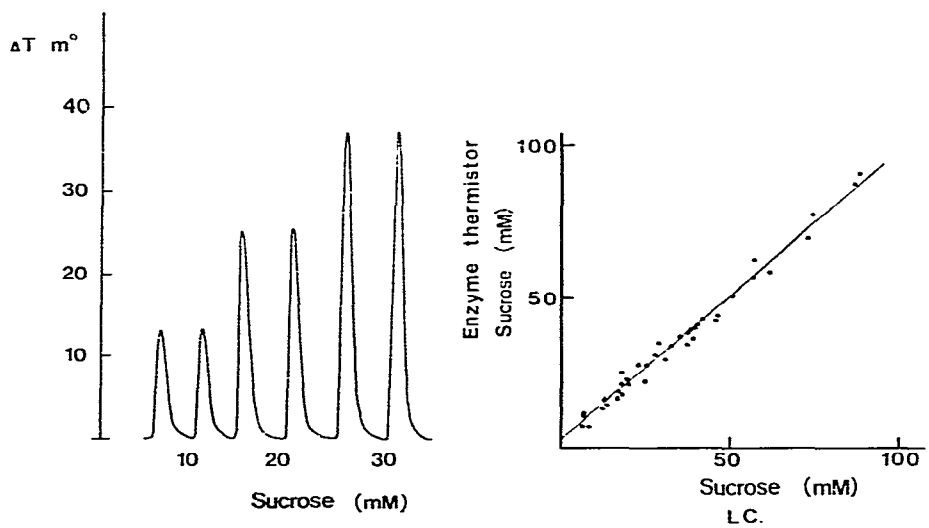

Fig. 2. Temperature responses obtained for pulses of different sucrose concentrations. Experimental details as described in the text. Total time needed for six analyses was 30 min.

Fig. 3. Correlation between sucrose concentrations as measured by the enzyme-thermistor technique and an l.c. technique[2]. $y = 0.96 \times + 4.6$; $r = 0.992$; and $n = 39$.

the temperature response increases (up to a volume giving thermal steady-state), but also the time required per determination increases.

Furthermore, the invertase system was tested for interference by any D-glucose present in the sample. No such interference was found, not even with concentrations of D-glucose as high as 50mM at 50mM sucrose.

Fig. 3 shows a comparison between concentration of sucrose determined by the enzyme thermistor, and a currently used l.c.-technique[2]. Although the samples used in the study were made up in buffer, we have previously demonstrated on repeated occasions that the enzyme-thermistor technique is applicable to crude samples[7,11]. The standard deviation was determined for 10 samples at 50 $\pm$1 mM.

In a recent study of the analysis of products, especially cellobiose, formed on enzymic hydrolysis of cellulose, the same situation was encountered with low evolution of heat of hydrolysis of the disaccharide[11]. In this instance, both cellobiose and D-glucose are present, and it would have been advantageous to base the analysis only on the primary reaction so as to avoid the need for discrimination between D-glucose formed from cellobiose and D-glucose already present in the sample. However, because of the low reaction-heat from the β-D-glucosidase-catalyzed reaction, D-glucose oxidase had to be used as the indicator enzyme.

For this purpose, immobilized β-D-glucosidase was placed in a precolumn and the immobilized D-glucose oxidase in the thermistor. By passing the solution to be analyzed through both columns, the total content of D-glucose and cellobiose could be measured, whereas if only the thermistor column were used, only the D-glucose present in solution prior to hydrolysis of cellobiose could be determined[11].

Interest has recently been focused on disaccharides in urine from patients suffering from malignant tumors[12,13]. It has been found that, when such patients have ingested a relatively large, standardized dose of disaccharides, they start excreting disaccharides in the urine. Lactose and sucrose have been assayed in urine, and good correlations have been found between excretion of the disaccharides and the presence of tumors. As both disaccharides produce D-glucose on hydrolysis, it is advantageous to measure only the heat of hydrolysis. Enzyme electrodes containing the immobilized enzymes invertase, mutarotase, and D-glucose oxidase have also been used for such analysis[14,15].

To simplify the handling of the samples, we tried as a semi-continuous procedure a direct change between the different samples without any intermittent wash with buffer (Fig. 4). Such a procedure assumes a good baseline stability, a characteristic of the new generation of enzyme thermistors. Fig. 4 also shows that the enzyme thermistor is well suited for continuous monitoring of substrate concentrations, as it responds quickly to a change in concentration. The stability of the measurements permits continuous registration over a long period (several h), which has been utilized in recent applications of the enzyme thermistor in process control[16].

D-Galactose and D-glucose were determined with the acid of D-galactose oxidase and D-glucose oxidase, respectively. D-Galactose oxidase initially showed low operational stability, but after regular treatment of the system with mM Cu^{2+} over-

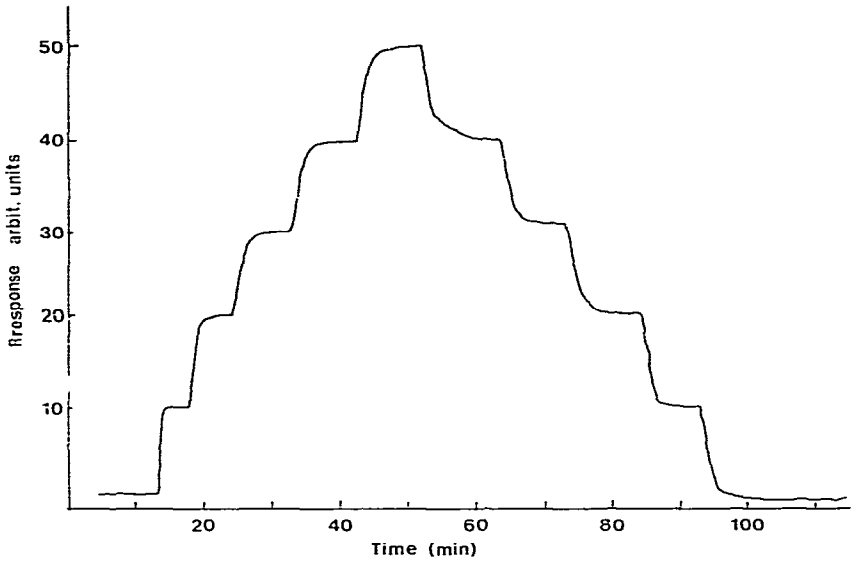

Fig. 4. Temperature recordings obtained when a semicontinuing procedure was applied involving direct change between the samples without any intermittent wash. Samples containing 10, 20, 30, 40, and 50 mmol of sucrose per L were analyzed. Each sample was administered for a period long enough to achieve thermal steady-state before next sample was introduced.

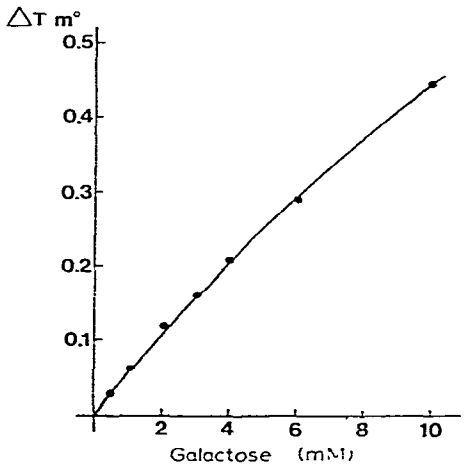

Fig. 5. Calibration curve for D-galactose with immobilized D-galactose oxidase.

night, a preparation gave reproducible responses for at least one week. The standard curve obtained is shown in Fig. 5.

In the analytical applications conducted thus far, D-glucose oxidase has shown extremely high operational stability. A glass-bound preparation has been kept at room temperature and used intermittently for 14 months with the same response to a standard solution. However, some recent reports claim that when the enzyme is used

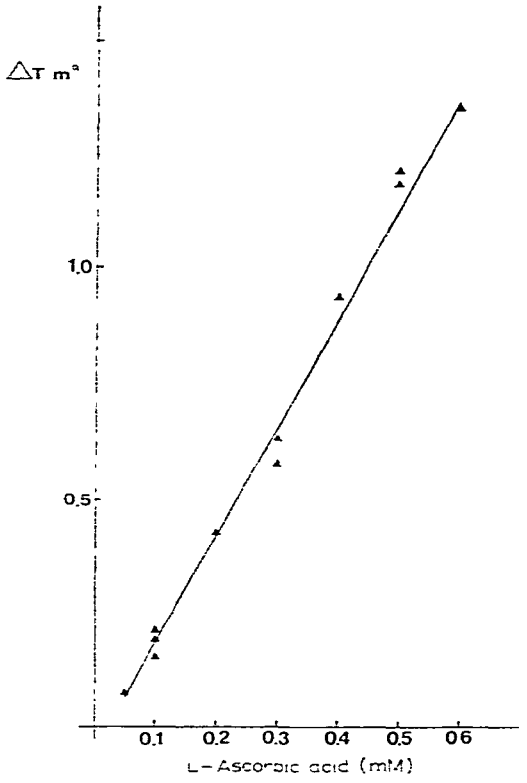

Fig. 6. Calibration curve for L-ascorbic acid, using biospecifically, reversibly immobilized L-ascorbate oxidase (4.5 U for the whole thermistor bed of 0.5 mL).

in continuous contact with the substrate, especially at high concentrations, a substantial decrease in stability takes place, as the hydrogen peroxide produced denatures the enzyme[17,18]. To compensate for this factor, either co-immobilization of catalase[19] or—more effectively—of superoxide dismutase[20] may be used.

Another alternative is to use biospecific, reversible immobilization where the enzyme is replaced by fresh enzyme[21] after a certain period, whether it is necessary or not. The specific interactions between lectins and glycoproteins may, therefore, reversibly immobilize carbohydrate-containing enzymes on a solid support. As, at least in the examples studied thus far, the lectin–glycoprotein interactions seem to be mainly or even exclusively protein–carbohydrate rather than protein–protein interactions[22], a very high retention of the specific activity is observed. As the protein part of the glycoproteins seems not to take part directly in the interaction, the catalytically active regions of the protein are influenced but little by the interactions[8,21,22].

In the present study, concanavalin A was used as a molecular anchor and D-glucose oxidase as well as invertase and L-ascorbate oxidase were immobilized.

In the example illustrated in Fig. 6, 4.5 U of L-ascorbate oxidase was used.

Testing the reproducibility of activity bound to the concanavalin A–Sepharose column, invariably identical (variation in response for a given substrate concentration was 1–2%) results were obtained during a ten-day period when 4.5 U of L-ascorbate oxidase was introduced into the system. The sensitivity of the analyses utilizing L-ascorbate oxidase bound to concanavalin A–Sepharose is satisfactory in standard solutions (Fig. 6).

The enzyme was used for one day, and the first step each morning was the washing and reloading of the enzyme reactor with fresh enzyme according to the procedures described earlier. Owing to the high retention of enzymic activity and the short time the enzyme preparation is used, it is sufficient to use rather small amounts of enzyme. This situation contrasts with that with covalently immobilized preparations containing a large excess of catalytic capacity to gain operational stability over long periods of time. Furthermore, in the analysis of large substrate molecules, which are sterically excluded from the interior of the carrier, the enzyme used should preferably be bound at the surface. The limited areas available place demands on high specific catalytic activity of the enzyme-molecules immobilized, to secure sufficient operational stability. Biospecific, reversible immobilization employing surface-bound lectins, for example, should be very useful for this purpose.

The present paper describes analysis of simple sugars with the aid of continuous-flow systems with immobilized enzymes. More-complex carbohydrates may also be quantified with the same equipment, but with the use of different procedures. One such example would be the use of immobilized lectins in a competitive-binding assay where a fixed amount of enzyme-labeled carbohydrate competes with free carbohydrate for the binding sites on the lectins. After binding has taken place, the amount of enzyme bound, which may be related to the amount of free carbohydrate present in the sample, is measured by addition of a pulse of substrate[24].

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REFERENCES

1 H. BAUER AND W. VOELTER, *Chromatographia*, 9 (1976) 433–439.
2 J. K. PALMER, *Anal. Lett.*, 8 (1975) 215–224.
3 T. TOBA AND S. ADACHI, *J. Chromatogr.*, 135 (1977) 411–417.
4 H. U. BERGMEYER, in H. U. BERGMEYER (Ed.), *Principles of Enzymatic Analysis*, 1st edn., Verlag Chemie, New York, 1978, p. 47.
5 H. E. PAULY AND G. PFLEIDERER, *Z. Physiol. Chem.*, 356 (1975) 1613–1623.
6 H. H. WEETALL AND A. M. FILBERT, *Methods Enzymol.*, 34 (1974) 59–72.
7 B. DANIELSSON, K. GADD, B. MATTIASSON, AND K. MOSBACH, *Anal. Lett.*, 9 (1976) 987–1001.
8 B. MATTIASSON AND C. BORREBAECK, *FEBS Lett.*, 85 (1978) 119–123.
9 F. KELLY-FALCOZ, H. GREENBERG, AND B. L. HORECKER, *J. Biol. Chem.*, 240 (1965) 2966–2970.
10 B. MATTIASSON, B. DANIELSSON, AND K. MOSBACH, *Anal. Lett.*, 9 (1976) 867–889.
11 B. DANIELSSON, E. RIEKE, B. MATTIASSON, F. WINQVIST, AND K. MOSBACH, *Appl. Biochem. Biotechnol.*, 6 (1981) 207–222.

12 R. A. FISCHER, B. M. ROSHOFF, J. H. ALTSHULER, W. R. THAYER, AND H. M. SPIRO, *Cancer*, 18 (1965) 1278–1284.
13 R. O. STASIV, A. B. PATEL, AND H. D. BROWN, *Biotechnol. Bioeng.*, 14 (1972) 629–635.
14 M. CORDONNIER, F. LAWNY, D. CHAPOT, AND D. THOMAS, *FEBS Lett.*, 59 (1975) 263–267.
15 I. SATOH, I. KARUBE, AND S. SUZUKI, *Biotechnol. Bioeng.*, 18 (1976) 269–272.
16 B. DANIELSSON, B. MATTIASSON, R. KARLSSON, AND F. WINQVIST, *Biotechnol. Bioeng.*, 21 (1979) 1749–1766.
17 J. E. PRENOSIL, R. CARTER, AND I. J. DUNN, *Chimia*, 31 (1977) 109.
18 R. CARTER, J. E. PRENOSIL, AND J. R. BOURNE, *Eur. Congr. Biotechnol., 1st, Interlaken*, Sept. 25–30, 1978, pp. 107–110.
19 M.-O. MÅNSSON, B. MATTIASSON, S. GESTRELIUS, AND K. MOSBACH, *Biotechnol. Bioeng.*, 18 (1976) 1145–1159.
20 R. P. CHAMBERS, K. O. MCELRATH, AND W. COHEN, poster at *Enzyme Eng. Conf. 4th, Bad Neunahr*, Sept 25–30, 1977.
21 B. MATTIASSON, *Appl. Biochem.*, 3 (1981) 183–194.
22 C. BORREBAECK AND B. MATTIASSON, *Eur. J. Biochem.*, 107 (1980) 67–71.
23 B. MATTIASSON, E. RIEKE, D. MUNNECKE, AND K. MOSBACH, *J. Solid-Phase Biochem.*, 4 (1979) 263–270.
24 C. BORREBAECK AND B. MATTIASSON, *Anal. Biochem.*, 107 (1980) 446–450.