Biochimica et Biophysica Acta, 397 (1975) 101—109

BBA 67550

ENZYMATIC RECYCLING OF COENZYMES BY A MULTI-ENZYME SYSTEM IMMOBILIZED WITHIN SEMIPERMEABLE COLLODION MICROCAPSULES

JOHN CAMPBELL and THOMAS MING SWI CHANG

Department of Physiology, McGill University, Montreal, Quebec (Canada)

(Received February 17th, 1975)

Summary

Hexokinase (ATP:D-glucose 6-phosphotransferase EC 2.7.1.2) and pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase EC 2.7.1.40) were co-immobilized within semipermeable collodion microcapsules. The resulting microcapsules displayed excellent hexokinase and pyruvate kinase activities, with the measured pyruvate kinase activity considerably greater than that measured for hexokinase. The co-immobilized enzymes, when used sequentially were capable of recycling both ATP and ADP when exposed to the appropriate conditions. Furthermore, when exposed to limiting amounts of coenzyme, the cycles were capable of reusing the total amount of coenzyme supplied at least three times in 90 min. The use of microencapsulation to produce partially "self sufficient" enzyme systems is discussed.

Introduction

There are a number of drawbacks to the more widespread use of enzymes in medical, analytical, and industrial fields. One major drawback is the high cost of pure enzyme preparations, while the immunological response initiated by foreign proteins, when introduced in vivo, limits the application of soluble enzymes in enzyme replacement therapy.

Recently, however, there has been a great advance in the technology of preparing immobilized enzyme derivatives [1—6], and hence the potential reusability of these enzyme derivatives helps to alleviate the high cost. Furthermore, it has been shown that immobilized enzymes can be used in vivo and that no immunological response is observed [6]. However, many enzymes require coenzymes for their successful operation, and hence, in spite of the economies introduced by immobilized enzymes, the cost of an enzyme based reaction may still be unacceptably high, due to the coenzyme requirement. In addition,

many enzyme systems applicable to enzyme therapy also require coenzymes. Consequently, new methods for the generation of coenzymes in situ, or some means of recycling coenzymes without their rapid depletion are also desired. Furthermore, in artificial cell models which contain complex enzyme systems [6,7], coenzymes are required in most cases. Consequently, there is an established requirement for the wider biomedical application of, and the more economical and flexible use of coenzymes.

There have been several different approaches to the problem of coenzyme conservation. (I) For example, Hornby et al. [8,9] have used immobilized enzymes to generate a desired coenzyme from a less expensive precursor (e.g. NADH from NAD^+). The coenzyme, once formed is then used in the normal manner [8,9]. (II) Another approach has been to covalently attach the coenzyme to a support material and thus make the coenzyme potentially reusable. There are many examples in the literature of the immobilization of coenzymes [10—14]. It has been shown that immobilized coenzymes usually retain only a fraction of their original activity [13,14]. The present types of immobilized coenzymes experience a number of drawbacks. For example, unlike proteins, coenzymes have only a very limited number of sites available for the attachment of the support material. Furthermore, the relatively small size of the coenzyme molecule necessitates that the site of attachment to the support material is fairly close to the enzymically active part of the coenzyme, thus introducing the complication of steric availability. This steric problem is likely to be even more severe when enzymes attached to macromolecular supports are used in conjunction with immobilized coenzymes. However, successful attempts have been made to increase the steric availability of immobilized coenzymes by introducing spacer arms to remove the coenzyme away from the support material [14]. (III) A third approach to coenzyme conservation is to recycle the coenzyme, once used, back to its original form, consequently reducing the amount of coenzymes required initially. This has been achieved in the recycling of NAD^+/NADH using a soluble enzyme reaction coupled to redox dyes [15]. The same result has also been achieved using malate dehydrogenase and alcohol dehydrogenase in free solution [16], but here problems arising from the use of enzymes in free solutions are encountered.

In the present report, the technique of microencapsulation is used as a combination of the latter two approaches (II and III). Microencapsulated enzymes have the advantage of being immobilized, and yet still soluble within the microcapsule. Furthermore, there is no limit to the number of different enzymes which can be included in the microcapsules [6]. In this work, it has been shown possible to cycle a coenzyme by way of an immobilized two-enzyme system and the practicality of the further incorporation of an immobilized coenzyme into this system is considered.

Materials and Methods

Enzymes

Type F-300 hexokinase (EC 2.7.1.2) from Baker's yeast (227 units/mg); type III pyruvate kinase (EC 2.7.1.40) from rabbit muscle (485 units/mg); type VI lactic dehydrogenase (EC 1.1.1.28) from porcine heart (390 units/mg); and

type VII glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from Baker's yeast (350 units/mg) were all obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. and were used without further purification.

Substrates and coenzymes

Grade I ADP from equine muscle; Grade I ATP; NADP (sodium salt); Grade V β-NADH from yeast; and phosphoenol pyruvate (trisodium salt) were all obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

The ATP, ADP, NADP, and phosphoenol pyruvate were all stored desiccated below 0°C, while the NADH was stored desiccated at room temperature. Solutions of all the above were made up in buffer as required and were used within 24 h of their preparation.

Solutions of glucose were made up in distilled water 24 h before use to ensure complete mutaroration and were always used within 2 days of their preparation.

All other reagents were of the highest available analytical grade.

Preparation of semipermeable microcapsules containing both hexokinase and pyruvate kinase

Semipermeable collodion microcapsules were prepared by the interfacial coacervation method [17,18] by employing the updated procedure [6]. 2.4 mg of hexokinase and 2.4 mg of pyruvate kinase were dissolved in 2.5 ml of a filtered hemoglobin solution (10 g/100 ml) and the microencapsulation procedure which generated microcapsules with a mean diameter of 80—100 μm was employed [6]. The resulting microcapsules were washed exhaustively with saline (4°C) and finally with a solution containing 0.1 M sodium phosphate, pH 7.5 in saline (4°C). The microcapsules were stored under the same phosphate buffer in a total volume of 30 ml, at 4°C.

Measurement of enzymic activity

The enzymic activity of the microcapsules was determined by incubating a 0.5 ml quantity of the microcapsules with 4.2 ml of the appropriate substrates, coenzymes, and cofactors. The reactions were performed in a shaking, thermostatted, water bath (American Optical Corporation, U.S.A.) at 110 rev./min. Reactions were performed at 25°C and were initiated by the addition of 0.5 ml of the microcapsule suspension. After the allotted time, reactions were terminated by quickly filtering the reaction mixtures through a sintered glass funnel under suction. Thus, the removal of the microcapsules terminated the reaction. In practice, the microcapsules were not allowed to dry, thus preventing any breakage of the microcapsules which might have been caused by dehydration. All absorbance readings were measured using a DBGT Beckman Spectrophotometer in 1 cm light path cuvettes.

Measurement of hexokinase activity alone

The hexokinase activity of the microcapsules was determined singly by coupling the reaction to that catalysed by glucose-6-phosphate dehydrogenase. Thus, the microcapsules were agitated in the presence of 0.116 M glucose, 0.64 mM ATP, 11.6 mM $MgSO_4$, 16 mM KCl, 0.21 mM $NADP^+$; 0.1 M sodium

phosphate, pH 7.5. After termination of the reaction, the extinction at 340 nm was noted, 7.8 μg glucose-6-phosphate dehydrogenase was added and the reaction was allowed to reach equilibrium, at which time, the new extinction at 340 nm was noted. The extent of the hexokinase reaction was proportional to the observed increase in extinction at 340 nm.

Measurement of pyruvate kinase activity alone

The pyruvate kinase activity of the microcapsules was determined singly by coupling the reaction to that catalysed by lactate dehydrogenase. In this case, the microcapsules were agitated in the presence of 0.64 mM phosphoenol pyruvate, 0.84 mM ADP, 11.6 mM KCl, 0.21 mM NADH, 100 μg lactate dehydrogenase, and 0.1 M sodium phosphate, pH 7.5. After termination of the reaction, the extinction at 340 nm was noted and comparison with a blank assay in which no lactate dehydrogenase had been added gave the total change in extinction at 340 nm. Thus, the extent of the pyruvate kinase reaction was proportional to the observed decrease in extinction at 340 nm.

Measurement of both hexokinase and pyruvate kinase activity of the microcapsules using recycling processes

The combined hexokinase/pyruvate kinase activity of the microcapsules was demonstrated by employing one or other of the recycling processes (A and B) shown in Fig. 1.

In the cycle A, the microcapsules were exposed to 0.116 M glucose, 0.64 mM ATP, 0.64 mM phosphoenol pyruvate, 11.6 mM $MgSO_4$, 16 mM KCl, 0.21 mM NADH, 100 μg lactate dehydrogenase and 0.1 M sodium phosphate, pH 7.5, and the extent of the reaction was determined as described previously for the demonstration of pyruvate kinase activity singly.

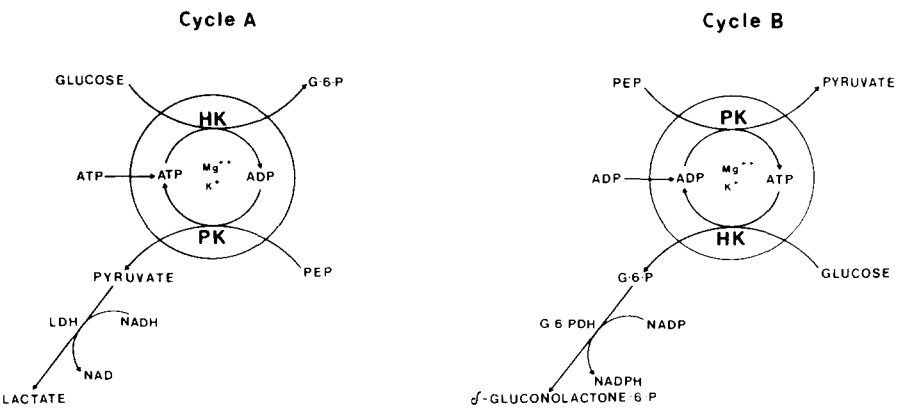

Fig. 1. Schematic representation of the recycling of coenzymes by co-encapsulated hexokinase/pyruvate kinase. Reactions are shown unidirectional for simplicity. Cycle A. The recycling system is supplied with glucose, ATP, and phosphoenol pyruvate, thus hexokinase is the first enzyme in the cycle, and the production of pyruvate indicates the operation of the complete cycle. Experimental details are given in the text. Cycle B. The recycling system is supplied with phosphoenol pyruvate, ADP, and glucose, thus pyruvate kinase is the first enzyme in the cycle, and the production of glucose 6-phosphate indicates the operation of the complete cycle. Experimental details are given in the text. HK, hexokinase; PK, pyruvate kinase; PEP, phosphoenol pyruvate; LDH, lactate dehydrogenase; G-6-PDH, glucose-6-phosphate dehydrogenase.

In cycle B, the microcapsules were exposed to 0.64 mM phosphoenol pyruvate, 0.84 mM ADP, 0.116 M glucose, 0.21 mM $NADP^+$, 11.6 mM $MgSO_4$, 16 mM KCl and 0.1 M sodium phosphate, pH 7.5, and the extent of the reaction was determined as described previously for the determination of hexokinase activity singly.

Results and Discussion

Individual enzyme activities

Many enzymes have been successfully immobilized by the various encapsulation techniques [6,7,17–20]. In the present study, however, two physiologically significant enzymes (hexokinase and pyruvate kinase) have been co-immobilized by their simultaneous inclusion within collodion microcapsules.

Permeability studies with microcapsules indicate that they are impermeable to macromolecules, such as enzymes and antibodies, but smaller molecules can equilibrate rapidly across the ultrathin membrane [6]. The results obtained in this work confirm this, and show that the permeability of the membrane towards the fairly large coenzyme molecules required in this study was such as to not greatly effect the catalytic properties of either of the enzymes while inside the microcapsules. Furthermore, no leakage of enzyme from the microcapsules was observed.

Figs 2(a) and 2(b) represent the progress curves for the generation of ADP from ATP by hexokinase and the generation of ATP from ADP by pyruvate kinase respectively. From these figures it is obvious that the pyruvate kinase catalysed reaction is significantly faster than that catalysed by hexokinase. Consequently, in any process involving sequential reactions catalysed by the enzymes within these microcapsules, the hexokinase reaction would be expected to be rate limiting.

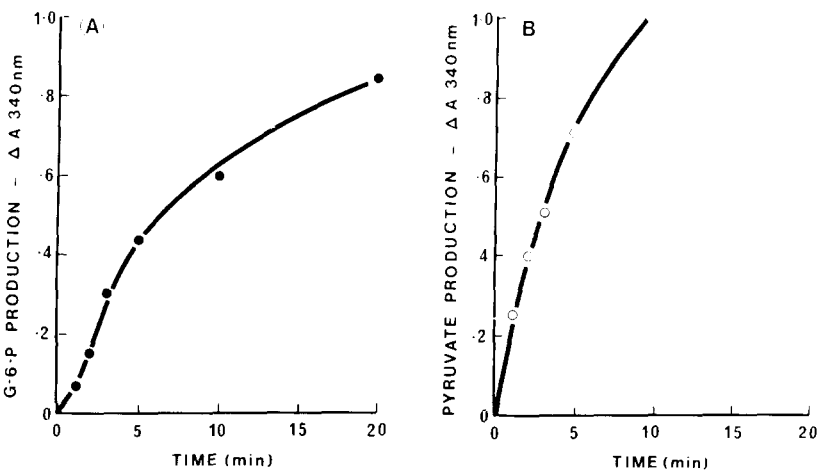

Fig. 2. The individual enzymic activities of the hexokinase/pyruvate kinase microcapsules. (a). The progress curve for the generation of glucose 6-phosphate and ADP from glucose and ATP by the hexokinase/pyruvate kinase microcapsules when assayed for hexokinase activity singly. (b). The progress curve for the generation of pyruvate and ATP from phosphoenol pyruvate and ADP by the hexokinase/pyruvate kinase microcapsules when assayed for pyruvate kinase activity singly. Experimental details are given in text.

Recycling of coenzymes within microcapsules

The results of exposing the co-encapsulated enzymes to cyclic processes A and B are shown in Figs 3(a) and 3(b), respectively. The cycles differed only in terms of which enzyme initiated the cycle and consequently, which coenzyme was the starting nucleotide. The operation of the second enzyme in each cycle was observed by employing the appropriate enzymic system to detect the second, non-cycled product of the second enzyme reaction. In other words, in cycle A, the production of each molecule of pyruvate (and in consequence, the oxidation of each molecule of NADH) indicated the regeneration of one molecule of ATP from ADP. The analogous argument is also applicable to cycle B. Consequently, Figs 3(a) and 3(b) indicate that the microencapsulated two-enzyme system studied here was capable of the regeneration of either ATP or ADP. Furthermore, the observed progress curves for cycles A and B were similar to that shown in Fig. 2(a) obtained for the assay of the hexokinase activity alone, thus confirming that the hexokinase catalysed reaction was the rate limiting step. In cycle A, the hexokinase reaction was the first event, and thus the subsequent production of pyruvate could not be faster than the production of ADP. In cycle B, the generation of ATP by the pyruvate kinase (Fig. 2(b)) was faster than the overall observed rate of production of glucose 6-phosphate (Fig. 3(b)), once again establishing the hexokinase reaction as overall rate limiting.

The progress curves of cycles A and B (Figs 3(a) and 3(b)) were fairly linear up to at least a 3 min reaction time, whereas, the progress curves for the individual enzymes during the same time scale were not (Figs 2(a) and 2(b)). In the individual assay systems, both the removal of coenzyme and the build up of product could have caused this to occur. In contrast, in the cyclic system, the coenzyme-substrate was continually regenerated and at the same time the coen-

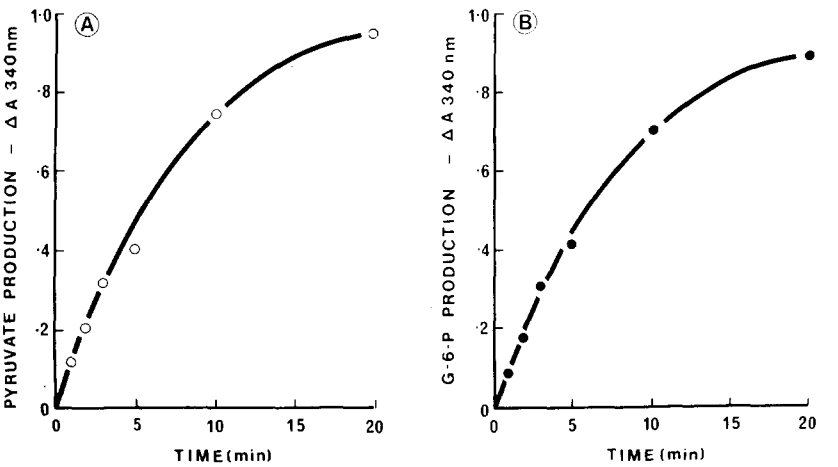

Fig. 3. The recycling of coenzymes by the hexokinase/pyruvate kinase microcapsules. (a). The progress curve for the generation of pyruvate by the hexokinase/pyruvate kinase microcapsules when exposed to the conditions described in cycle A. (b). The progress curve for the generation of glucose 6-phosphate by the hexokinase/pyruvate kinase microcapsules when exposed to the conditions described in cycle B. Experimental details are given in text.

zyme-product was removed, hence promoting a more linear response with time. These preliminary observations suggest the potential of such cyclic systems although more detailed work is required to clearly establish this result.

"Multiplication" of coenzymes

The cyclic processes A and B were repeated but with a low concentration of coenzyme (0.032 mM ATP for cycle A and 0.0425 mM ADP for cycle B). In addition, these low concentration assays were performed in the absence of the second substrate for the second enzyme in both cycles (e.g. phosphoenol pyruvate absent in cycle A and glucose absent in cycle B). The results of this are shown in Figs 4(a) and 4(b). In both cases, by employing the coenzyme regeneration cycles, degrees of conversion beyond the theoretical maximum (if no cycle had been employed) were observed. In contrast, by blocking the coenzyme regeneration cycle (by the omission of a necessary substrate) the degree

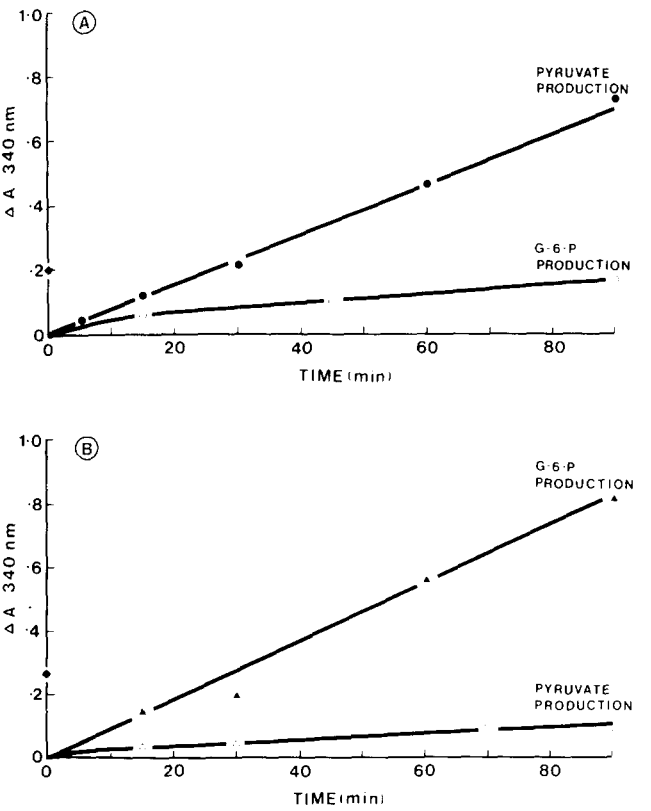

Fig. 4. The 'multiplication' of coenzymes by the hexokinase/pyruvate kinase microcapsules. (a) (•) The progress curve for the generation of pyruvate by the hexokinase/pyruvate kinase microcapsules when exposed to the conditions described in cycle A, with low ATP concentration. (○) The similar progress curve (for glucose-6-phosphate production) but in the absence of phosphoenol pyruvate, thus blocking the cycle. (b). (▲) The progress curve for the generation of glucose 6-phosphate by the hexokinase/pyruvate kinase microcapsules when exposed to the conditions described in cycle B, with low ADP concentration. (△) The similar progress curve (for pyruvate production) but in the absence of glucose, thus blocking the cycle. (◆) Represents the theoretical maximum obtainable ΔA. 340 nm for both cycles when the low coenzyme concentrations were supplied. All experimental details are given in text.

of conversion always stayed below this theoretical maximum. In both coenzymes regeneration cycles, after a 90 min reaction time, each coenzyme molecule had been used at least 3 times.

In this study, the recycling of both ATP and ADP within semipermeable microcapsules was clearly established. Recently much attention has been paid to the concept of the regeneration of coenzymes. As previously mentioned, an approach to economy in the use of coenzymes can be achieved in a number of ways. Firstly, the coenzyme can be generated from a less expensive precursor [8,9] and then used normally. A second method, of more recent interest, is to make the coenzyme potentially reusable by rendering it insoluble [10—14] and thirdly, the coenzyme can be recycled, resulting in the requirement of less coenzyme initially [15,16,21,22]. The second method mentioned above is perhaps the most attractive and at the same time the most difficult to achieve in that there are two main drawbacks. Firstly, insoluble coenzymes have been prepared which retain enzymically recognisable activity. However, this activity is usually only a fraction of the starting activity [13,14]. Secondly, the use of immobilized coenzymes would seem to preclude the simultaneous use of enzyme molecules bound to macromolecular supports, as the steric problems so introduced could prove insurmountable. However, microencapsulation could resolve these two drawbacks. Firstly, as shown in this work, the recycling of coenzymes within microcapsules is feasible, thus making large amounts of the coenzyme unnecessary. Consequently, immobilized coenzyme derivatives with low activity may prove workable propositions when employed in a microencapsulated system. The microencapsulation of even insoluble compounds has been demonstrated previously [6], hence, this would pose no problem if the coenzyme derivative of choice was insoluble. Secondly although microencapsulated enzymes have all the advantages of being immobilized in nature, the actual enzymes within the microcapsules remain in solution. This would give the interaction of an active immobilized coenzyme (if available) with the appropriate enzyme(s) the largest possible chance of success.

Work is now proceeding in this laboratory to include immobilized cofactors in this, and in similar encapsulated enzyme systems.

In conclusion, it seems likely that the microencapsulation of enzymes and coenzyme derivatives is one of the most likely systems to succeed in an attempt to obtain a partly "self-sufficient" enzymic system. Furthermore, by isolating enzymes from the external environment and by recycling coenzymes within the microcapsules, these artificial cells resemble extremely primitive biological cells.

Acknowledgements

This research is supported by grant MRC-MT-2100 from the Medical Research Council of Canada. J.C. is a Postdoctoral fellow supported by MRC-MT-2100.

References

1 Silman, I.H. and Katchalski, E. (1966) Annu. Rev. Biochem. 35, 873—908
2 Melrose, G.J.H. (1971) Rev. Pure Appl. Chem. 21, 83—119

3 Goldman, R., Goldstein, L. and Katchalski, E. (1971) in Biochemical Aspects of Reactions on Solid Supports (Stark, Y.R. ed.), pp. 1—78, Academic Press, London and New York
4 Stanley, W.L. and Olson, A.E. (1974) J. Food Sci. 39, 660—666
5 Mosbach, K. (1971) Sci. Am. 244, 26—33
6 Chang, T.M.S. (1972) Artificial cells, 1st edn, p. 207, Charles C. Thomas, Publisher, Springfield, Ill., U.S.A.
7 Chang, T.M.S. (1967) Sci. J. 1, 62—67
8 Hornby, W.E., Inman, D.J. and McDonald, A. (1972) FEBS Lett. 23, 114—116
9 Hornby, W.E., Campbell, J., Inman, D.J. and Morris, D.L. (1974) in Enzyme Engineering (Pye, K. and Wingard, L. Jr., eds), Vol. 2, pp. 401—407, Plenum Publishing Corporation, New York
10 Mosbach, K., Guilford, H., Ohlsson, R. and Scott, M. (1972) Biochem. J. 127, 625—631
11 Lowe, C.R. and Dean, P.D.G. (1971) FEBS Lett. 14, 313—316
12 Ikeda, S. and Fukui, S. (1973) Biochem. Biophys. Res. Commun. 52, 482—488
13 Weibel, M.K., Weetall, H.H. and Bright, H.J. (1971) Biochem. Biophys. Res. Comm. 44, 347—352
14 Larsson, P.-O. and Mosbach, K. (1971) Biotech. Bioeng. 13, 393—398
15 Kato, T., Berger, S.J., Carter, J.A. and Lowry, O.H. (1973) Anal. Biochem. 53, 86—97
16 Rokosh, D.A., Kurz, W.Y.W. and LaRue, T.A. (1973) Anal. Biochem. 54, 477—483
17 Chang, T.M.S (1964) Science 146, 524—525
18 Chang, T.M.S., MacIntosh, F.C. and Mason, S.G. (1966) Can. J. Physiol. Pharmacol. 44, 115—128
19 Siu Chong, E.D. and Chang, T.M.S. (1974) Enzyme 18, 218—239
20 Poznansky, M.J. and Chang, T.M.S. (1974) Biochim. Biophys. Acta 334, 103—115
21 Srere, P.A. et al. (1973) in Proc. Nat. Acad. Sci. U.S. 70, 2534—2538
22 Chambers, R.D. et al. (1974) in Enzyme Engineering (Pye, E.K. and Wingard, Jr, L.B., eds), Vol. 2, 195—202, Plenum Press, New York