

主論文目録

1. 題目 Studies on Allosteric Effects of Phosphoenolpyruvate Carboxylase from *Escherichia coli*

(大腸菌フォスファエリルピルビン酸カルボキシラーゼにおけるアロステリック効果に関する研究)

2. 公表の方法・時期

1部 主論文題目 Kinetic Studies on the Allosteric
Nature of Phosphoenolpyruvate Carboxylase from
Escherichia coli (大腸菌のフォスファエリルピルビン酸カルボキシラーゼ
のアロステリックな性質に関する反応速度論的研究)

発表雑誌名 The Journal of Biochemistry (日本
生化学会刊行誌), 68巻 227頁 (1970年刊行) 掲載

2部 主論文題目 Effects of High Pressure on the
Stability and Activity of Allosteric Phosphoenol-
pyruvate Carboxylase from *Escherichia coli*
(大腸菌のアロステリックなフォスファエリルピルビン酸カルボキシラーゼの安
定性と活性におよぼす高圧の影響)

発表雑誌名 The Journal of Biochemistry (日本
生化学会刊行誌), 73巻 3号 (1973年刊行) 掲載予定

3. 冊数 1冊

EFFECTS OF HIGH PRESSURE ON THE STABILITY AND ACTIVITY OF
ALLOSTERIC PHOSPHOENOLPYRUVATE CARBOXYLASE FROM ESCHERICHIA COLI

By KATSURA IZUI

(From the Department of Chemistry, Faculty of Science,
Kyoto University, Kyoto)

EFFECTS OF HIGH PRESSURE ON THE STABILITY AND ACTIVITY OF
ALLOSTERIC PHOSPHOENOLPYRUVATE CARBOXYLASE FROM ESCHERICHIA COLI*

By KATSURA IZUI

(From the Department of Chemistry, Faculty of Science,
Kyoto University, Kyoto)

Received for publication, August 2, 1972.

Effects of hydrostatic pressure up to 3,000 atm on phosphoenolpyruvate (PEP) carboxylase [EC 4.1.1.31] were studied. The enzyme in the absence of its ligands lost some activity by the compression at 1,000 atm for 15 min at 22°C and was inactivated completely by the compression at 2,000 atm for 15 min in complete nonreversibility upon release of pressure. An activation volume for the inactivation was evaluated as about -120 ml/mole of the enzyme based on the dependency of inactivation rate on the magnitude of pressure. Investigation of the effect of each ligand on the pressure inactivation of the enzyme revealed the following facts: (i) PEP, one of the substrates, and the allosteric activators such as acetyl-CoA and fructose-1,6-diphosphate showed no effect; (ii) L-Aspartate, one of the allosteric inhibitors, showed a marked protective effect. The extent of

* A preliminary account of this work appeared in Seikagaku, 40, 385 (1968) (in Japanese).

protection increased with increasing concentrations of L-aspartate and was saturated at 10 mM; (iii) Long chain fatty acid such as laurate, the other allosteric activator, markedly accelerated the pressure inactivation.

Discussion was done on the conformational states induced by each ligand based on these results. In addition, the response of enzyme to the effectors at 500 atm (where the native subunit structure was possibly retained,) was investigated with the intension of getting some information on the change in partial molar volume of the enzyme associated with the allosteric transition. Although no significant pressure effect was observed in this regard, a possible usefulness of this approach was discussed.

PEP* carboxylase [EC 4.1.1.31] is a key enzyme in Enterobacteriaceae, supporting the biosynthetic activity of the tricarboxylic acid cycle by replenishing oxaloacetate to it (1,2). The enzyme activity is known to be under the control of multiple allosteric effectors, reflecting important roles of the enzyme in intermediary metabolism: The enzyme from E. coli is inhibited by L-aspartate (3,4) and activated by CoASAc (5), FDP (6) and by long chain fatty acids or their CoA derivatives (7). Furthermore, the enzyme is markedly activated by high concentrations of various

* Abbreviations used are: PEP, phosphoenolpyruvate; FDP, fructose-1,6-diphosphate; CoASAc, acetyl-CoA; DTT, dithiothreitol; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid).

organic solvents such as dioxane and alcohols (8) which are presumed to bind at the same site of the enzyme that for fatty acids (7). At least four distinct regulatory sites have been shown to be inherent in the enzyme for binding with these effectors by the technique of genetic desensitization (9). These effectors seem to affect the catalytic activity by causing conformational changes of the enzyme protein. Therefore, it is of interest to inquire the nature of conformational changes of such an enzyme having multiple effectors induced by each effector. A series of studies in this laboratory have provided some pieces of evidence supporting that multiple conformational states are accessible to the enzyme (10, 11) and that the state induced by binding with aspartate is remarkably different from those by other effectors (6,11).

As a new approach to this problem the effect of each allosteric ligand on the enzyme stability under high hydrostatic pressure was studied. Since pressure is known to be one of the agents for destruction of folded structure of native proteins, an alteration of pressure-stability of the enzyme caused by effector is supposed to be indicative of some conformational change of the enzyme associated with an allosteric transition. Furthermore, the response of the enzyme activity to the effectors under pressure was investigated from the following point: An increase in pressure, in general, shifts the equilibrium in the direction which reduces the volume of the system as a whole. If a change in the partial molar volume of the enzyme protein is associated with an allosteric transition, the application of hydrostatic pressure on the enzyme may shift the allosteric equilibrium

defined by Monod et al. (12) as if it were a kind of "effector". Such a study will provide some information about the thermodynamic properties of allosteric transition, and consequently about the nature of chemical bonds involved in the transition.

EXPERIMENTAL

PEP carboxylase was partially purified from E. coli W by the method described previously (6,13). The enzyme preparation, capable of catalyzing 5 to 20 μ moles of oxaloacetate formation per min per mg protein under the standard reaction conditions (13), was used throughout this study. It was stored at 0°C in suspension in 60%-saturated $(\text{NH}_4)_2\text{SO}_4$ solution containing 10 mM aspartate, 10 mM Mg^{++} and 0.1 M Tris-HCl, pH 7.4. Before use, the enzyme was collected by centrifugation, dissolved in 0.1 M Tris- H_2SO_4 , pH 8.5, containing 1 mM DTT, and the solution was passed through the column of Sephadex G-50 equilibrated with 0.1 M Tris- H_2SO_4 , pH 8.5, containing 0.1 mM DTT. In the experiment of sucrose density gradient centrifugation, the Tris- H_2SO_4 -DTT buffers containing 1 mM and 0.1 mM EDTA were used for dissolution and for elution of the enzyme, respectively.

The enzyme activity was determined by photometric method (13) or isotopic method (10). The former was used for the measurement of residual enzyme activity after application of pressure, and the latter for the measurement of the enzyme activity under pressure. The standard reaction mixture for photometric assay contained the following constituents in μ moles per one ml: Tris- H_2SO_4 buffer, pH 8.5, 100; potassium PEP, 1.0;

KHCO_3 , 10; MgSO_4 , 10; dioxane, 1,170; NADH, 0.1; and 1.8 I.U. of malate dehydrogenase [EC 1.1.1.37]. The standard reaction mixture for isotopic assay contained the following constituents in μmoles per one ml: Tris- H_2SO_4 buffer, pH 8.5, 100; potassium PEP, 4.4; $\text{KH}^{14}\text{CO}_3$ (0.5 $\mu\text{Ci}/\mu\text{mole}$), 10; MgSO_4 , 10; NADH, 1.8; and 10 I.U. of malate dehydrogenase. Malate dehydrogenase was assayed according to the method of Ochoa (14).

The pressure equipment used was of the same type as described by Suzuki and Suzuki (15). A pressure vessel was a cylinder, 10 cm in diameter and 13 cm in height with a hole of 1.6-cm diameter at the center. The hole was filled with liquid paraffin and the reaction tubes, at most 7, were immersed together into the liquid paraffin in the hole. Pressure was applied by pressing the bar fitted in the hole by the use of hydro-pressure alignment. The reaction tube was a plasticized polyvinylchloride tube having a dimension of 2 mm in inner diameter and 4 cm in height, stoppered with glass rods on both sides. The tube was washed thoroughly with detergent and ether before use*.

* The enzyme activity in the absence of any allosteric activator was found always higher by about 3-fold when the reaction was allowed to proceed in a water-washed tube of plasticized polyvinylchloride than when it was done in a glass test tube. This phenomenon was ascertained to be due to the activation by trace amounts of some plasticizer which exuded from the tube of polyvinylchloride to the reaction mixture. To avoid this unfavorable effect the tube had to be washed as described. Among the → 次頁へ続<

Zone sedimentation of the enzyme in sucrose density gradients was carried out as described by Martin and Ames, taking yeast alcohol dehydrogenase ([EC 1.1.1.1], $s_{20,w} = 7.4$ S) and bovine liver catalase ([EC 1.11.1.6], $s_{20,w} = 11.3$ S) as internal standards (16). A 100- μ l sample solution containing PEP carboxylase (10 or 100 μ g protein), alcohol dehydrogenase (140 μ g) and catalase (37 μ g) was layered on a linear 5 to 20% (w/v) sucrose gradient of total volume of 4.6 ml. Both the sample solution and the gradient contained 0.1 M Tris- H_2SO_4 buffer, pH 8.5, 0.1 mM EDTA, 0.1 mM DTT and, when indicated, the compound which was to be tested as ligand. After centrifugation at 40,000 rev/min for 8 hr at 20°C in a RPS 50A rotor of Hitachi model 65P ultracentrifuge, the gradient was fractionated. PEP carboxylase was assayed spectrophotometrically at 30°C with a 10- μ l portion of each fraction in the standard reaction mixture. Alcohol dehydrogenase and catalase were assayed spectrophotometrically with a 5- μ l portion of each fraction according to the methods of Racker (17), and Chance and Maehly (18), respectively.

Protein concentration was determined by the method of Lowry et al. (19) with crystalline bovine serum albumin as a standard.

CoASAc was prepared by the method of Simon and Shemin (20) and was

前頁より続く → authentic samples of plasticizers tested such as dioctyladipate, dioctylphthalate and tetraethyleneglycoldimethylether, the last compound was effective in the activation. Presumably it causes an activation by binding to the allosteric site for free fatty acids (7).

determined by the method with citrate synthase [EC 4.1.3.7] and DTNB (21). Bovine liver catalase, bovine serum albumin, DTT, FDP and DTNB were purchased from Sigma Chemical Co., yeast alcohol dehydrogenase, malate dehydrogenase, citrate synthase and CoASH were from Boehringer Mannheim. All other chemicals were prepared or purchased as described previously (6).

RESULTS

Enzyme inactivation by pressure — Figure 1 shows time course of the enzyme inactivation under various magnitudes of pressure. The enzyme lost 50% of its original activity in 30-min compression at 1,000 atm of pressure at 22°C. The linearity of semilogarithmic plots of residual

Fig. 1

activity against time shows that the inactivation by pressure is of first order with respect to the enzyme concentration. The influence of pressure on the inactivation rate constant (k) is related by Eq. (1), where P is

$$\frac{\Delta \ln k}{\Delta P} = - \frac{\Delta V^\ddagger}{RT} \quad (1)$$

the magnitude of pressure, R is the gas constant, T is the absolute

temperature and $\Delta V^\ddagger$ is the molar volume change of activation. From the plots according to Eq. (1) (inset in Fig. 1), $\Delta V^\ddagger$ was evaluated to be about -120 ml/mole. Inactivation was irreversible, and seemed not to be attributable to the oxidation of essential sulfhydryl groups of the enzyme (11) accelerated by compression, since no protective effect of sulfhydryl compound such as 5 mM mercaptoethanol or 5 mM glutathione was observed.

The influence of allosteric effectors and substrates on the enzyme stability under high pressure was then investigated in order to obtain

Fig. 2

some information about the nature of conformational states of the enzyme induced by binding with each ligand. As seen from Fig. 2 and Table I,

Table I

15 mM aspartate strongly protected the enzyme against pressure inactivation. Since 15 mM glutamate, an analogue of aspartate but not an allosteric inhibitor of the enzyme, showed no protection, the effect of aspartate may be attributable to the specific allosteric interaction with the enzyme.

The degree of protection was dependent on the concentration of aspartate and saturated at the concentration of 10 mM, as shown in Fig. 3. The

Fig. 3

reason for a significant departure of "protection curve" from "inhibition curve" is not clear at present (Fig. 3). Unlike to aspartate, 1.1 M di-oxane as well as 0.2 mM laurate, both of which are known to bind to the same site on the enzyme to exert their activating effect (7), remarkably accelerated the enzyme inactivation by pressure (Fig. 2 and Table I). However, it seems not reasonable to ascribe these effects primarily to the conformational change caused by allosteric interaction of the effectors with the enzyme, since non-specific denaturing effect of these compounds cannot be excluded. The apparent synergism of them with pressure in the effect on the enzyme inactivation suggest that both may have something in common with respect to the mode of action on the protein structure, e.g. labilization of hydrophobic bond (22,23). The other effectors such as CoASAc (0.4 mM) and FDP (10 mM) caused no significant effect on the enzyme stability under high pressure, and 10 mM Mg^{++} , one of the reaction components, with or without 18 mM PEP showed only a slight protection (Table I). From these results it may be supposed that a gross conformational change is induced in the enzyme at least upon binding with aspartate.

Effect of pressure generated by centrifugal force on subunit structure

— As known with most allosteric proteins PEP carboxylase is also an oligomeric protein which is composed of 4 subunits of equal molecular weight (88,200 daltons*) (cf. 13). Since pressure is generally known to facilitate depolymerization of polymeric proteins to their subunits (24-26), it is highly probable that PEP carboxylase reversibly dissociates under several hundred atm of pressure where the allosteric effect was investigated as described in the following section. In order to test this

Fig. 4

possibility the sedimentation profile of the enzyme in the pressure gradient from 1 to 600 atm, which was expected to be generated by centrifugal force, was examined. In addition, the influences of effectors on the profile were also examined. Figure 4 and Table II show the results of sucrose density gradient centrifugation in the absence of effectors

* T. Yoshinaga, H. Teraoka, K. Izui and H. Katsuki, presented at the 44th Annual Meeting of the Japanese Biochemical Society held at Sendai in October, 1971.

Table II

and in the presence of 10 mM aspartate, 10 mM Mg^{++} or 10 mM FDP plus 0.82 mM CoASAc. In all runs the symmetrical single peak of the enzyme activity was observed, and all $s_{20,w}$ values estimated from the internal standards were almost the same (12.8-13.1 S) within experimental errors. These results indicate that the enzyme does not dissociates to its subunits even under several hundred atm of pressure, and that the effectors do not cause any change in subunit structure of the enzyme under these conditions. The observations with the enzyme from E. coli W described here seem to be significantly different from those with the enzyme from E. coli B which is similar to the former enzyme in allosteric properties (27).

Allosteric effect under high pressure — It is generally known that by the application of pressure, the rate of enzyme-catalyzed reaction is accelerated or retarded owing to the volume change associated with the formation of enzyme-substrate complex (28). In the case of allosteric enzymes, the following additional pressure effect is expected to occur, if the volume change is associated with the allosteric transition of enzyme protein: The enzyme activity is extraordinarily enhanced or depressed, or the cooperativity of each allosteric effector is increased or decreased by the application of pressure. From this point of view, PEP carboxylase seemed to be a favorable enzyme for studying whether or not pressure acts on enzyme as if it were a kind of "allosteric activator or inhibitor".

Because our previous kinetic studies which were done under the atmospheric pressure (10,29) had established that the effects of CoASAc (activator) and aspartate (inhibitor) are antagonistic to one another and that the cooperativity of the one becomes remarkable by increasing the concentration of the other.

The applied pressure was 500 atm, under which irreversible inactivation of the enzyme was negligible during 15-min compression (cf. Fig. 2). Under the conditions, malate dehydrogenase which was contained in the reaction mixture for trapping of oxaloacetate as malate was fairly stable: It only lost 5% and 11% of its original activity in 0.1 M Tris-HCl, pH 8.5, by 15-min compression at 1,000 and 3,000 atm, respectively. The results are given in Fig. 5. A pressure of 500 atm caused no significant effect

Fig. 5

on the allosteric properties of PEP carboxylase with respect to both aspartate and CoASAc, namely, on half-saturation concentration, maximal extent of activation or inhibition, and on the shape of saturation curve of the effector. Furthermore, pressure caused no remarkable effect on the enzyme activity in the absence of an effector as seen from the plots on the ordinates of both figures ~~A and~~ B. These results indicate that a pressure of 500 atm did not act on the enzyme as if it were a kind of

"effector".

DISCUSSION

PEP carboxylase was found to be inactivated at rather lower pressure, compared with other enzymes so far studied (30,31) which were reported to be inactivated at pressures above 4,000 atm. Furthermore, the activation volume ($-\Delta V^\ddagger$) of inactivation of PEP carboxylase was shown to be significantly larger than those described on other enzymes (31). Since previous studies on the stability of enzymes under high pressure were rather confined to monomeric enzymes or the ones with lower molecular weight (below 5×10^4 daltons), it is uncertain whether the instability of PEP carboxylase under high pressure is an inherent nature of oligomeric enzymes composed of subunits of higher molecular weights (above 5×10^4 daltons) or this fact reflects one of the characteristics of the allosteric enzymes in which conformational flexibility plays an important role for their function.

Aspartate, one of the allosteric effectors, was found to be markedly protective against pressure inactivation of the enzyme, whereas PEP and Mg^{++} , the reaction components, were not so effective. This implies that aspartate causes, by binding at its allosteric site, a conformational change of the enzyme protein through which the catalytic site of the enzyme acquires a more stability against high pressure. In accordance with this observation, previous studies by us have revealed the distinguished effect of aspartate among other ligands on the enzyme conformation:

Aspartate was shown strongly protective against inactivation by heat (6)

and by SH-modification with N-ethylmaleimide (11), and it caused 40% depression of the quantum yield of fluorescence due to 1-anilino-8-naphthalenesulfonate bound to the enzyme*. Since aspartate did not exert its effect on the subunit structure of the enzyme as observed by sedimentation velocity in sucrose density gradient centrifugation, the above-mentioned effects of aspartate may be ascribed to some marked change in tertiary structure of the enzyme. However, it remains to be elucidated whether aspartate has any effect on the stability of subunit structure of the enzyme in order to get more information on the mechanism for protective effect of aspartate against pressure inactivation of the enzyme. Since pressure is generally known to facilitate depolymerization of polymeric proteins to their subunits as mentioned already, it is highly probable that the dissociation precedes the irreversible inactivation of the enzyme at high pressures ($>1,000$ atm) as seen in the case of F-actin (32). Therefore, if aspartate stabilizes the tetrameric structure of the enzyme, the protecting effect of aspartate may mainly be attributable to the antagonistic action against the dissociating action of pressure.

No significant effect of pressure (500 atm) was detected on the allosteric nature of PEP carboxylase. Unfortunately the instability of the enzyme under pressure did not permit the investigation at higher

* T. Yoshinaga, K. Izui and H. Katsuki, presented at the 19th Annual Meeting of Kinki-branch of the Japanese Biochemical Society held at Osaka in June, 1972.

pressures (> 500 atm). In general, if the change in partial molar volume (ΔV) of an enzyme is associated with its allosteric transition, the effect of pressure on the allosteric constant (L) defined by Monod et al. (12) may be estimated according to the following thermodynamic equation, where

$$\ln L_p/L_1 = - (V_T - V_R) \Delta P/RT = - \Delta V \Delta P/RT \quad (2)$$

V_T and V_R are the partial molar volume of an enzyme in T-state (inactive state) and R-state (active state), respectively. If, for example, at least one hydrophobic bond is involved in the transition, about 20 ml per mole of volume change is expected (33) and hence L may be increased or decreased by about 5-fold by the application of 2,000 atm of pressure. On the other hand, a remarkable effect of pressure may be expected in those allosteric enzymes, in which the dissociation-association processes of subunits are closely associated with the allosteric transition (cf. 34). Because ΔV accompanied with polymerization of subunits are usually known to be larger than 100 ml/mole of subunit (25, 35-37) so that L may be changed by about 3,000-fold at 30°C by the application of 2,000 atm as estimated according to Eq. (2). Recent our study extended to the system of human hemoglobin showed that application of 2,000 atm of pressure increased the binding affinity of hemoglobin for ethylisothiocyanide by about 7-fold but caused no effect on the ligand cooperativity (38). Although no significant effect of pressure was observed on the allosteric properties with these systems, it seems necessary to apply this approach to many other allosteric systems in order to obtain more insight into the allosteric transition.

ACKNOWLEDGEMENT: I would like to express my sincere thanks to Prof. J. Osugi (Kyoto University) for the use of the pressure equipment, to Drs T. Asano, Y. Kitamura, Profs H. Katsuki (Kyoto University), K. Suzuki (Ritsumeikan University), and S. Asakura (Nagoya University) for their encouragement and useful discussions during the course of this work.

REFERENCES

1. J. M. Ashworth and H. L. Kornberg, Proc. Roy. Soc., B, 165, 179 (1966).
2. T. S. Theodore and E. Englesberg, J. Bacteriol., 88, 946 (1964).
3. T. Nishikido, K. Izui, A. Iwatani, H. Katsuki and S. Tanaka, Biochem. Biophys. Res. Commun., 21, 94 (1965).
4. T. Nishikido, K. Izui, A. Iwatani, H. Katsuki and S. Tanaka, J. Biochem., 63, 532 (1968).
5. J. L. Canovas and H. L. Kornberg, Biochim. Biophys. Acta, 96, 169 (1965).
6. K. Izui, T. Nishikido, K. Ishihara and H. Katsuki, J. Biochem., 68, 215 (1970).
7. K. Izui, T. Yoshinaga, M. Morikawa and H. Katsuki, Biochem. Biophys. Res. Commun., 40, 949 (1970).
8. H. Katsuki, T. Nishikido, K. Izui, A. Iwatani and S. Tanaka, 7th International Congress of Biochemistry, Tokyo (1967).
9. M. Morikawa, K. Izui and H. Katsuki, Biochem. Biophys. Res. Commun., 45, 689 (1971).
10. K. Izui, J. Biochem., 68, 227 (1970).
11. H. Teraoka, S. Naito, K. Izui and H. Katsuki, J. Biochem., 71, 157 (1972).
12. J. Monod, J. Wyman and J.-P. Changeux, J. Mol. Biol., 12, 88 (1965).
13. T. Yoshinaga, K. Izui and H. Katsuki, J. Biochem., 68, 747 (1970).
14. S. Ochoa, "Methods in Enzymology," ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, Vol I, p.735 (1955).

15. C. Suzuki and K. Suzuki, J. Biochem., 52, 67 (1962).
16. R. G. Martin and B. N. Ames, J. Biol. Chem., 236, 1372 (1961).
17. E. Racker, "Methods in Enzymology," ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, Vol I, p.500 (1955).
18. B. Chance and A. C. Maehly, "Methods in Enzymology," ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, Vol II, 764 (1955).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. Biol. Chem., 193, 265 (1951).
20. E. J. Simon and D. Shemin, J. Am. Chem. Soc., 75, 2520 (1953).
21. P. K. Tubbs and P. B. Garland, "Methods in Enzymology," ed. by J. M. Lowenstein, Academic Press, New York and London, Vol XIII, p.535 (1969).
22. C. Tanford, J. Am. Chem. Soc., 84, 4240 (1962).
23. W. Kauzmann, Advances in Protein Chemistry, 14, 1 (1959).
24. M. Maruyama, Science, 153, 145 (1966).
25. R. Josephs and W. F. Harrington, Biochem., 7, 2834 (1968).
26. G. Kegeles, L. Rhodes and J. L. Bethune, Proc. Nat. Acad. Sci., U. S., 58, 45 (1967).
27. T. E. Smith, Arch. Biochem. Biophys., 128, 611 (1968).
28. K. J. Laidler, "The Chemical Kinetics of Enzyme Action," Oxford, London, p.210 (1958).
29. K. Izui, A. Iwatani, T. Nishikido, H. Katsuki and S. Tanaka, Biochim. Biophys. Acta, 139, 188 (1967).

30. K. Suzuki, Seibutsu-buttsuri, 3, 194 (1964) (in Japanese).
31. K. Kitamura, Rev. Phys. Chem. Japan, 36, 44 (1966).
32. T. Ikkai and T. Ooi, Biochem., 5, 1551 (1966).
33. D. L. Caspar, "Principles of Biomolecular Organization," ed. by G. E. W. Woltensholme and M. O'Conner, J. and A. Churchill Ltd., London, p.7 (1966).
34. A. Levitzki and D. E. Koshland, Jr., Biochem., 11, 247 (1972).
35. C. L. Stevens and M. A. Lauffer, Biochem., 4, 31 (1965).
36. K. E. VanHolde and L. B. Cohen, Biochem., 3, 1803 (1964).
37. B. R. Gerber and H. Noguchi, J. Mol. Biol., 26, 197 (1967).
38. K. Suzuki, Y. Taniguchi and K. Izui, J. Biochem., 71, 901 (1972).

TABLE I. The effect of reaction components and allosteric effectors on the stability of PEP carboxylase under high pressure. The experimental conditions were the same as described in Fig. 2.

Addition (mM)		Residual enzyme activity (%) after the compression at				
		0	500	1,000	1,200	1,500 atm
Exp. 1	None	100	104	70		11
	L-Aspartate(15)	100	98	100		70
	L-Glutamate(15)	100	100	65		14
	Mg ⁺⁺ (10)	100	102	82		35
	Mg ⁺⁺ (10) + PEP(18)	100	100	80		39
Exp. 2	None	100	102	62		5
	CoASAc(0.4)	100	105	70		7
	FDP(10)	100	100	68		12
Exp. 3	None	100			25	
	L-Aspartate(10)	100			79	
	FDP(10)	100			37	
	Laurate(0.2)	100			5	

TABLE II. Sedimentation coefficients of PEP carboxylase under various conditions. The experiments were carried out as described in "EXPERIMENTAL".

Addition to centrifugation medium (mM)	Enzyme amount used (μ g protein)	Estimated 1) $s_{20,w}$
1 None	100	13.1
2 None	10	12.9
3 Mg^{++} (10)	100	12.8
4 L-Aspartate(10)	100	12.9
5 FDP(10) plus CoASAc(0.82) 2)	100	12.9

- 1) The values were obtained on the assumption that the partial specific volumes of the three enzymes shown in Fig. 3 were 0.725 ml/g of protein (16).
- 2) The concentration of CoASAc after the centrifugation was 0.47 mM.

Legends to Figures

Fig. 1. Time course of the inactivation of PEP carboxylase under various magnitudes of pressure. Pressure was applied to the enzyme solution (0.1 mg protein/ml) in 0.1 M Tris- H_2SO_4 buffer, pH 8.5, at 22°C for indicated periods. The residual enzyme activity was measured with a 10- μl portion of each sample. A control experiment for spontaneous inactivation of the enzyme (ca. 5% loss of the activity after an hour at 22°C), was carried out under exactly the same conditions except for the omission of pressure-application. Inset shows plots of the relation according to Eq. (1) given in the text.

Fig. 2. Curves of pressure inactivation of PEP carboxylase in the presence of aspartate or dioxane. The enzyme solutions (0.1 mg protein/ml of 0.1 M Tris- H_2SO_4 buffer, pH 8.5) containing 15 mM aspartate (—●—) or 1.17 M dioxane (—△—), and not containing any one of them (—○—) were incubated at 22°C for 15 min under increasing magnitudes of pressure as indicated and the residual enzyme activities were measured.

Fig. 3. Protection of PEP carboxylase against pressure inactivation by various concentrations of aspartate. The enzyme solutions (0.1 mg protein/ml of 0.1 M Tris- H_2SO_4 buffer, pH 8.5) containing aspartate of indicated concentrations were incubated at 1,200 atm at 22°C for 15 min, and the residual enzyme activity was measured. As ordinate in the figure was logarithmically plotted the ratio of the original activity (V_0) to the

residual activity ($\underline{V}$) so that the plots reflect the inactivation rate constant ($\underline{k}$); $\underline{k} = \frac{1}{0.4343\underline{t}} \log\left(\frac{V_0}{\underline{V}}\right)$, where $\underline{t}$ indicates time.

For comparison, the inhibition of the enzyme activity by increasing concentrations of aspartate was also plotted. The reaction mixture used for photometric assay in the inhibition experiment was the same as described in "EXPERIMENTAL" except for the omission of dioxane. The enzyme amount used was 10 μ g protein per 1 ml of the reaction mixture and the assay temperature was 30°C.

Fig. 4. Sedimentation of PEP carboxylase in sucrose density gradient centrifugation in the absence of the ligand. The experiment was carried out as described in "EXPERIMENTAL". The activities of PEP carboxylase and alcohol dehydrogenase are expressed in terms of the changes in absorbance at 340 nm/min, and that of catalase in terms of the change in absorbance at 240 nm/min.

Fig. 5. Response of PEP carboxylase activity to various concentrations of aspartate (A) and CoASAc (B) under 500 atm of pressure. The reaction mixture for isotopic assay as described in "EXPERIMENTAL" contained indicated concentrations of aspartate or CoASAc, and the enzyme concentrations were 60 and 12 μ g protein per ml, respectively. As soon as the reaction mixture was completed by the last addition of the enzyme, it was quickly syringed into the tube at 22°C, and the tube was inserted into the

pressure cell pre-equilibrated at the same temperature. After the incubation at 500 atm for 15 min, the reaction was substantially arrested by the immersion of the tubes into ice bath. Control experiments were done under the same conditions except for the performance of the reaction outside of the pressure cell both at 22°C and 0°C. After the reaction an aliquot (20 μ l) of each mixture was withdrawn and transferred to the scintillation vial containing 0.5 ml of 0.02 N HCl. The radioactivity incorporated into malate was measured according to the procedures as described previously (6). The radioactivities which were incorporated during the exposure of the sample at 22°C and 0°C (less than 2 and 10 min, respectively) under the atmospheric pressure were estimated from their corresponding controls and were subtracted from the total radioactivities on each sample. Under these conditions the enzyme reaction proceeded linearly with time up to 20 min at 22°C.

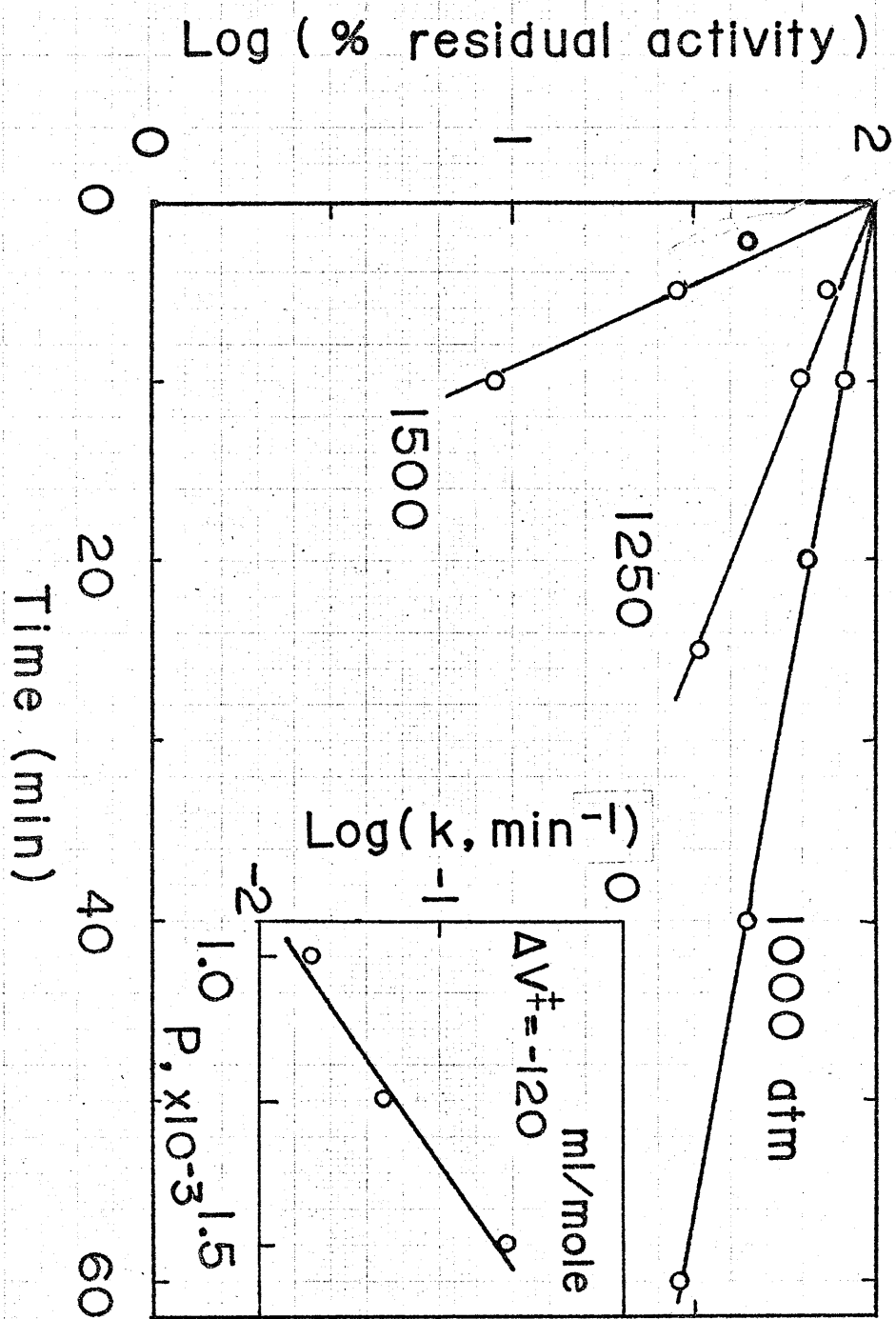


Fig. 1

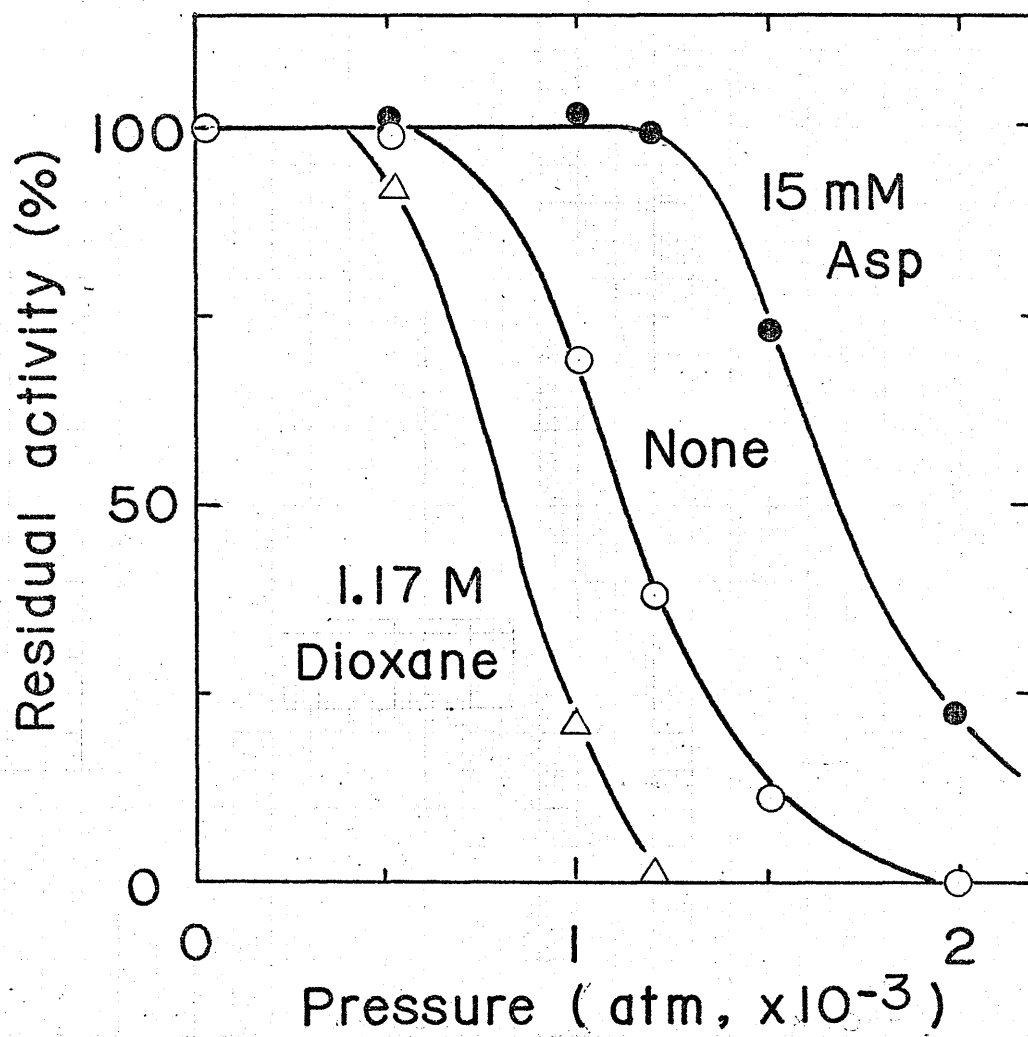


Fig. 2

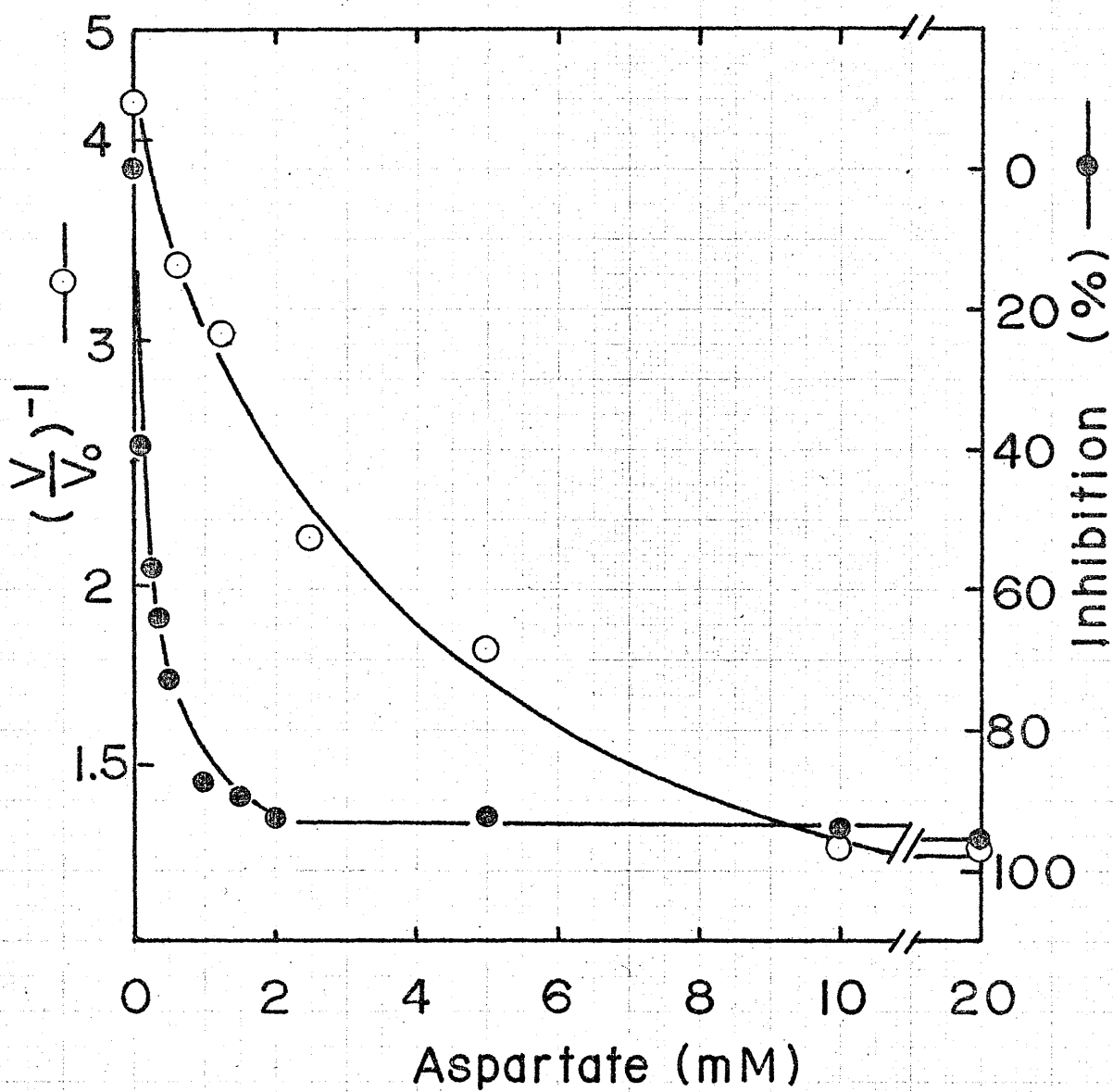
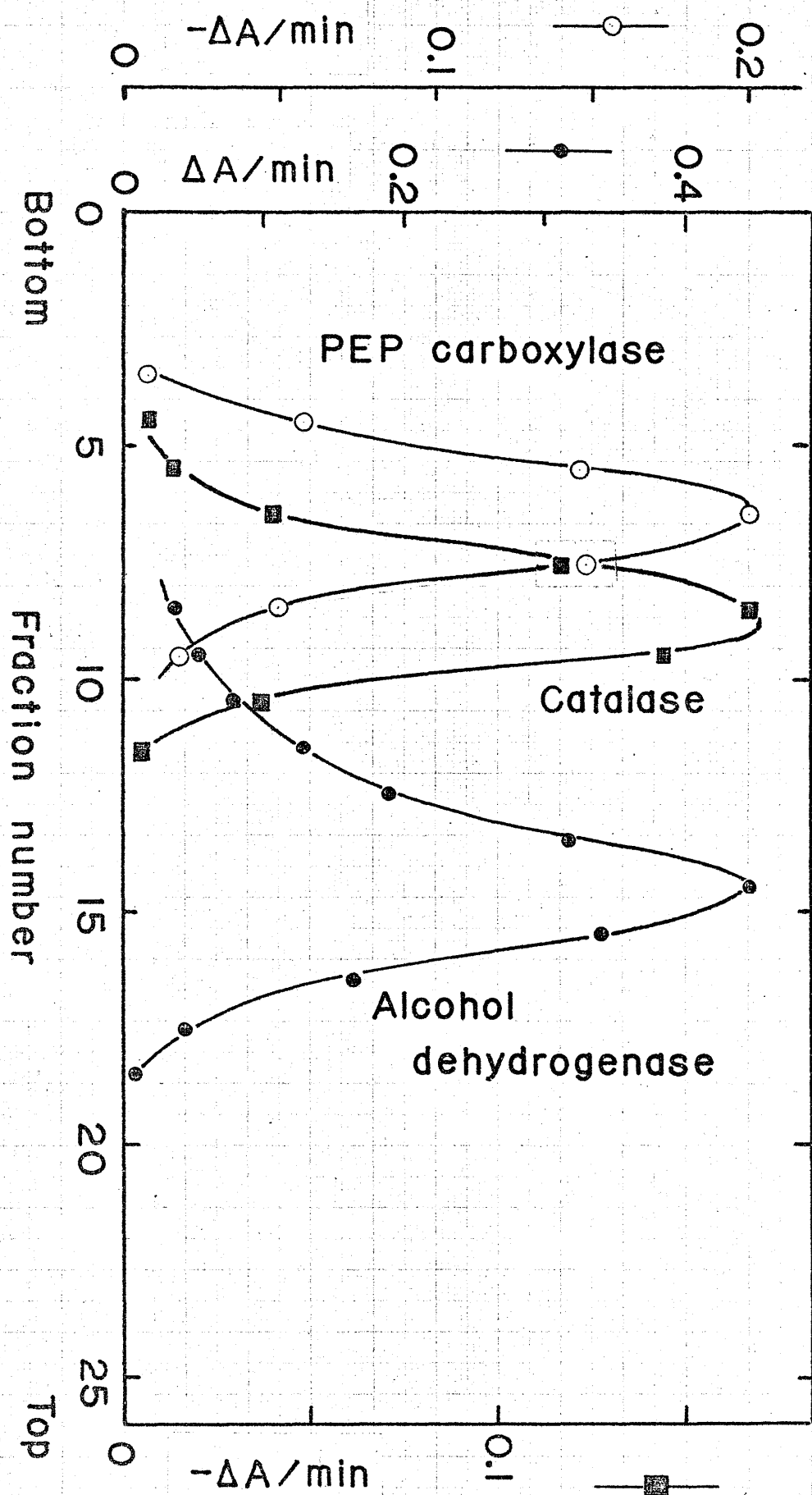


Fig. 3

Fig. 4



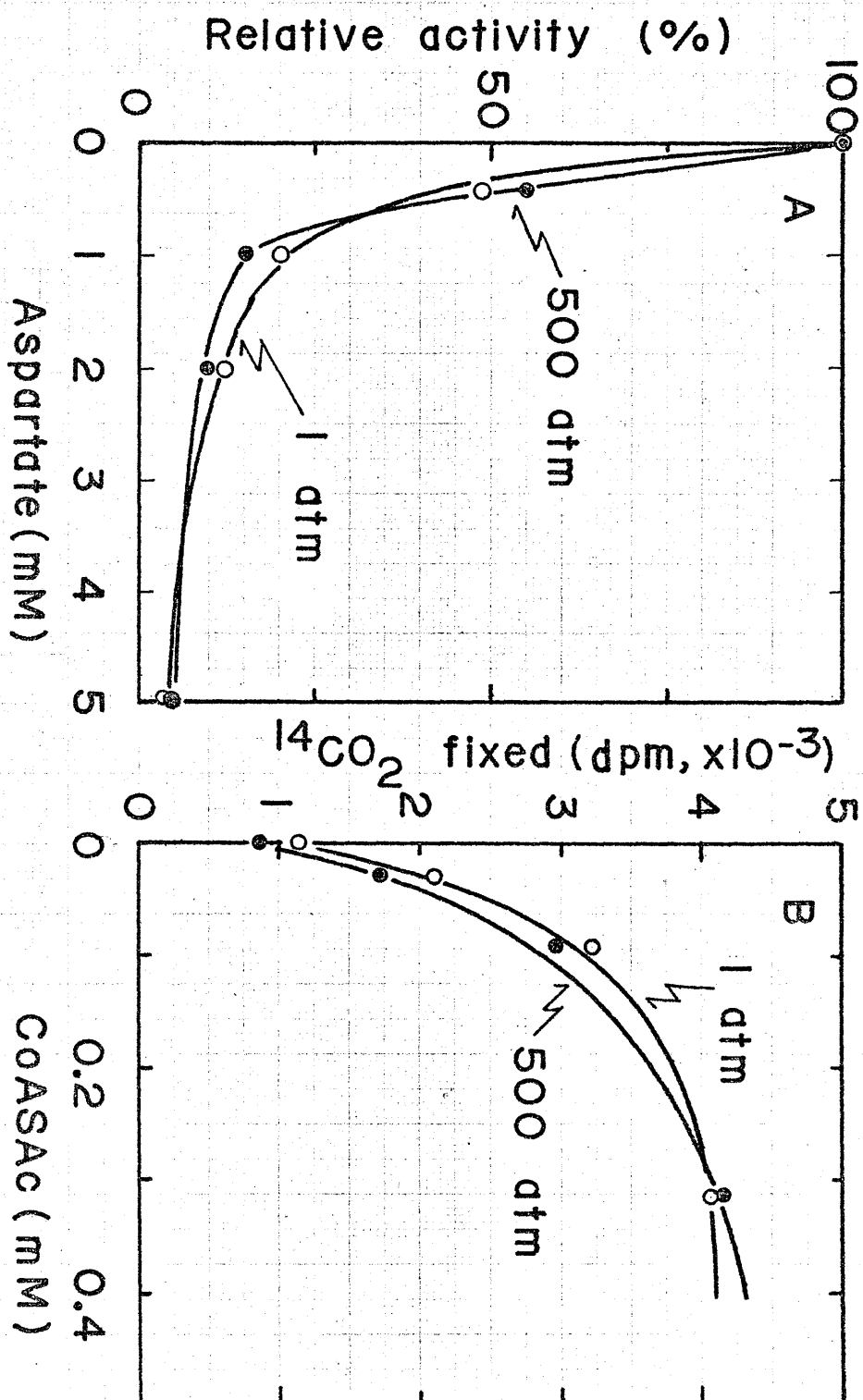


Fig. 5.