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IMMOBILIZED GLUTAMATE OXALOACETATE TRANSAMINASE

STEADY STATE KINETIC ANALYSIS AND STABILITY STUDIES

J. CAMPBELL* and W.E. HORNBY

Department of Biochemistry, University of St. Andrews, St. Andrews, Fife (U.K.)

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Summary

1. Glutamate oxaloacetate transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) was immobilized on amino ethyl cellulose using the bifunctional reagent diethyl adipimidate.

2. The steady state kinetic analysis was performed for the particulate and the free enzyme, and the Michaelis constants measured for the amino ethyl cellulose derivative were not greatly different from those measured for the free glutamate oxaloacetate transaminase, while the latter were in good agreement with values in the literature.

3. The amino ethyl cellulose-glutamate oxaloacetate transaminase was slightly more stable than the free enzyme at 65°C, but was stabilised less by polyethylene glycol than the free enzyme.

Introduction

In recent years the study of immobilized enzymes has intensified due to the wide variety of situations in which these artefacts can be employed. For example, immobilized enzymes have been used as preparative tools [1,2], and as analytical devices [3,4]. In addition, it is possible to recognise a therapeutic role for immobilized enzymes [5], and recently it has been suggested that immobilized enzymes may serve as models for the study of the action of membrane bound enzymes in vivo. Thus, several very important areas for the application of immobilized enzymes are well established.

When an enzyme is immobilized however, changes may occur in the enzyme's observed behaviour. For example, alterations in the pH optimum [6],

* Present address: Physiology Department, P.O. Box 6070, McGill University, Montreal H3G 1Y6, Quebec, Canada.

thermal stability [7], and measured Michaelis constants [8] have all been reported for several enzymes upon immobilization. Several excellent discussions of the kinetics of immobilized enzymes do exist [9—11], but it is surprising in the light of the above mentioned alterations in enzymic behaviour that detailed kinetic analysis of immobilized enzyme derivatives has been attempted only rarely [12—14]. Consequently, in the present study a physiologically important enzyme, with a well established mechanism was immobilized, and an attempt was made to characterise the immobilized derivative.

Materials and Methods

Enzyme

Type I glutamate oxaloacetate transaminase (EC' 2.6.1.1) from porcine heart was obtained from Sigma London Chemical Co. Ltd, Norbiton Station Yard, Kingston-upon-Thames, Surrey, U.K. as an ammonium sulphate suspension. Unless otherwise stated, solutions of glutamate oxaloacetate transaminase were prepared immediately prior to use by dissolving 300 μl of the ammonium sulphate suspension (1.6 mg) in 3.7 ml of 0.1 M *N*-ethyl morpholine, pH 8.5 containing 10 mg/ml polyethylene glycol 400 (BDH Chemicals Ltd, Poole, Dorset, U.K.) and 0.1 mM with respect to pyridoxal 5'-phosphate (Sigma Chemical Co., Ltd). The enzyme solution was then dialysed for 3 h at 4° C against 2 × 5 litres of the same composite *N*-ethyl morpholine buffer.

Immobilization procedure

Pretreatment of amino ethyl cellulose. Cellex AE with a degree of substitution of 0.315 mequiv/g was obtained from Bio Rad Laboratories, 32nd Griffin, Richmond, U.S.A. Prior to use, the cellulose powder was washed successively with 0.5 M NaOH and 0.5 M HCl, and finally with water to remove all trace of acid or alkali. The cellulose powder was stored as a 40 mg/ml suspension in water at 4° C.

Coupling of glutamate oxaloacetate transaminase to amino ethyl cellulose. Amino ethyl cellulose, pretreated as described above, was activated for the subsequent immobilization of enzyme by preparing the imidate derivative of the support material, and then exposing this to the dialyzed glutamate oxaloacetate transaminase solution [15]. The reaction sequence is shown in Fig. 1.

Prior to activation, 160 mg of amino ethyl cellulose was washed with 100 ml of absolute ethanol on a sintered-glass funnel to ensure completely anhydrous conditions. The amino ethyl cellulose pellet was then transferred to a solution containing 20 ml of absolute ethanol, 5 ml of 100% (v/v) *N*-ethyl morpholine, and 100 mg of ethyl adipimidate (obtained from Pierce Chemical Co., Rockford, Ill., U.S.A. or alternatively prepared by the synthesis of Pinner [16]). This mixture was stirred at room temperature for 2 h, after which excess ethyl adipimidate was removed by thoroughly washing the activated cellulose with absolute ethanol. The activated amino ethyl cellulose was then quickly transferred to the dialyzed enzyme solution and the slurry was stirred at 4° C for 2 h. The amino ethyl cellulose-enzyme was washed free of unbound protein on a sintered-glass funnel with 0.5 M NaCl (500 ml), and then 0.1 M sodium arsenate, pH 7.4, containing 10 mg/ml polyethylene glycol and 1.0 mM with

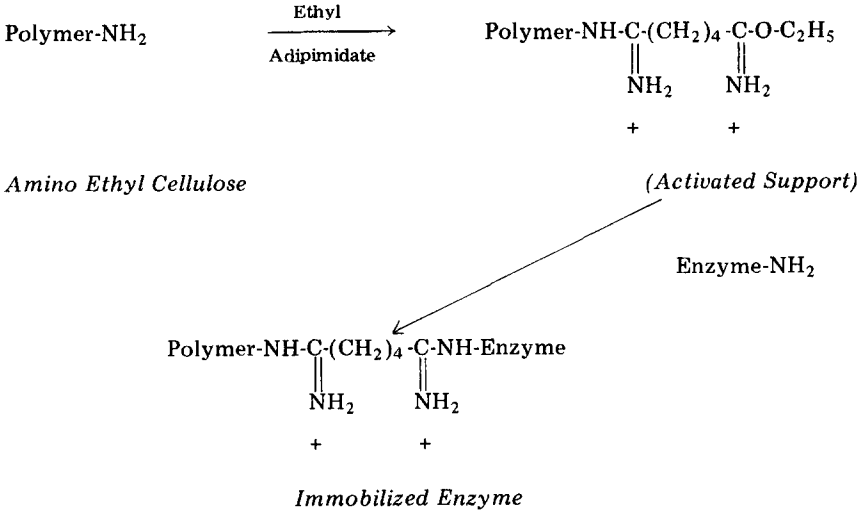


Fig. 1. The immobilization of enzymes on amino ethyl cellulose using ethyl adipimidate.

respect to pyridoxal 5′-phosphate (500 ml). The immobilized glutamate oxaloacetate transaminase derivative was stored as a 5 mg/ml suspension in the composite arsenate buffer at 4° C.

Progress of coupling glutamate oxaloacetate transaminase to activated amino ethyl cellulose. The progress of the coupling of glutamate oxaloacetate transaminase to activated amino ethyl cellulose was studied by withdrawing 100 μl aliquots of the coupling mixture at 30 min intervals. The aliquots were immediately centrifuged and the supernatant decanted. The pellets were washed as described above and suspended in 100 μl of the composite arsenate buffer. 50 μl of these suspensions and 10 μl of the decanted supernatants were assayed for immobilized enzyme activity and residual soluble enzyme activity respectively as described below.

Estimation of bound protein

An estimation of the enzyme bound in any coupling procedure was achieved by assaying the enzymic activity of the coupling solution before and after coupling.

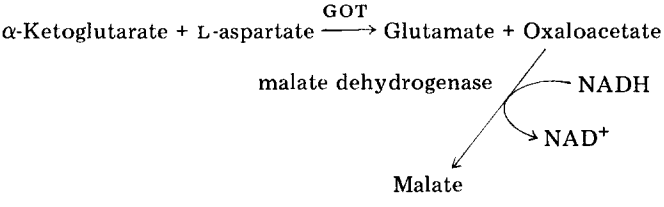
Generally, a small aliquot of the enzyme solution, for coupling, was extracted from the bulk solution immediately prior to the initiation of the coupling by the addition of the activated support. This aliquot was subjected to exactly the same conditions as the enzyme in the coupling mixture, apart from the presence of the activated support. After the allotted coupling time, the enzyme solution was removed from the support, and the latter was washed free of unbound protein with 0.1 M NaCl.

The initial washings were collected, mixed with the post-coupling enzyme solution, and the volume measured. The enzymic activity of this solution, and also the activity of the aliquot of the precoupling enzyme solution were then measured as described below and the difference gave an estimation of the amount of enzyme immobilized when the volumes of the solutions were taken

into account. The presence of any free ethyl adipimidate would also have caused a reduction of the residual enzyme activity, but as the support material was thoroughly washed before exposure to the enzyme solution this can be discounted here.

Measurement of glutamate oxaloacetate transaminase activity

Glutamate oxaloacetate transaminase activity was followed by linking the reaction to that catalyzed by malic dehydrogenase as shown below:



Soluble enzyme. Soluble activity was determined by measuring the rate of decrease of absorbance at 340 nm in the presence of α-ketoglutarate and L-aspartate (both from Sigma London Chemical Co., Ltd), using a Beckman DBGT spectrophotometer (Beckman Instruments Ltd, Glenrothes, Fife , U.K.). Unless otherwise stated, assays were carred out in a 3.56 ml reaction volume, contained in a 1 cm light path cuvette at 25° C. Reactions were performed in the presence of 0.1 M sodium arsenate, pH 7.4, 0.14 mM NADH and 0.1 mg malate dehydrogenase, and were initiated by the addition of 10 μl of a glutamate oxaloacetate transaminase solution in the composite arsenate buffer.

Amino ethyl cellulose-enzyme. Amino ethyl cellulose-glutamate oxaloacetate transaminase activity was assayed in a similar fashion to soluble enzyme activity, however, in order to assay the insoluble enzyme, it was necessary to stir the assay cuvette in the spectrophotometer. This was done to prevent the cellulose-enzyme derivative from settling out of suspension during the assay. The stirring cuvette assay technique was basically the system described by Mort et al. [17], using the modification of Bayne [18]. Much lower concentrations of suspension were employed , however, and this resulted in an extremely low 'noise' level.

Assays were initiated by the addition of 50 μl of the amino ethyl cellulose-enzyme suspension. Stirring was commenced immediately, and all other parameters were as described for the soluble enzyme assay.

Effect of substrate concentration

Soluble glutamate oxaloacetate transaminase. The effect of the α-ketoglutarate concentration on the activity of soluble glutamate oxaloacetate transaminase was studied at fixed levels of L-aspartate. The concentration of α-ketoglutarate was varied from 1.16 mM to 0.028 mM at three L-aspartate concentrations, (39.4 mM, 5.91 mM, and 1.97 mM).

The effect of the L-aspartate concentration on the activity of soluble glutamate oxaloacetate transaminase was studied at fixed levels of α-ketoglutarate. The concentration of L-aspartate was varied from 40.0 mM to 0.85 mM at three α-ketoglutarate concentrations, (1.16 mM, 0.23 mM, and 0.061 mM).

Amino ethyl cellulose-glutamate oxaloacetate transaminase. The effect of the α-ketoglutarate concentration on the activity of amino ethyl cellulose-glutamate oxaloacetate transaminase was studied at fixed L-aspartate levels. The concentration of α-ketoglutarate was varied from 4 mM to 0.047 mM at three L-aspartate concentrations, (20.3 mM, 10.15 mM, and 2.97 mM).

The effect of the L-aspartate concentration on the activity of amino ethyl cellulose-glutamate oxaloacetate transaminase was studied at fixed α-ketoglutarate levels. The concentration of L-aspartate was varied from 40 mM to 0.77 mM at three α-ketoglutarate concentrations, (1.17 mM, 0.234 mM, and 0.058 mM).

Stability

Thermal inactivation of soluble glutamate oxaloacetate transaminase. 5.0 ml of a 0.05 mg/ml enzyme solution in 0.1 M sodium arsenate, pH 7.4, was incubated at 65° C. At known intervals of time 10 μl aliquots were removed, and assayed for residual glutamate oxaloacetate transaminase activity as previously described.

Thermal inactivation of amino ethyl cellulose-glutamate oxaloacetate transaminase. 5.0 ml of a 5 mg/ml suspension of amino ethyl cellulose-enzyme in 0.1 M sodium arsenate, pH 7.4, was incubated at 65° C. At known intervals of time 50 μl aliquots were removed, and assayed for residual insoluble glutamate oxaloacetate transaminase activity as previously described.

Effect of polyethylene glycol and pyridoxal 5′-phosphate on the thermal inactivation of both soluble and amino ethyl cellulose-glutamate oxaloacetate transaminase. The thermal inactivation of both soluble and amino ethyl cellulose-glutamate oxaloacetate transaminase at 65° C was repeated in the presence of both 10 mg/ml polyethylene glycol and 0.1 mM pyridoxal 5′-phosphate.

Results and Discussion

Amino ethyl cellulose-glutamate oxaloacetate transaminase

The progress of the coupling of glutamate oxaloacetate transaminase to adipimidate-activated amino ethyl cellulose was studied as previously described in Methods, and Fig. 2 shows the simultaneous disappearance of soluble enzyme activity and appearance of insoluble enzyme activity in the coupling mixture. These results indicate that the coupling was essentially complete after 60 min (under the conditions employed). 8 mg of enzyme were coupled per g of support, and the bound enzyme retained 7.5% of its original activity. The amount of bound protein and the retention of enzymic activity are both fairly low, however other standard coupling techniques, such as those employing glutaraldehyde and diazotisation, failed to produce active, immobilized enzyme derivatives.

Effect of substrate concentration on the reaction rate of free and amino ethyl cellulose-glutamate oxaloacetate transaminase

Several workers have described how the effect of substrate concentrations on the initial reaction velocity can be used to distinguish between different

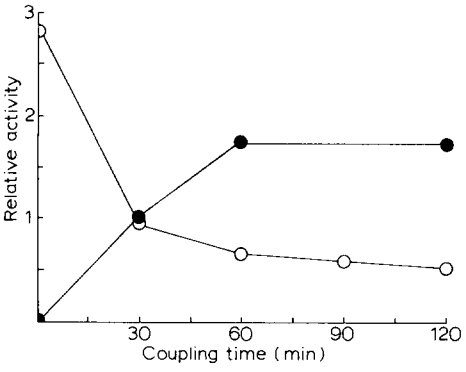


Fig. 2. The time course of the coupling of glutamate oxaloacetate transaminase to activated amino ethyl cellulose. •, insoluble amino ethyl cellulose-enzyme activity; ○, soluble enzyme activity remaining in the supernatant.

mechanisms of enzyme action [20,21]. The patterns obtained when this data is presented in the form of double reciprocal plots [21] helps to assign a mechanism to the enzyme under investigation.

The enzyme glutamate oxaloacetate transaminase is particularly abundant in cardiac muscle, and in the event of heart damage (e.g. coronary thrombosis) there is an elevation of the level of glutamate oxaloacetate transaminase detectable in the blood, due to leakage of the enzyme from damaged cells in the myocardium. There is much evidence in the literature which suggests that glutamate oxaloacetate transaminase functions via a Ping Pong Bi Bi mechanism [22—24]. The effect of varying the concentrations of both substrates on the initial reaction velocity of amino ethyl cellulose-glutamate oxaloacetate transaminase is shown in Figs 3 and 4, while the results of similar experiments with the free enzyme are shown as inserts in the appropriate figure. The patterns obtained for the free enzyme (Figs 3 and 4, inserts) are characteristic of an enzyme operating with a Ping-pong mechanism [19]. This is in full agreement with the bulk of the evidence in the literature [22—24]. However, the double-reciprocal plots obtained for the amino ethyl cellulose derivative are curved concave downwards (Figs 3 and 4).

Several factors could have been responsible for this phenomenon. For example, if one or other of the substrates activated the immobilized enzyme at high concentration, then shapes similar to those obtained might have been expected. However, as the soluble enzyme exhibited no trace of such a phenomenon, this is rather unlikely. Furthermore, α-ketoglutarate forms a dead end complex with the enzyme at high concentrations [24], and this rules out any activation by α-ketoglutarate.

Another possible cause of downward curving double reciprocal plots is that the immobilized enzyme may exist in a variety of different microenvironments, and consequently may exhibit a range of Michaelis constants depending on the microenvironment of the enzyme. This would manifest itself as downward curving double reciprocal plots [25]. In the case in question, the reagent used to couple the enzyme to amino ethyl cellulose (ethyl adipimidate) is specific for amino groups, however, the enzyme was almost certainly immobi-

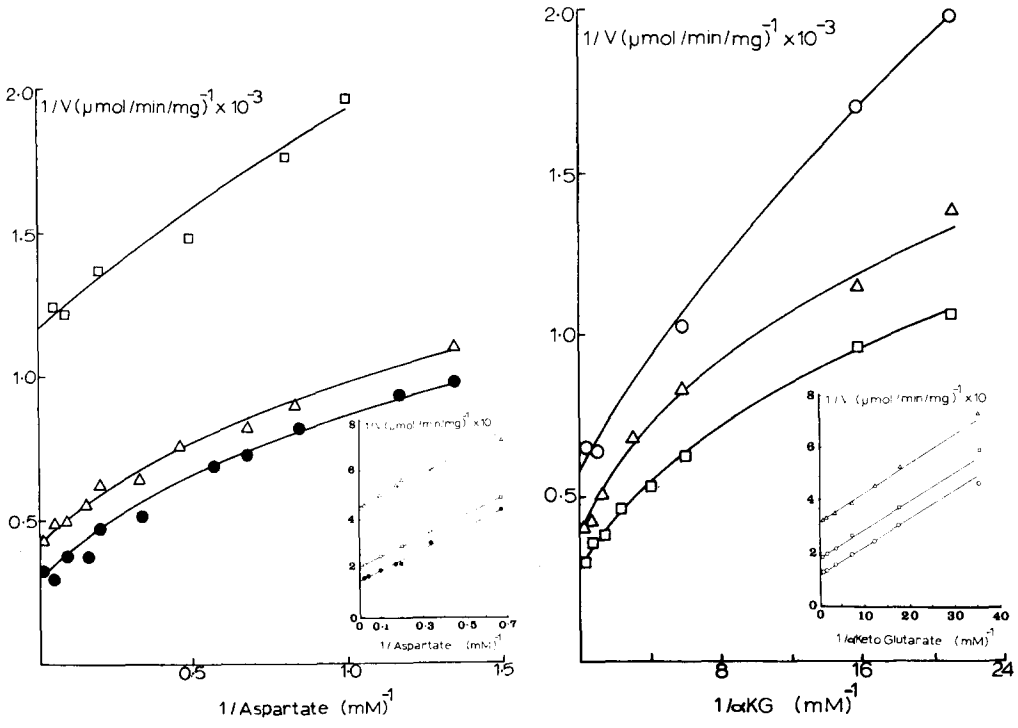


Fig. 3. The effect of α-ketoglutarate concentration on the activity of amino ethyl cellulose-glutamate oxaloacetate transaminase. Assays were performed as described in the text at three fixed aspartate concentrations. •, 2.97 mM; △, 10.15 mM; and □, 20.3 mM L-aspartate. Insert: The effect of α-ketoglutarate concentration on the activity of soluble glutamate oxaloacetate transaminase. Assays were performed as described in the text at three fixed aspartate concentrations. △, 1.97 mM; □, 5.91 mM; and •, 39.4 mM L-aspartate.

Fig. 4. The effect of aspartate concentration on the activity of amino ethyl cellulose-glutamate oxaloacetate transaminase. Assays were performed as described in the text at three fixed α-ketoglutarate concentrations. □, 0.058 mM; △, 0.234 mM; and ○, 1.17 mM α-ketoglutarate. Insert: The effect of aspartate concentration on the activity of soluble glutamate oxaloacetate transaminase. Assays were performed as described in the text at three fixed α-ketoglutarate concentrations. △, 0.061 mM; □, 0.23 mM; and ○, 1.16 mM α-ketoglutarate.

lized in a variety of different ways, depending on which residue or residues were involved in the binding to the support material. Thus, a mixed population of immobilized enzymes probably existed with a range of Michaelis constants, and this may have contributed to the observed shape of the double reciprocal plots.

A third possible cause of downward curvature in the double reciprocal plots is the existance of diffusional limitations. It has been pointed out recently [26,27] that this type of curvature in Lineweaver-Burk plots for immobilized enzymes can be explained in terms of diffusional barriers. Furthermore, it appears likely that in the case of reactions catalysed by immobilized enzymes, only very slow reactions completely escape the effects of diffusion [28]. Thus, it appears that diffusional barriers introduced by the support material may be responsible for the distorted double reciprocal plots shown in Figs 3 and 4.

In addition it has been shown that diffusional limitations can amplify the

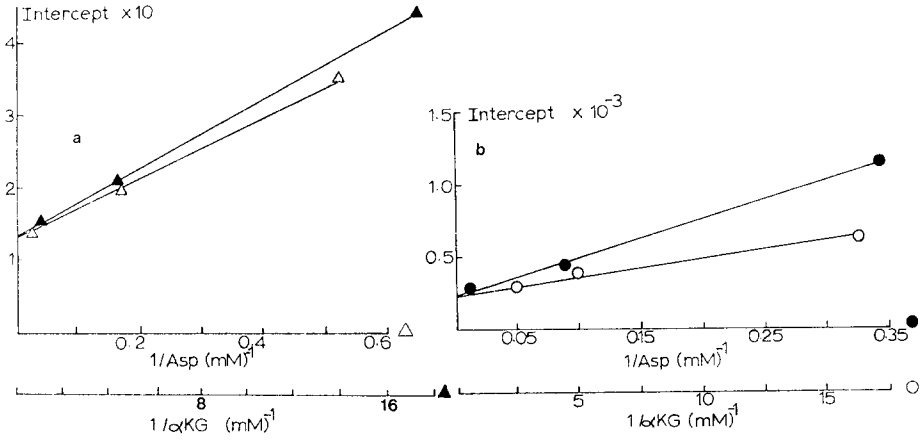


Fig. 5. (a) Replot of the intercept on the ordinate versus the reciprocal of the fixed substrate concentration for the data shown as inserts in Figs 3 and 4. (b) Replot of the intercept on the ordinate versus the reciprocal of the fixed substrate concentration for the data shown in Figs 3 and 4.

effect of product inhibition on heterogeneously catalysed reactions, and that this can lead to the observation of curved kinetic patterns [29]. Consequently, this also may be a contributing factor to the observed results.

The extraction of meaningful data from curved double-reciprocal plots is of questionable significance. In this case, however, the use of sufficiently high substrate concentrations makes the determination of the intersection with the ordinate quite accurate. Consequently, secondary plots of the intercepts on the ordinate versus the reciprocals of the fixed substrate concentrations are shown in Fig. 5b.

For the amino ethyl cellulose-glutamate oxaloacetate transaminase. The calculated Michaelis constants are listed in Table I. The corresponding replots of the results obtained for the free enzyme are shown in Fig. 5a. and the calculated Michaelis constants are also listed in Table I.

From Table I it can be seen that the Michaelis constants calculated here for soluble enzyme are in fair agreement with those reported in the literature [24]. Furthermore, the results also show that the measured Michaelis constants for glutamate oxaloacetate transaminase are not greatly affected by the enzyme's immobilization onto amino ethyl cellulose.

TABLE I

MICHAELIS CONSTANTS MEASURED FOR FREE AND AMINO ETHYL CELLULOSE-GLUTAMATE OXALOACETATE TRANSAMINASE

Enzyme	K_m aspartate (M)	K_m α-ketoglutarate (M)
Soluble	$3.6 \cdot 10^{-3}$	$1.7 \cdot 10^{-4}$
Soluble*	$3.9 \cdot 10^{-3}$	$4.3 \cdot 10^{-4}$
Amino ethyl cellulose-enzyme	$3.5 \cdot 10^{-3}$	$2.4 \cdot 10^{-4}$

* From ref. 24.

Thermal inactivation of free and amino ethyl cellulose-glutamate oxaloacetate transaminase

The time courses of the thermal inactivations of free and amino ethyl cellulose-glutamate oxaloacetate transaminase at 65° C are shown in Fig. 6. The results indicate that the immobilized enzyme derivative was slightly more resistant to thermal inactivation at 65° C than the free enzyme. Similar stabilizations of enzymes on immobilization have been reported in the literature [9]. The semi-log plot of the thermal inactivation of soluble glutamate oxaloacetate transaminase (not shown here) was a straight line, indicating a normal, first-order rate of decay for the activity of the enzyme. The half life was 6 min at 65° C. The semi-log plot of the thermal inactivation of amino ethyl cellulose-glutamate oxaloacetate transaminase (not shown here) was, however, biphasic. Initially, the rate of decay of enzyme activity was similar to that of the soluble enzyme, but after about 12 min incubation at 65° C, the rate of decay of enzyme activity slowed considerably. The reason for this is not clear, but it has been postulated that biphasic semi-log plots, similar to that reported here may be due to the influence of a mixed population of enzymes which probably exist in the heterogeneously catalysed reaction. Fig. 6 also shows the effect of 10 mg/ml polyethylene glycol and 0.1 mM pyridoxal 5'-phosphate on the thermal stability of free and amino ethyl cellulose-glutamate oxaloacetate transaminase at 65° C. These results show that a greater increase in the thermal stability of the soluble enzyme was obtained in comparison with that obtained for the amino ethyl cellulose derivative. This was probably due to the support material sterically preventing the polyethylene glycol from coming into close contact with the immobilized enzyme, and hence restricting the protective properties of the polyethylene glycol.

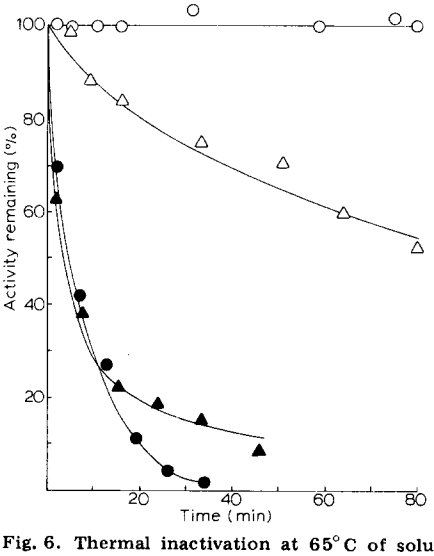


Fig. 6. Thermal inactivation at 65° C of soluble (●), and amino ethyl cellulose-glutamate oxaloacetate transaminase (▲). Open symbols denote inactivation in the presence of 10 mg/ml polyethylene glycol and 0.1 mM pyridoxal 5'-phosphate.

In conclusion, the immobilized glutamate oxaloacetate transaminase derivative described in this work exhibited enzymic parameters which were not vastly different from those of the free enzyme, indicating the usefulness of diethyl adipimidate as a coupling reagent.

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