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Enzymic Activity of Whole Cells Entrapped in Reversed Micelles

Studies on α-Amylase and Invertase in the Entrapped Yeast Cells

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ABSTRACT

Studies have been conducted on the enzymic activity of Baker's yeast and also of Brewer's yeast entrapped into the reversed micelles formed by cetyl pyridinium chloride (CPCl) in *n*-hexane. The activities of α-amylase and invertase enzymes in the entrapped cells have been estimated and compared with those in the control experiments where there was no entrapment. The following significant observations have been made: 1. except for invertase, enzymes in Brewer's yeast, the entrapped yeast cells showed enhanced enzymic activities; 2. when the yeast cells were entrapped inside the reversed micelles along with substrates of the two enzymes, α-amylase, and invertase, the activity of each of these enzymes showed a further enhancement in comparison to that showed in the experiments in which substrates of the individual enzymes alone were entrapped—the phenomenon of synergism; 3. when the yeast cells and the respective substrates were entrapped inside separate reversed micelles and the solutions containing entrapped cells and entrapped substrates were mixed, the activities of the individual enzymes, α-amylase and invertase, showed further enhancement in comparison to the case in which the cells and the substrates were entrapped inside the same reversed micelle (in this case also the phenomenon of synergism was observed); and (4) In the case of experiments in which there was no

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entrapment, it was observed that the presence of substrates induced more release of enzymes from the yeast cells.

These observations on yeast cells, which to the best of our knowledge have not been reported before, should be biotechnologically relevant.

Index Entries: Entrapped yeast cells; α-amylase; invertase; reversed micelles; synergistic effect.

INTRODUCTION

Solubilization of enzymes in apolar solvents via reversed micelles has opened up the possibility of carrying out enzyme catalyzed reactions in nonaqueous media *(1,2)*. The discovery that enzymes can function in apolar solvents has dramatically expanded the range of reactions that can be approached through biocatalysis *(1–13)*. In a good number of cases, reverse micelles have been shown to act as efficient microreactors for enzymic reactions; the activity and stability of the enzymes have been shown to enhance several-fold when entrapped inside reverse micelles. The question of what is the maximal molecular size that can be hosted in reversed micelles has also been attended to *(1)*. Surprisingly, molecules as large as plasmids, with molecular weights of the order of 3×10^6, can readily be solubilized in organic solvents with the help of reverse micelles *(1,14)*. Even the whole cells of *Escherichia Coli (E. coli)* and of *Acintobacter calcoaceticus* have been solubilized by a reverse micellar system containing Tween-85 and water in isopropylpalmitate *(15)*.

In view of these observations, it is tempting to investigate reactions catalyzed by the enzymes released by the whole cells entrapped inside the reverse micelles. In this article, therefore, we report our investigations on the enzymic activity of yeast cells entrapped in the reverse micelles formed by cetyl pyridinium chloride (CPCl) in *n*-hexane. In these studies, we have worked with Baker's yeast and Brewer's yeast and in each case investigated the activities of two enzymes, namely α-amylase and invertase. Several interesting observations have been made, e.g., except for invertase enzyme in Brewer's yeast, in all other cases enhancement in the activities of the enzymes has been observed. It has also been observed that when α-amylase and invertase were made to act simultaneously, the activity of each of these enzymes was found to be enhanced in comparison to the situation when they were acting alone—the phenomenon of synergism. In order to rule out the possibility of the occurrence of side reactions, the synergistic effect has been confirmed using purified enzymes.

It has also been observed that if the yeast cells and the substrates are entrapped separately inside the reverse micelles, and the solutions containing entrapped cells and entrapped substrates are mixed, the activities

of the enzymes are enhanced further in comparison to the case when cells and substrates are entrapped inside the same (reversed) micelle.

Experiments have also been conducted on the enzymic activity of the yeast cells in the usual aqueous medium in the absence of reversed micelles. The significant observation in these experiments was that the presence of substrates induced more release of enzymes from the yeast cells.

MATERIALS AND METHODS

Materials

Brewer's yeast and Baker's yeast, α-amylase (CAS 9000-90-2), invertase from bakers yeast (CAS 9001-57-4), potato starch (CAS 9005-84-9), sucrose (CAS 50-1-54), CPCl (CAS 6004-24-6), *n*-hexane (CAS-110-54-3), BSA (CAS 9049-46-8), all from Sigma, and hydrochloric acid Analar grade were used in this study. Water distilled twice in an all Pyrex glass still was used for preparing solutions.

Methods

Two types of experiments were performed; one in which the reactants—yeast cells and or substrate were entrapped inside the reversed micelles and the other in which the reactions were conducted in the usual aqueous medium in the absence of reverse micelles. In the case of the former, the procedure similar to the one described in previous publications was adopted *(8,9,13)*.

To 20 mL of *n*-hexane contained in a glass beaker, 0.0193 g of Cetyl pyridinium chloride was added. To the turbid solution thus obtained, 0.1 mL of yeast cell suspension—prepared by adding known amount of the yeast cells (8×10^{-4} g of the cells in case of Brewer's yeast and 5×10^{-4} g in case of Baker's yeast) to 1.0 mL of appropriate buffer—was added slowly with vigorous stirring until the system became transparent, indicating the formation of reversed micelles and the solubilization of the yeast cells inside them. When activity of invertase was intended to be assessed, known volume of its substrate–sucrose solution of known strength, prepared in the appropriate buffer, was also solubilized, along with the yeast cells inside the reversed micelles. Similarly, when activity of α-amylase was intended to be assessed, known volume starch solution of known strength was solubilized inside the reversed micelles along with the yeast cells. Sodium acetate buffer, 10 m*M*, pH 4.6, and 0.1 m*M*, pH 4.8, were used for estimating the activity of invertase and α-amylase, respectively. For assessing the activities of both invertase and α-amylase when both enzymes act simultaneously, known volumes of the solutions of known strength of the substrates of both enzymes were solubilized simultaneously along with the yeast cells inside the reversed micelles.

Experiments were also performed by entrapping yeast cells and the substrate of the enzymes inside separate reversed micellar-water pools and then mixing known volumes of the solubilized yeast cells and the solubilized substrates. In these experiments, concentrations of the yeast cells, substrates, the surfactants, and the volume of the organic solvents were kept the same as those in the experiments when yeast cells and the substrates were entrapped inside the same reversed micellar-water pools.

In all these experiments, the reaction mixture—after incubating for known intervals of time at the desired temperature—was vigorously shaken with 5 mL of aqueous phase containing suitable amount of the killing agent of the enzymic reaction; for invertase 0.5 mL of 0.1*M* Na_2HPO_4 solution and for α-amylase 1 mL of 6*N* HCl solution was contained in the aqueous phase as a killing agent. It was expected that the products formed along with the unused reactants would come out in the aqueous phase, because on extraction with the aqueous phase, the reversed micelles either acquire the configuration of regular micelles or get dissociated into monomers. In either case, the materials solubilized inside the reversed micelles became available for estimation in the aqueous phase. In fact, it was confirmed through control experiments that the method of aqueous extraction gave reliable results.

Experiments were also conducted in the usual aqueous medium in the absence of reversed micelles. Yeast cells were suspended in the appropriate buffer and incubated at desired temperatures for different intervals of time. The suspensions were then centrifuged at 4000 rpm for 15 min and the activity of the two enzymes viz. α-amylase and invertase were estimated in the supernatant and also in the centrifugate. The enzymic activity in the centrifugate was estimated by suspending it in appropriate volume of buffer.

Enzyme Assay

Invertase

Invertase was assayed by the method described in literature *(16,17)*. In the case of reactions in the usual aqueous medium, the following procedure was adopted. Reaction mixture in a total volume of 1 mL contained 0.1 mL yeast cell suspension, 0.2 mL 10 m*M* sodium acetate buffer (pH 4.6) and 0.7 mL of sucrose solution of appropriate strength such that its final concentration was 0.4*M*. The reaction mixture was incubated for 30 min at 35°C. The reaction was then stopped by adding 0.1*M* Na_2HPO_4 solution, and boiling in a water bath for 10 min. To a 0.02 mL of this mixture water was added to increase volume to 0.50 mL. Reducing sugars produced were estimated by Nelson–Somogyi method *(17)*. Absorbance was recorded at 560 nm. Glucose served as standard. Specific activity of invertase is defined as μg of reducing sugars produced/min/mg protein.

α-Amylase

Activity of α-amylase was assayed using the method described in literature *(18, 19)*. For assay of the enzymic activity in the usual aqueous medium, 1.0 mL yeast cell suspension in 0.1*M* sodium acetate buffer (pH 4.8) and 1.0 mL starch solution of strength 10 mg/mL in 0.15*M* NaCl were incubated at 30°C for 30 min. Reaction was stopped by adding 1 mL of 6*N* HCl. To estimate unhydrolyzed starch, 1 mL aliquote was transferred to 25 mL volumetric flask and 15 mL water was added followed by 0.5 mL I-KI solution (0.2% I_2 in 2% KI). Volume was made up to 25 mL with water and absorbance was read at 660 nm. HCl when added immediately before incubation in the assay mixture served as zero-time sample. Suitable blanks for substrate and enzyme were also incubated and measured. The amount of starch hydrolyzed was estimated from the difference between the values from the blank and the actual experimental run. The specific activity of the α-amylase enzyme is defined as mg starch hydrolyzed/min/mg protein.

Protein Estimation

Protein was estimated by the method of Lowry et al. *(20)* using bovine serum albumin (BSA) as standard.

Estimation of Maximum Velocity (V_{max}) and Michaelis Constant (K_m)

Lineweaver–Burk (LB) plots were prepared for all the systems in aqueous phase and in the reversed micellear medium for the determination of maximum velocity (V_{max}) and Michealis constant (K_m). Illustrative LB plots in case of Brewer's yeast for invertase and α-amylase in two cases, namely when the yeast cells were suspended in the usual aqueous medium in the absence of reversed micelles and when they were entrapped inside the reversed micelles, are shown in Fig. 1A,B.

RESULTS AND DISCUSSION

In the experiments conducted in the absence of reversed micelle, in which the yeast cells were suspended in the appropriate buffer and centrifuged after incubation at desired temperatures for different intervals of time, the activity of the two enzymes, α-amylase and invertase were estimated in both the supernatant and the centrifugate. The supernatant did not show any activity, whereas the centrifugate did, which remained the same in all samples incubated up to 10 h (Table 1). The activity shown by the centrifugate was found more or less equal to the activity shown by the whole suspension incubated for the same interval of time. These observations indicate that the enzymes are not released by the cells in the supernatant at least up to 10 h. This inference is also corroborated by values of protein estimated in the supernatant (Table 1).

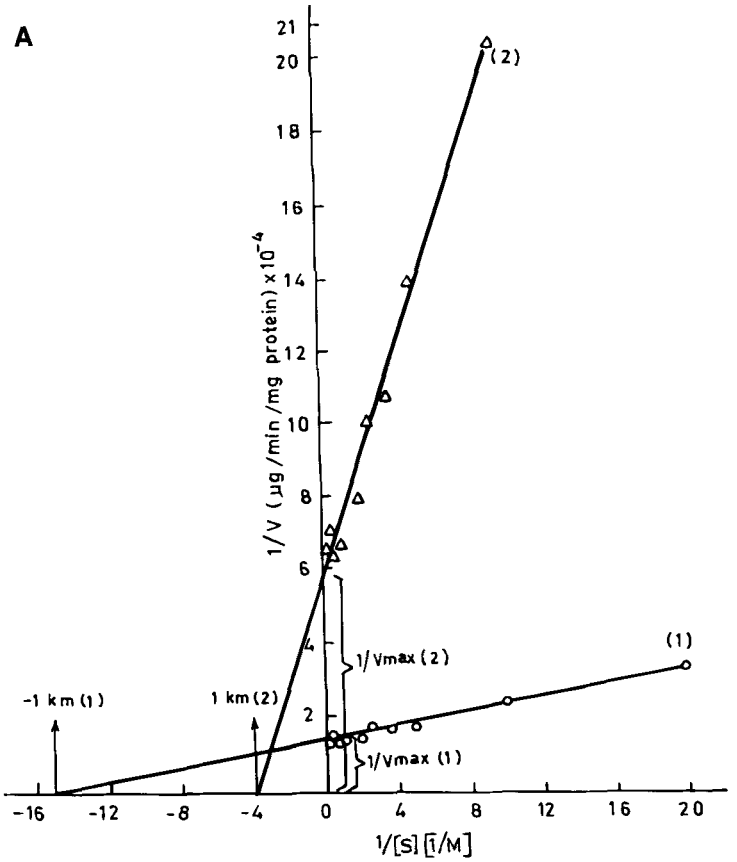

Fig. 1. Lineweaver and Burk (LB) plots in case of Brewer's yeast. V is the initial velocity, and S is the substrate concentration. **(A)** LB plot for invertase: (1) in aqueous system without surfactants and (2) after entrapment in the reversed micelles of CPCI in *n*-hexane. For aqueous system 0.1 mL yeast-cell suspension and 0.7 mL sucrose solution of strengths varying from 0.05 to 5.0*M* were incubated for 20 min at 35°C, whereas in micellar system, 0.1 mL yeast-cell suspension and 0.3 mL sucrose solution of strengths varying from 0.05 to 5.0*M* were incubated for 20 min at 35°C. **(B)** LB plot for α-amylase: (1) in aqueous system without surfactants and (2) after entrapment in the reversed micelles of CPCI in *n*-hexane. For aqueous system 0.1 mL of yeast-cell suspension and 1.0 mL of starch solution of strengths varying from 15 to 150 mg/mL were incubated for 20 min at 30°C, whereas in micellar system, 0.1 mL yeast-cell suspension and 0.3 mL starch solution of strengths varying from 15 to 150 mg/mL were incubated for 20 min at 30°C.

The yeast cells were then suspended in the appropriate buffer and incubated at desired temperature for 20 min in the presence of known amount of substrate. This suspension was divided into several parts of 0.1 mL each. In one of them, the reaction was stopped immediately and the activities of the enzymes were estimated in the whole-cell suspension. These data served as control.

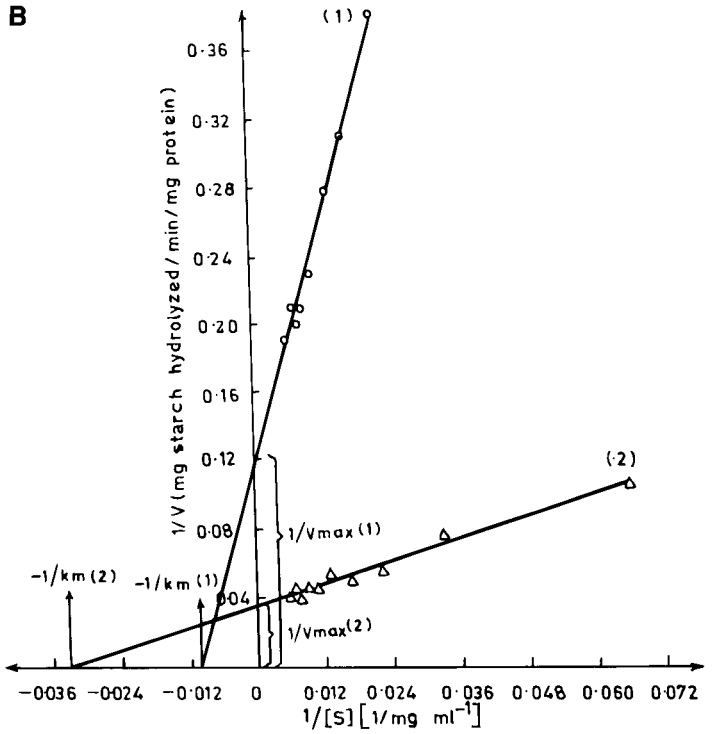

Fig. 1. *Continued*

To the remaining 0.1 mL samples, additional substrate solution (0.7 mL of 0.6*M* sucrose in the case of invertase and 1 mL of 20 mg/ml starch in case of α-amylase) was added and incubated for different intervals of time. The reaction was then stopped in each of these samples and the activity of the enzymes was estimated. The activities of the enzymes in these samples (Table 2), which remained almost constant up to 10 h of incubation, were found to be higher than in the samples where the yeast cells were initially incubated without substrates (Table 1). These observations (Tables 1 and 2) indicate that the presence of substrate induces the release of more enzyme from the yeast cell. This observation was further corroborated by the higher values of protein estimated in the supernatant of the samples preincubated with substrate. Similar trends were observed with Baker's yeast and Brewer's yeast. Quantitatively, however, Brewer's yeast showed more activity than Baker's yeast.

Experiments with Entrapped Enzymes

Kinetic data on activities of invertase and α-amylase in case of Brewer's yeast cells entrapped into the reversed micelles of CPCl in *n*-hexane along with the data in the usual aqueous system in the absence of reverse micelles, obtained from the LB plots are recorded in Table 3(A–F).

Table 1
Activities of Invertase (μg Products Formed/min/mL) and α-Amylase (mg Starch Hydrolyzed/min/mL) When Yeast Cells Were Incubated in Buffer Alone for Different Intervals of Time Along with the Corresponding Values for the Whole Suspension[a,b]

		Brewer's Yeast			Baker's Yeast		
Enzyme	Incubation Time (min)	Centrifugate	Supernatant	Whole Suspension	Centrifugate	Supernatant	Whole Suspension
Invertase	0	1111.11		1111.11	625.00		625.00
	5	1111.11		1111.11	625.00		625.00
	10	The values remained the same up to 600 min	Undetectable	The values remained the same up to 600 min	The activity remained the same up to 600 min	Undetectable up to 600 min	The activity remained the same up to 600 min
	600						
α-Amylase	0	1.20		1.20			
	5	1.20	Undetectable	1.20	Undetectable	Undetectable	Undetectable
	10	1.20		1.20			
		The values remained the same up to 600 min		The values remained the same up to 600 min			

[a] Protein in the supernatant was found to be nil whereas in centrifugate it was 0.150 mg/mL in Brewer's yeast and 0.250 mg/mL in case of Baker's yeast.

[b] Final concentration of sucrose for invertase was 0.4*M*.

Table 2
Activities of Invertase (μg Products Formed/min/mL) and α-Amylase (mg Starch Hydrolyzed/min/mL) in the Whole Cell Suspension When Yeast Cells Were Incubated with Substrate for Different Intervals of Time[a]

Enzyme	Incubation Time (min)	Brewer's Yeast	Baker's Yeast
Invertase	5	8852.11	2987.59
	10	9511.88	3210.27
	15	10285.31	3505.08
	600	Remained same up to 600 min	Remained same up to 600 min
α-Amylase	5	5.7	Undetectable up to 360 min
	10	7.08	
	15	8.25	
	360	The values remained the same up to 600 min	The values remained the same up to 600 min
	600		

[a] Values of protein in supernatant in case of Brewer's yeast was 0.37 mg/mL and it remained constant up to 600 min of incubation. Similarly, in case of Baker's yeast the value was found to be 0.40 mg/mL, which also remained constant up to 600 min of incubation.

The situations A–F for which the kinetic data are recorded in Table 3 are diagrammatically depicted in Fig. 2.

As it can be seen from the illustrative LB plot (Fig. 1B), in the case of enzyme α-amylase when yeast cells were entrapped inside the reversed micelles, the value of K_m decreased in comparison to that in the usual aqueous medium. This trend indicates that the affinity of α-amylase towards its substrate, starch, is enhanced when the whole cells and the substrate are entrapped into the reversed micellar water pools. The activity of invertase, however, showed a decline (Fig. 1A). In Baker's yeast, the activities of both invertase and α-amylase showed enhancement when the yeast cells were entrapped into the reversed micelles, the activity of α-amylase in the control experiment—i.e., the usual aqueous system in the absence of reverse micelles—was undetectably low, whereas in the reversed micellar system it rose to a measurable finite value (Table 3A and B).

Yeast Cells and Substrates in Separate Reversed Micelles

Reversed micellar entrapped water pools are in dynamic equilibrium *(21)*. It has been demonstrated that the substrate entrapped in one set of water pools has the ability to communicate with another substrate located in a different set of surfactant-entrapped water pools *(21)*. It is therefore desirable to see how the activities of the enzymes are affected if the yeast

Table 3
V_{max} and K_m Values for Invertase and α-Amylase in Various Reversed Micellar Systems Along with Values in Control Experiment in Both Brewer's Yeast and Baker's Yeast[a]

		A	B	C	D	E	F
				Brewer's Yeast			
Invertase	V_{max} (μg/min/mg protein)	7142.86	1785.72	9090.91	20,000.00	3448.28	60606.06
	K_m	0.067*M*	0.263*M*	0.051*M*	0.025*M*	0.136*M*	0.0084*M*
α-Amylase	V_{max} (mg/min/mg protein)	8.34	27.78	9.09	35.72	71.43	181.82
	K_m (mg/mL)	100	30.30	90.91	24.39	11.91	4.59
				Baker's Yeast			
Invertase	V_{max} (μg/min/mg protein)	2500.00	3333.33	5500.00	18666.65	13888.89	62500.00
	K_m	0.20*M*	0.166*M*	0.091*M*	0.027*M*	0.039*M*	0.008*M*
α-Amylase	Specific activity[g] (mg/min/mg protein)	Undetectable	0.67	0.73	2.87	1.139	14.063

[a] Situations A–F are diagrammatically depicted in Fig. 2.

A: Control experiments, i.e., the usual aqueous system when no surfactant was used and yeast cells were suspended in buffer alone. B: Reversed micellar system where substrate of only one enzyme was solubilized in reversed micellar-water pool along with the yeast cells. C: Synergistic effect when the substrates of invertase and α-amylase were used in the usual aqueous system without surfactants. D: Synergistic effect when substrates of invertase and α-amylase were solubilized inside same reversed micellar-water pool along with yeast cells. E: Reversed micellar system where yeast cells and substrate were solubilized into separate reversed micelle. F: Synergistic effect in (*E*) above. G: Because the activity of α-amylase was very low with control experiments, LB plots could not be constructed. Therefore data on specific activity has been utilized for comparison.

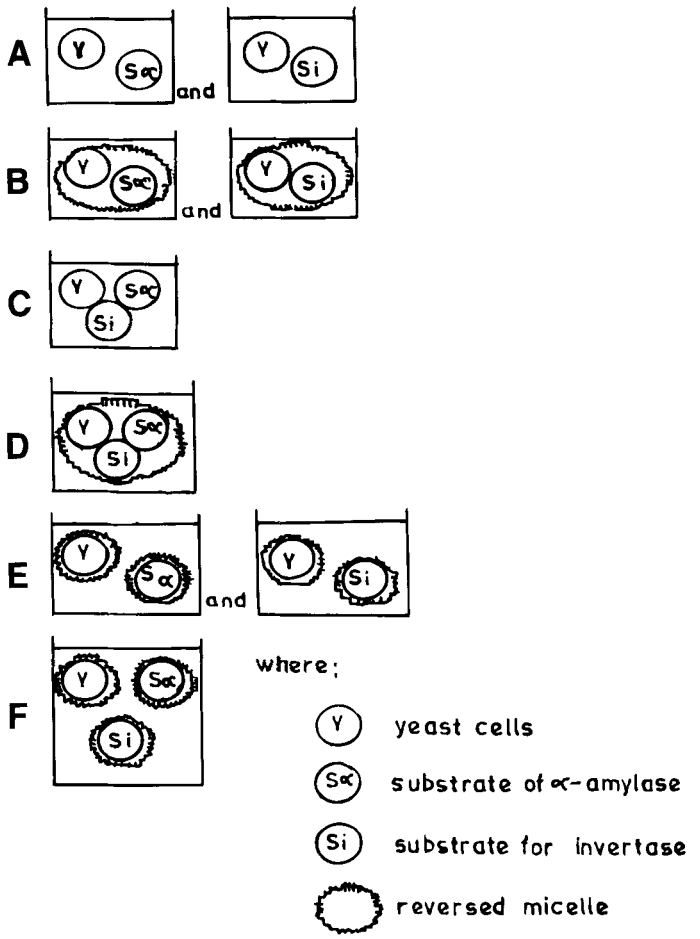

Fig. 2. Diagrammetic depiction of the different situations **A–F** for which the kinetic data are recorded in Table 3.

cells and the substrates of the particular enzyme are entrapped in separate reversed micellar-water pools and then mixed. Kinetic data summarized in Table 3 is quite encouraging. Enhancement in the activity of the enzymes in these experiments can be seen to be much more than the enhancement when the yeast cells and the substrates of the respective enzymes were entrapped in the same reversed micellar-water pools.

Synergism

In nature, it never happens that one particular enzyme acts in isolation; in fact, several enzymic reactions go on in the vicinity of each other and therefore should influence each other. Because yeast cells are the source of several enzymes, in this study we have focused attention on invertase and α-amylase, it is desirable to investigate how the activities of

these two enzymes are affected when they act simultaneously in the vicinity of each other. In fact, experiments were performed to discover whether or not the two enzymes influenced each other synergistically when they acted in close proximity to each other, i.e., whether or not the activity of each of two enzymes is enhanced when they act in the vicinity of each other in comparison to when they act separately in isolation. This study was carried out both in purely aqueous systems in the absence of reversed micelles and also in the reversed micellar system, by first introducing the substrates of any one of the enzymes and then substrates of both enzymes (invertase and α-amylase) simultaneously in the reaction systems. It should be mentioned that in the experiments on synergistic effects where the activities of invertase and α-amylase were estimated in the presence of each other, incubation temperature was maintained at 33°C, which is midway of the optimum temperatures for α-amylase (30°C) and of invertase (35°C). The kinetic data recorded in Table 3(A–F) clearly indicate the synergistic effect. The synergism is much more in the reversed micellar system than in the purely aqueous system (Table 3 A–F). This observation, which is quite significant from a technological point of view, also calls for an indepth study of the enzyme-enzyme interaction leading to the phenomenon of synergism.

Synergistic effect was also studied when yeast cells and the substrates of invertase and α-amylase were entrapped in separate reversed micellar-water pools and then mixed. Enhancement in the activities of invertase and α-amylase was found to be maximum (Table 3F) in these experiments; the enhancement was even greater when the yeast cells and the substrates of the two enzymes were entrapped in the same reversed micellar-water pool (Table 3A–F).

Because the possibility of side reactions influencing the expression of the enzyme activities is not completely ruled out, the synergistic effect was also studied using purified enzymes; α-amylase and invertase both from Sigma (CAS numbers given in the Materials section). Here again, two types of experiments were performed: one in which the reactants (i.e., purified enzymes and their substrates) were entrapped inside the reversed micelles and the other in which the reactions were conducted in the usual aqueous medium in the absence of reversed micelles. In both types of experiments, the same conditions of temperature and pH were maintained as in the corresponding experiments with yeast cells. In the two types of experiments, enzymes were assayed following the methods described in the experimental section. The enzyme α-amylase was also assayed by estimating reducing sugars using dinitrosalicylic acid (DNSA) reagent *(22)*.

Kinetic data (V_{max} and K_m) for both enzymes, α-amylase and invertase when they acted in isolation and also when they acted in close vicinity of each other, both in the usual aqueous medium and in the reversed micellar medium, were obtained by constructing the LB plots and are recorded in

Table 4
V_{max} and K_m Values for Purified Invertase and α-Amylase in Reversed Micellar Systems Along with Values in the Corresponding Control Experiments[a]

		A	B	C	D
Invertase	V_{max} (μg/min/mg protein)	23260	33330	37040	58820
	K_m (M)	0.204	0.143	0.120	0.083
α-Amylase	V_{max} (mg/min/mg protein)	5.26	7.14	8.33	16.67
	K_m (mg/mL)	0.227	0.200	0.182	0.154

[a] Different situation a–d are diagrammatically depicted in Fig. 3.

A: Control experiments, i.e., the usual aqueous system when no surfactant was used. B: Invertase and α-amylase in the reversed micellar system when they acted in isolation from each other. C: Synergistic effect in the usual aqueous medium, i.e., when invertase and α-amylase act in the vicinity of each other in the same aqueous medium in the absence of surfactants. D: Synergistic effect when invertase and α-amylase act in close proximity in the reversed micellar medium.

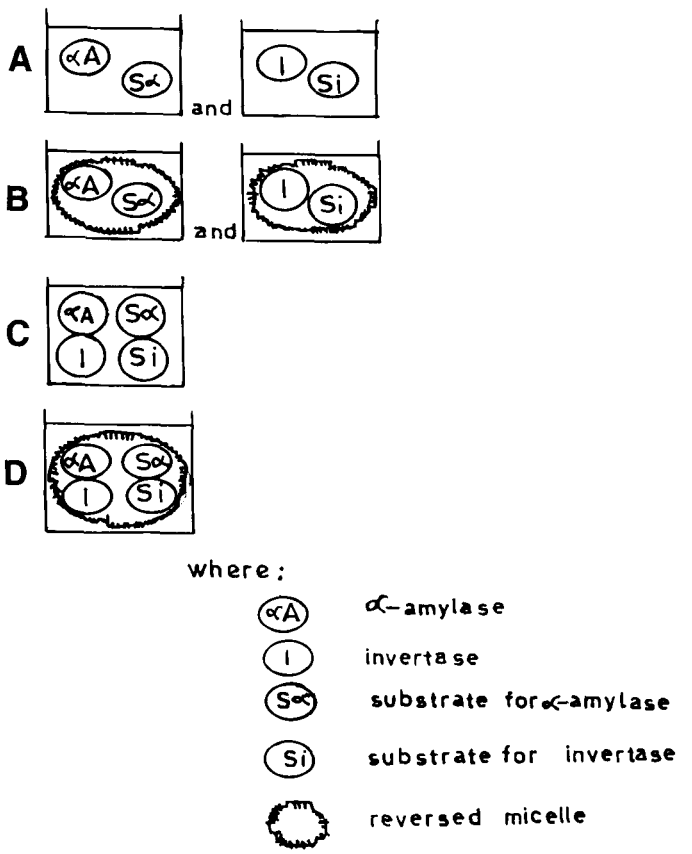

Fig. 3. Diagrammetic depiction of the different situations **A–D** for which the kinetic data are recorded in Table 4.

Table 4. The various situations, A-D, for which the kinetic data are recorded in Table 4 are diagrammatically depicted in Fig. 3. A perusal of the kinetic data in Table 4 confirms the synergistic effect; the synergism being maximum in the reversed micellar medium.

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