Kinetics in microemulsion V. Glucose oxidase catalyzed oxidation of β-D-glucose in aqueous, micellar and water-in-oil microemulsion media

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The oxidation of β-D-glucose by the enzyme glucose oxidase was studied in aqueous medium, in solutions of surfactants AOT (2-ethylhexylsulfosuccinate, sodium salt) TX-100 (polyethylene glycol p-tert octyl phenyl ether) and in w/o microemulsion medium (water/AOT/decane) at different water/AOT mole ratio (ω), pH, temperature and in presence of additives. The time-dependent activities of the enzyme in aqueous and microemulsion media were determined. The catalytic process was retarded in the presence of TX-100 and AOT. In microemulsion medium, $k_{cat}$ values exhibited a deformed $W$-shaped profile with $\omega$. At pH 7, a maximum value of $k_{cat}$ was observed at $\omega = 10.6$. The $k_{cat}$ values were found to be higher in microemulsion medium than in aqueous medium at both pH’s 7 and 8. Activation parameters for the kinetic process were evaluated together with the thermodynamics of the enzyme-substrate Michaelis complex. The $\Delta G^*$ was lower, whereas $\Delta H^*$ and $\Delta S^*$ were higher in microemulsion than in water. The Michaelis constant, $K_M$ was also lower in microemulsion. The inhibition effects of the additives, NaNO$_3$ and NaC were studied in both aqueous and microemulsion media by examining their influences on catalytic constant, $k_{cat}$ and Michaelis constant $K_M$. In microemulsion, both the additives NaNO$_3$ and NaC produced non-competitive inhibition.

Keywords: microemulsion, enzyme activity, inhibitor, anisotropy

The dispersed water droplets of water-in-oil microemulsion can function as unique micro reactors; the energetic and mechanistic courses of many reactions may be significantly altered or affected when performed in such micro reactors. In such compartmentalized media, chemical processes are subjected to the influence of inherent liquid-liquid interface. Adsorption of materials at interface may impart ordering of water droplets normally not encountered in bulk solutions. Rate of a particular reaction may be influenced by several factors, such as migration of reactants to the interface, nature (polarity) of compartment’s interior or core, size of water pool, nature of head groups of surfactants, which congregate at interfacial region, etc. The physical properties of the solubilized water in w/o microemulsions are quite different from the properties of bulk water, especially at low pool size, where water molecules essentially solvate surfactant head groups and availability of free water is minimum. At higher [water]/[surfactant] mole ratio (ω), water pool shows resemblance to bulk water. There are instances, where water pools at low ω have distinct effects on the chemical reactions, compared to that at high ω. Enzyme reaction in reverse micelles and in w/o microemulsions are considered models that mimic the catalytic activity of enzymatic reactions in vivo, especially that of membrane enzymes and enzymes working in different organelles.

Though the effects of water pool-size, pH, surfactants and inhibitors on catalytic behaviour of different enzymes in compartmentalized media have been well explored, the glucose oxidase-catalyzed oxidation of glucose is a rarely studied system. In the present work, oxidation of β-D-glucose by glucose oxidase in aqueous medium and also in presence of surfactants AOT and Triton X-100 was studied at concentrations at critical micellar concentration (CMC) and below and above CMC. The reaction was also studied in water-in-oil microemulsion comprising water/AOT/decane at various [water]/[AOT] mole ratios ω. The effects of temperature, pH and additives NaNO$_3$ and NaC were also explored.
Materials and Methods

AOT (2-ethylhexylsulphosuccinate, sodium salt, purity 99% and having 3% moisture content determined by Karl Fischer titration method), TX-100 (polyethylene glycol p-tert octyl phenyl ether), β-D-glucose, aminonaphthalene sulphonate (ANS), dye o-dianisidine dihydrochloride, sodium cholate (NaC) and enzymes, glucose oxidase (GOD), peroxidase (POD) type 1, and type X-S from Aspergillus niger (activity 128000 units/g solid) were obtained from Sigma Chemical Co., USA. The oil n-decane (G.R) was obtained from E. Merck (Germany). NaNO₃ was a product of SD Fine Chemicals, India.

Preparation of solutions for aqueous and microemulsion media

Solution I—Buffer enzyme solution was prepared with requisite amounts of Na₃HPO₄, 2H₂O (0.08 mmdm⁻³) and NaH₂PO₄, 2H₂O, (0.05 mmdm⁻³) and glucose oxidase (GOD) and peroxidase (POD), such that the concentrations of stock solutions of POD and GOD were 1.16 units/ml and 0.76 units/ml in buffer, respectively. The solutions were stored in a refrigerator and used within 2 weeks after their preparation; Solution II—Stock solution of o-dianisidine dihydrochloride was 0.08 mmdm⁻³. The solution was stored in a refrigerator and used within a week; Solution III—Solutions II and I were mixed in 1/100 (v/v) and shaken vigorously to prepare solution III. It was always freshly prepared and used. Final strength of the dye in solution was maintained at 0.6 m mol dm⁻³; Solution IV—Standard glucose solution was prepared in 2.5% (v/v) perchloric acid. Final concentration of glucose in stock solution was kept at 0.51 m mol dm⁻³.

All stock solutions mentioned above were prepared ten times concentrated for experiments in microemulsion medium to adjust for the involved dilution effect during the sample preparation.

Spectrophotometric measurements

Kinetics of the reaction was studied in a Shimadzu 1601 UV-VIS Spectrophotometer in the usual way²² for aqueous and micellar medium. For microemulsion medium, nine ternary mixtures of wt% compositions of decane/AOT/water as 55/35/10 (S₁), 60/30/10 (S₂), 45/40/15 (S₃), 65/25/10 (S₄), 50/35/15 (S₅), 55/30/15/(S₆), 45/35/20 (S₇), 50/30/20 (S₈), 40/35/25 (S₉) were used. Aqueous part contained buffer enzyme-chromogen mixture, substrate and also additives. The concentrated stock solutions of enzyme, buffer, chromogen and substrate were injected into AOT/decane medium to form the aqueous pool of microemulsion. Preparations were thoroughly mixed and the kinetics was studied in the same way. Oxidation of β-D-glucose was not observed in the absence of the enzyme.

Determination of extinction co-efficient (ε) of the dye in solvent environments

The λₘₐₓ of oxidized form of dye determined spectrophotometrically for aqueous medium and in the presence of AOT, NaC and TX-100 were found to be at 450, 438 and 445 nm, respectively. In microemulsion medium, λₘₐₓ of the dye was at 425 nm and was independent of ω for the range of composition used in this study. Different concentrations of oxidized form of dye were generated by diluting the enzyme-dye-buffer mixture (solution III) with the enzyme-buffer mixture (solution I) in presence of maximum amount of substrate in aqueous, micellar and microemulsion media and ε values were estimated at the respective λₘₐₓ in accordance with Beer’s Law. The values are presented in Table 1.

Enzyme assay in aqueous medium and micellar medium

A fixed amount of buffer-enzyme-chromogen mixture was used with five different concentrations of the substrate (0.6 mmd⁻³-3.0 mmd⁻³) in aqueous medium in 5 ml test tube. Final concentrations of GOD and POD were 0.21 units/ml and 0.29 units/ml, respectively. Final ionic strength of buffer was 0.02 M. Reaction mixtures were allowed to equilibrate for 30 min at 303 K with constant stirring. The liberated H₂O₂ oxidized the dye resulting in coloured solutions with λₘₐₓ at 450 or 425 or 438, nm whose absorbances were monitored spectrophotometrically. The corresponding concentrations were estimated using the exponent of oxidized form of dye determined spectrophotometrically for aqueous medium and in the presence of AOT, NaC and TX-100 were found to be at 450, 438 and 445 nm, respectively. In microemulsion medium, λₘₐₓ of the dye was at 425 nm and was independent of ω for the range of composition used in this study. Different concentrations of oxidized form of dye were generated by diluting the enzyme-dye-buffer mixture (solution III) with the enzyme-buffer mixture (solution I) in presence of maximum amount of substrate in aqueous, micellar and microemulsion media and ε values were estimated at the respective λₘₐₓ in accordance with Beer’s Law. The values are presented in Table 1.

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<table>
<thead>
<tr>
<th>Medium</th>
<th>[Surfactant] nm³</th>
<th>λₘₐₓ/nm</th>
<th>ε/mol⁻¹ cm⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>0.2-2.0</td>
<td>438</td>
<td>8800</td>
</tr>
<tr>
<td>AOT</td>
<td>0.03-3.0</td>
<td>425</td>
<td>9600</td>
</tr>
<tr>
<td>Microemulsion</td>
<td></td>
<td>425</td>
<td>2560</td>
</tr>
</tbody>
</table>
Enzyme assay in microemulsion medium

Five different concentrations of substrate were incorporated in microemulsion medium by injecting microlitre quantities of substrate. A fixed amount of enzyme-buffer-chromogen mixture was added to each solution such that the overall substrate and enzyme-buffer solutions were of same concentration as that in aqueous medium. The spectrophotometric measurements were performed in the same way as in aqueous medium at \( \lambda_{\text{max}} = 438 \text{ nm} \).

Determination of percentage enzyme activity

Solutions of enzyme and dye \( o \)-dianisidine dihydrochloride was prepared in \( \text{Na}_2\text{HPO}_4 \) and \( \text{NaH}_2\text{PO}_4 \) buffer medium and spectrophotometrically assayed at 303 K at intervals of 2 min for the first 10 min and then at intervals of 10 min for 1 hr and subsequently at the intervals of 3, 24 and 48 hr (i.e. for 3 consecutive days).

The same reaction mixture was prepared in microemulsion medium at \( \omega = 10.6 \) with same overall concentration as in aqueous medium. The enzyme activity was monitored at 303 K for 3 consecutive days at the intervals described above.

Fluorescence anisotropy measurement

Fluorescence anisotropy measurements were taken with Hitachi F-4500 Spectrofluorimeter, Hitachi Ltd, Tokyo, Japan, using fluorophore ANS, as it resides at the oil-water interface. The measurements were taken at 298 K, using excitation and emission wavelength \( \lambda_{\text{ex}} = 330 \text{ nm} \) and \( \lambda_{\text{em}} = 480 \text{ nm} \), respectively. Concentration of the probe was kept fixed at 1.0 \( \mu \text{mol dm}^{-3} \). Compositions \( S_1, S_2, S_3, S_4, S_5, \) and \( S_6 \) were chosen for the anisotropy measurements. Each run was duplicated to ascertain reproducibility.

The reaction

\[
\beta\text{-D-Glucose} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{GOD}} \text{D-Gluconic acid} + \text{H}_2\text{O}_2 \text{ (Step 1)}
\]

\[
\text{H}_2\text{O}_2 + \text{O}-\text{Dianisidine dihydrochloride (colourless)} \xrightarrow{\text{POD}} \text{o-Dianisidine dihydrochloride (coloured)} + \text{H}_2\text{O} \text{ (Step 2)}
\]

GOD is found to be most active between \( \text{pH} \ 5-6 \), while POD is most active between \( \text{pH} \ 4-8 \). The \( \text{pH} \) in the present study was maintained with a phosphate buffer at 7, measured with a Elico pH meter (India) of accuracy ± 0.01, following the suggestion of Bergmeyer and Brant.

Results and Discussion

Activity of glucose oxidase in aqueous and microemulsion media

The percent activity of glucose oxidase was studied as a function of time in both aqueous and microemulsion media. In aqueous medium, up to initial 10 min, the amount of product formed increased from 24 to 30 \( \mu \text{mol} \) and then diminished to 10 \( \mu \text{mol} \) on 3rd day. However, in microemulsion medium, an almost constant amount of 110 \( \mu \text{mole} \) product formation was observed for 3 consecutive days (Fig. 1A). These observations indicate that the catalytic process is favoured in the compartmentalized environment over that in the bulk medium. Fig. 1B [100 × (\( \mu \text{mol} \) of product formed in microemulsion medium)/(\( \mu \text{mol} \) of product formed in aqueous medium)] derived from above observation thus shows an initial plateau, followed by an upward trend.

There are variable reports in literature on the enzyme activity in micellar and microemulsion media. Enzymatic super and subactivity in reverse micelles is reported by Ruckenstein and Karpe for acid phosphatase-catalyzed hydrolysis of \( p \)-nitrophenyl phosphate in Brij 56 (polyoxyethylene-10-cetyl ether)/octane solution and \( \alpha \)-chymotrypsin-catalyzed hydrolysis of N-glutaryl-L-phenylalanine-p-nitroanilide in tetraethylene glycol mono-n-dodecyl ether/heptane micellar solution. Gupta et al. observed an initial decline in the activity of the enzyme alkaline phosphatase in water/AOT/decane microemulsion medium, which was restored later. In AOT/vegetable oil/water microemulsion, however, alkaline phosphatase lost its activity. The deactivation process of \( \alpha \)-chymotrypsin in compartmentalized medium was different in presence of different surfactant...
observations were reported on other enzymes, such as protease and α-amylase. In water/AOT/heptane microemulsion, lipase retained 60% stability for 6 days, but the activity was reduced by a factor of 20 in water/heptane/chloroform/CTAB. The activity of liver alcohol dehydrogenase was reported to be influenced by cationic (hexadecyl trimethyl ammonium bromide) and anionic surfactant (sodium dodecyl sulphate, SDS) in the opposite fashion. In anionic microemulsion, the enzyme quickly lost its activity, while in cationic microemulsion, the enzyme activity was more stable. In reverse micelles, a decrease in the specific activity of α-amylase was found to depend on [water]/[surfactant] mole ratio, ω. Enzyme superactivity was also reported by some workers. The maximum activity that can be sometimes much higher than that in aqueous buffered medium, often coincides with diameter of inner cavity of w/o microemulsion, which is roughly of same dimension as that of the macromolecule. The stability of invertase in reverse micelles is found to be more than that in bulk aqueous medium. The activity of cholesterol oxidase for the catalyzed oxidation of cholesterol has shown two maxima depending on the composition of the detergent-less microemulsion.

Quantification of kinetic results

The kinetic results are processed using Lineweaver-Burk (L-B) equation based on Michaelis-Menten proposition:

\[ v^{-1} = \left( k_{cat} [E]_T \right)^{-1} + K_M \left( k_{cat} [E]_T \right)^{-1} [S]^{-1} T \]  

where, \( v \) is the rate of reaction of the substrate (dS/dt), \( k_{cat} \) is the catalytic rate constant, \( K_M \) is the Michaelis constant (instability constant of enzyme-
substrate complex), \([E]_T\) is total concentration of the enzyme and \([S]_T\) is concentration of substrate used.

Figs. 2A and B represent the L-B plots in aqueous medium, at different concentrations of AOT and TX-100, respectively. The reaction was studied in presence of non-ionic surfactant TX-100 at above and below CMC. But, in AOT, the reaction could only be performed at and below CMC for solubility problem. The \(K_M\) and \(k_{cat}\) values are presented in Table 2. The results show a 40% fall in \(k_{cat}\) value in presence of TX-100 and AOT, as compared to that in aqueous medium. This behaviour was independent of surfactant type and concentration. The \(K_M\) values are different in AOT and TX-100, indicating that the enzyme-substrate complexation process is different in TX-100 and AOT. The effect of cationic surfactant CTAB could not be studied due to solubility problem. The enzymatic process was studied in microemulsion medium of nine different compositions with \(\omega\) ranging from 7 to 17.6. Fig. 2C is a representative L-B plot in five different microemulsion compositions.

Both \(k_{cat}\) and \(K_M\) showed a non-symmetric \(W\)-shaped dependence on the \([\text{water}]/[\text{AOT}]\) mole ratio \(\omega\) (Fig. 3A). The rising trend after \(\omega=16.5\) is a striking feature. The \(k_{cat}-\omega\) and \(K_M-\omega\) profiles in Fig. 3A have shown two maxima: one may be for GOD and the other for POD. To clarify further, the following experiment was performed. POD was added to \(\text{H}_2\text{O}_2\) solution having same strength as in the buffered reaction medium. The conversion of colourless \(\sigma\)-dianisidine dihydrochloride to its colored oxidized form was found to be instantaneous and the absorbance did not change with time as observed for 30 min. The first step of the reaction shown above was thus the rate-limiting step. Since all the reactants and products were water soluble, the kinetic events were restricted to the water pool of microemulsions and was insignificantly (if at all) influenced by the exchange rates between the droplets. The reaction could not be studied at \(\omega>17.6\), due to the stability problem of microemulsion.

A bell shaped dependence of \(k_{cat}\) on \(\omega\) is normally observed for enzyme-catalyzed processes in different microemulsion systems\(^{15,18-20,30}\). The non-symmetric \(W\)-shaped dependence in the present study is a deviation from the normal trend. Fluorescence anisotropy measurements at different \(\omega\) have showed a reverse

<table>
<thead>
<tr>
<th>Medium</th>
<th>[Surfactant]/mmoldm(^{-3})</th>
<th>(k_{cat}/\text{sec}) (^{-1})</th>
<th>(10^5K_M/\text{mol dm}^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>0.300</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td>AOT</td>
<td>0.2</td>
<td>0.168</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.168</td>
<td>9.8</td>
</tr>
<tr>
<td>TX-100</td>
<td>0.03-3.0</td>
<td>0.168</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Table 2 — Rate constant \((k_{cat})\) and Michaelis constant \((K_M)\) in aqueous and micellar media for the oxidation of \(\beta\)-D glucose by glucose oxidase at 303 K at \(pH\) 7

Fig. 3 — (A): Dependence of \(k_{cat}\) and \(K_M\) on [Water]/[AOT] ratio \((\omega)\) at 303 K; (B): Variation of \(k_{cat}\) and anisotropy with [Water]/[AOT] ratio \((\omega)\) at 298 K
trend. The anisotropy-ω profile is opposite of $k_{cat}$-ω profile, with a minor displacement (Fig. 3B). The $k_{cat}$ decreased with increase in anisotropy of the system. The variation in microviscosity of interface is reflected on anisotropy-ω profile, accounting for the deviation from the normal trend in variation between $k_{cat}$ and ω. For α-chymotrypsin-catalyzed process with three different substrates in water/AOT/heptane system, $k_{cat}$ values were similar to that in bulk aqueous solution whereas the $K_M$ values were found to be greater\(^{26}\). Earlier, Gupta et al.\(^{2}\) observed a maxima for $k_{cat}$ at ω=18 for alkaline phosphatase in decane/AOT/water system. Thus, the $k_{cat}$ and $K_M$ values may be dramatically different in compartmentalized medium or may remain almost the same as in bulk water.

**Effect of pH and temperature**

The glucose oxidase aided oxidation of glucose was studied at pH 7 and 8 in aqueous and in microemulsion media at ω=10.6 (Table 3). At both pHs, a 3-4-fold increase of $k_{cat}$ values was observed in microemulsion medium, while $K_M$ values diminished. However, $k_{cat}$ and $K_M$ values showed increase with rise in temperature. There are several reports in the literature on the pH dependence of the catalytic rate constant in microheterogeneous and aqueous media. The alkaline phosphatase-catalyzed process showed a bell shaped dependence on pH for AOT containing microemulsion\(^{2,18}\), both in aqueous and microemulsion media, but the effect was more pronounced in microemulsion medium\(^2\). A sharp increase in $k_{cat}$ value at pH 8 over pH 9 was found at ω = 30\(^{15}\). A sigmoidal rate-pH profile was reported for α-chymotrypsin-catalyzed process of N-acetyl-L-tryptophan methyl ester in water pools of heptane/AOT/water system\(^6\).

The pH-dependent enzyme activity is guided by the electrolytic nature of the enzyme. Although often the reaction rate follows a bell shaped dependence on pH, but more information is required to propound a general theory. An enzyme can exist in different forms (states of ionization). In the simplest case, three forms are considered depending on pH, of which one state is capable of binding substrates and catalyzing the reaction\(^{27}\). A search for finding different forms as a function of pH is thus required to explain the pH dependence of rate.

**Energetics of the catalytic process**

The enthalpy of activation, $\Delta H^*$ and entropy of activation $\Delta S^*$ were calculated from the slope and the intercept of the linear relation:

$$\ln k_{cat} = (\ln kT/h + \Delta S^*/R) - \Delta H^*/RT$$  \hspace{1cm} (2)

where $h$ is the Plank’s constant and $k$ is the Boltzmann constant. The free energy of activation $\Delta G^*$ then followed from the relation:

$$\Delta G^* = \Delta H^* - T \Delta S^*$$  \hspace{1cm} (3)

The change in the enthalpy and the entropy for Michaelis complex formation were obtained from the slope and intercept of the linear relation:

$$\ln K_M = \Delta S^*/R - \Delta H^*/R$$  \hspace{1cm} (4)

The standard free energy of Michaelis complex formation (with reference to the standard state of unit molality) was obtained from the relation

$$\Delta G^\ominus = -RT \ln K_M$$  \hspace{1cm} (5)

The results are presented in Table 4.

The Gibbs free energy, $\Delta G^\ominus$ for Michaelis complex formation in aqueous and in o/w microemulsion me-

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp/K</th>
<th>Aqueous medium</th>
<th>Microemulsion medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$k_{cat}$/sec(^{-1})</td>
<td>$10^5 K_M$/mol dm(^{-3})</td>
</tr>
<tr>
<td>7</td>
<td>293</td>
<td>0.115</td>
<td>2.69</td>
</tr>
<tr>
<td></td>
<td>298</td>
<td>0.130</td>
<td>2.78</td>
</tr>
<tr>
<td></td>
<td>303</td>
<td>0.300</td>
<td>10.6</td>
</tr>
<tr>
<td>8</td>
<td>293</td>
<td>0.130</td>
<td>3.74</td>
</tr>
<tr>
<td></td>
<td>298</td>
<td>0.187</td>
<td>6.00</td>
</tr>
<tr>
<td></td>
<td>303</td>
<td>0.214</td>
<td>6.57</td>
</tr>
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</table>
dia is found to be of comparable magnitude, whereas $\Delta H^0$ and $\Delta S^0$ for the same complex formation in two media have been observed to be different; both are higher in the microheterogeneous environment than in water. The free energy of activation $\Delta G^*$ of kinetic process is lower in microemulsion medium than in water (the process was favourable in the former); the evaluated enthalpy of activation $\Delta H^*$ and entropy of activation $\Delta S^*$ have been both found to be higher in microemulsion medium. Earlier, Gupta et al.\textsuperscript{2} reported that although $\Delta G^*$ for alkaline phosphatase-catalyzed hydrolysis of $p$-nitrophenyl phosphate in aqueous and in w/o microemulsion media differed slightly, the $\Delta H^*$ and $\Delta S^*$ were higher in microenvironment. Activation energy of 60 kJ mol\textsuperscript{-1} has been reported for oxidation of cholesterol by cholesterol oxidase\textsuperscript{29}. For lipase-catalyzed process, the activation energy in compartmentalized condition was found to be comparable with that in aqueous medium\textsuperscript{28}.

The $\Delta H^0$ and $\Delta S^0$ have quantitatively and favourably compensated each other. The phenomena has produced a compensation temperature of 295 K from the slope of the plot shown in Fig. 4. This temperature (295 K) is required to multiply with the entropy of the process to dimensionally equate it with the difference between the enthalpy and the free energy. For a good compensation case, the magnitude of free energy is reasonably lower than enthalpy. For both kinetic and equilibrium processes in aqueous solution are very frequently proportional to changes in entropy\textsuperscript{33,34}. In accordance with Lumry and Rajinder\textsuperscript{35}, this effect is essentially a consequence of displacement of water from active cavity of the enzyme and the rearrangement of the surrounding water structure. It should be, however, noted that the temperature-affected structural rearrangement of the enzyme may have a contribution on the overall thermodynamics of the process, and consequently on the compensation phenomenon\textsuperscript{36}.

**Inhibitory effects of additives, NaNO\textsubscript{3} and NaC**

The reactions catalyzed by enzymes are often influenced by additives. Inhibition of reaction rates by the additives may be competitive and non-competitive. In competitive inhibition, the inhibitor forms a complex with the enzyme by binding to the

<table>
<thead>
<tr>
<th>Temp/K</th>
<th>$\Delta G^*/$kJmol\textsuperscript{-1}</th>
<th>$\Delta H^*/$kJmol\textsuperscript{-1}</th>
<th>$\Delta S^*/$JKmol\textsuperscript{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>303</td>
<td>25.4</td>
<td>22.4</td>
<td>70.4(73)</td>
</tr>
<tr>
<td>298</td>
<td>26.2</td>
<td>23.9</td>
<td>99.6</td>
</tr>
<tr>
<td>293</td>
<td>26.6</td>
<td>24.4</td>
<td></td>
</tr>
</tbody>
</table>

A, Aqueous; B, microemulsion at $\omega=10.6$; a, $(\Delta E^*)=\Delta H^*+RT$ (where $T$ is the average temp., 298 K)

![Fig. 4—Enthalpy-entropy compensation plot. $\Delta H^0$, $\Delta H^*$ enthalpy of complexation and enthalpy of activation, respectively and $\Delta S^0$, $\Delta S^*$ entropy of complexation and entropy of activation respectively. ([\(\lambda\)], aqueous medium; [O], microemulsion medium)](image)
active centers; as a result, the effective concentration of the enzyme is reduced. This has an effect on Michaelis constant \( (K_M) \) and the slope of the L-B plot is altered, and the intercept remaining unchanged. In non-competitive inhibition, the inhibitor may form complexes with both the enzyme and Michaelis enzyme-substrate combination and both intercept and slope of the L-B plot get affected. L-B eqn. then takes the following form:

\[
\frac{1}{v} = \left( \frac{k_{cat} [E]_T}{T} \right)^{-1} (1 + [I] K_I^{-1}) + K_M \left\{ 1 + [I] K_I^{-1} \right\} \left\{ \frac{[S]}{T} \right\}^{-1} \quad \cdots (6)
\]

where \( I \) and \( K_I \) represent the inhibitor and inhibitor constant, respectively, on the assumption that both complexes of the inhibitor with the enzyme and enzyme-substrate combination have equal binding strengths. From equations (1) and (6), \([I]\) and \(K_I\) can be related with the intercepts of (1) and (6) as:

\[
\frac{\text{(Intercept)}_2}{\text{Intercept)}_1} = \frac{1 + [I]}{K_I} \quad \cdots (7)
\]

The role of additives (NaNO\(_3\) and NaC) was studied in aqueous buffered and microemulsion media (\( \omega = 10.6 \)). NaNO\(_3\) was found to inhibit the reaction at concentrations 5, 10 and 20 m mol dm\(^{-3}\) in aqueous and microemulsion media (Fig. 5A and B). Both \( k_{cat} \) and \( K_M \) values changed; the nature of inhibition was thus non-competitive. In aqueous medium, addition of 0.1 mmol dm\(^{-3}\) and 10 mmol dm\(^{-3}\) NaC enhanced the kinetic process, while 1.0 m mol dm\(^{-3}\) NaC caused retardation. In microemulsion medium, NaC acted as an inhibitor at all concentrations and the inhibition was concentration-dependent. The nature of inhibition was also non-competitive. The results are shown in Fig. 6A and B. For alkaline phosphatase-catalyzed hydrolysis of \( p \)-nitrophenyl phosphate, the surfactants NaC, AOT and CTAB exhibited complex non-competitive inhibition\(^2\). But for lipase and \( \alpha \)-chymotrypsin-catalyzed processes, competitive inhibition was observed with SDS and CTAB and non-competitive inhibition with AOT\(^{25}\). According to Lee and Chang\(^{37}\), the substrate inhibition in w/o microemulsion is similar to that in aqueous medium. All these divergent reports indicate the complexity of the reaction media.

Our observations of non-competitive inhibitions by NaC on alkaline phosphatase-catalyzed splitting of \( p \)-nitrophenyl phosphate\(^2\) and glucose oxidase-catalyzed oxidation of glucose (this study) although consistent, are contrary to the reports on other systems cited above. The ultimate effect rests on how NaC can interact with the enzyme and enzyme-substrate complex. Until more kinetic results are at hand, a generalization may be premature and faulty.

Mechanistic comprehension

The reaction catalyzed by glucose oxidase:
D-Glucose + O₂ → D-Glucono-δ-lactone + H₂O₂

utilizes co-enzyme FAD (flavinadenine dinucleotide) as a prosthetic group and O₂ as hydrogen acceptor in two stages:

GOD-FAD + D-Glucose ⇌ GOD-FADH₂ + D-Glucono-δ-lactone

and

GOD-FADH₂ + O₂ ⇌ GOD-FAD + H₂O₂

The rate of production of H₂O₂ was monitored as the rate of the reaction by its fast conversion into H₂O by reacting with the leuco dye-catalysed by POD, shown as step 2 in the end of ‘Materials and Methods’ section.

Considering a single substrate (glucose) single binding site reaction in excess O₂:

\[ \text{GOD} + \text{D-Glucose} \xrightleftharpoons[k_M^{-1}]{k_{\text{cat}}} \text{GO:glu} \xrightarrow{k_{\text{cat}}} \text{GOD} \]

products

under steady state conditions using the Michaelis Menten constant \( K_M \) with Lineweaver-Burk formalism, Eq. 1 was used.

The efficacy of the process was controlled by the enzyme configuration and environmental factors viz, pH, medium polarity, ionic strength, salt or additive, temperature, etc. These factors are different in the microreactor compartment (i.e., water pool in w/o microemulsion) than in bulk water. The uptake of O₂ and its conversion into H₂O₂ was thus different (faster) in microemulsion medium. Whether this was the consequence of the effect of a single factor or a concerted effect of several factors remains a matter for future investigation.

Conclusion

The rate of the enzymatic oxidation of β-D-glucose was enhanced in the aqueous microreactor of the compartmentalized w/o medium (at \( \omega = 10.6 \)) than in bulk aqueous medium. The activity of glucose oxidase was retained for more than three days in the water pool of the microemulsion, while a 30% loss in activity of the enzyme occurred in aqueous medium during that period. The \( k_{\text{cat}} \) and the interfacial fluorescence anisotropy of the microwater pool were inversely related with \( \omega \). NaNO₃ and NaC produced non-competitive inhibitions in microemulsion, whereas only NaN₃ produced such an effect in aqueous medium.

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