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Enzyme Reactions in Polymer Media

Torvard C. LAURENT

Medicinsk-Kemiska Institutionen, Uppsala Universitet

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The chemical potential of a compound often increases when a neutral polymer is added to the solution. This has been interpreted as an exclusion of the compound from part of the solvent. The present paper describes how the exclusion influences some enzyme reactions. A theoretical prediction based on the Michaelis-Menten treatment is presented. Three enzyme systems have been examined experimentally, *i.e.*: (a) the degradation of hyaluronic acid by hyaluronate lyase in the presence of polyethylene glycol; (b) the lactate dehydrogenase reaction in dextran solution; (c) the cleavage of benzoyl DL-arginine *p*-nitroanilide by trypsin and its inhibition by serum albumin in dextran solution. In all cases, the presence of the polymer produced a decrease of the apparent K_m value for the substrates and of the apparent K_i value for the inhibitor. The magnitude of this decrease correspond with that calculated theoretically. The effects were moderate in dextran solutions as concentrated as 0.20–0.25 g/ml.

The reason for studying enzyme reactions in polymer media may not be immediately obvious. It represents, however, an initial attempt to describe the environment in which intracellular enzymes function. The macromolecular concentration in the cell is high and furthermore, many enzymes are bound to membranes. The incorporation of enzymes in membranes and gels for preparative and analytical applications has also attained considerable importance.

Previous work has directed attention to the fact that in a mixed solution of two components *e.g.* a linear polymer and a protein the chemical potentials of the components often are higher than when the substances are present individually [1–3]. The effect is usually interpreted as a mutual exclusion of the macromolecules from part of the solution and can be discussed in terms of increased activity coefficients of the components [1–5]. The exclusion hypothesis is based on the observation that the change in chemical potential is a function of the size of the excluded molecules.

The change in activity of a protein in the presence of a polymer can be measured by equilibrium dialysis between a polymer phase and a buffer phase. The protein is partitioned between the phases, and the partition coefficient (*i.e.* the ratio concentration in buffer/concentration in polymer) is determined by the relative increase in activity coefficient of the protein in the polymer medium [6,7]. Most commonly equilibrium experiments have been performed by gel chromatography [5,7–9].

This paper is No. 18 in the series "Interaction between polysaccharides and other macromolecules".

Enzymes. Hyaluronate lyase (EC 4.2.99.1); lactate dehydrogenase (EC 1.1.1.27); trypsin (EC 3.4.4.4).

The chemical activity of enzymes, their substrates and inhibitors should accordingly be increased when a polymer is added to the system in which they are present and one should also expect the kinetics of an enzyme reaction to be affected by polymers. The present investigation was undertaken to verify such effects and to estimate their magnitudes.

In addition to enhancing chemical potentials, polymers also decrease diffusion coefficients of other compounds, a sieving effect [10,11]. This leads to a decrease in collision frequency between reactants and thus to a decrease in reaction rates. The sieving effect should not, however, affect the equilibrium constants of the reactions.

THEORY

Equilibrium of a Chemical Reaction

The effect of a porous network (*e.g.* a polymer network) on the equilibrium constant of a reaction has already been discussed by Giddings [12]. The equilibrium constant, K_{eq}, of the reaction:

$$n \cdot A + m \cdot B + \ldots . \rightleftharpoons p \cdot X + q \cdot Y + \ldots . \quad (1)$$

(where A, B, ... and X, Y, ... are reactants and products respectively and n, m, ... and p, q .... are the number of moles of each compound which takes part in the reaction) is described by

$$K_{eq} = \frac{(\gamma_A \cdot C_A)^n (\gamma_B \cdot C_B)^m \ldots .}{(\gamma_X \cdot C_X)^p (\gamma_Y \cdot C_Y)^q \ldots .} \quad (2)$$

where C_A, C_B, ... C_X, C_Y .... denote concentrations of the compounds and γ_A, γ_B .... γ_X, γ_Y, ... their activity coefficients in the presence of polymer. The activity coefficients in a polymer medium can

be obtained in equilibrium dialysis experiments as discussed above if it is assumed that the activity coefficients of the compounds are unity in pure buffer. γ is equal to $1/K_{av}$, where K_{av} is the form of partition coefficient earlier used in gel chromatography [4].

The apparent equilibrium constant (K_{app}) calculated from the concentrations of the reactants and products in a polymer medium is thus related to the true equilibrium constant by the equation:

$$K_{app} = \frac{C_A^n \cdot C_B^m \cdot \ldots}{C_X^p \cdot C_Y^q \cdot \ldots} = K_{eq} \cdot \left(\frac{\gamma_X^p \cdot \gamma_Y^q \cdot \ldots}{\gamma_A^n \cdot \gamma_B^m \cdot \ldots} \right) \quad (3)$$

In a polymer medium exerting exclusion, the magnitude of the last factor, which incorporates the activity coefficients, is determined by the relative sizes of the reactants and the products. In many enzymatic reactions, the reactants and products are of approximately the same molecular size and the polymer network will therefore affect the equilibrium only to a minor extent.

Kinetics of a One-Substrate Reaction

A one-substrate enzyme reaction can be described by (see *e.g.* [13, 14]):

$$E + S \rightleftharpoons ES \rightarrow E + P \quad (4)$$

where E is enzyme, S substrate, ES enzyme-substrate complex and P product. The Michaelis-Menten constant, K_m, may be regarded as the dissociation constant of the enzyme-substrate complex:

$$K_m = \frac{\gamma_E \cdot C_E \cdot \gamma_S \cdot C_S}{\gamma_{ES} \cdot C_{ES}} \quad (5)$$

where C_E, C_S and C_{ES} are the concentrations of free enzyme, substrate and enzyme-substrate complex respectively and γ_E, γ_S and γ_{ES} their activity coefficients in a polymer medium.

The Michaelis-Menten equation can be written:

$$v = \frac{V}{1 + (K_m/C_S) \cdot \frac{\gamma_{ES}}{\gamma_E \cdot \gamma_S}} \quad (6)$$

or in the modification suggested by Lineweaver and Burk [15]:

$$\frac{1}{v} = K_m \cdot \frac{\gamma_{ES}}{\gamma_E \cdot \gamma_S} \cdot \frac{1}{V} \cdot \frac{1}{C_S} + \frac{1}{V} \quad (7)$$

where v is the reaction rate and V the maximal reaction rate which occurs at substrate saturation.

Thus, when the reaction is performed in a polymer medium the value of K_m as derived from a Lineweaver-Burk plot will be influenced by a change in chemical activity of the reactants due, for example, to exclusion. As mentioned in the introduction, V can be also influenced by other factors, *e.g.* the sieving effect in a polymer network.

Kinetics of a Two-Substrate Reaction

Only one example of a two-substrate reaction will be discussed, *i.e.* a two-substrate reaction in which a ternary complex is formed by ordered addition of the substrates. It may be written:

$$\begin{aligned} E + S_1 &\underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES_1 \\ ES_1 + S_2 &\underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} ES_1S_2 \\ ES_1S_2 &\underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} EP_1 + P_2 \\ EP_1 &\underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} E + P_1 \end{aligned} \quad (8)$$

k_{+1}, k_{-1} *etc.* are rate constants in the individual reactions.

According to Alberty [13] the initial velocity of the forward reaction at steady-state rate is:

$$v = \frac{V}{1 + K_1/C_{S_1} + K_2/C_{S_2} + K_3/C_{S_1} \cdot C_{S_2}} . \quad (9)$$

K_1, K_2 and K_3 are constants. If one assumes that the rate constants are all similarly effected by sieving *etc.* and that $k_{+4} \gg k_{+3}$ then K_1 and K_2 have the following values in a polymer medium:

$$\begin{aligned} K_1 &= \frac{k_{+3}}{k_{+1}} \cdot \frac{\gamma_{ES_1S_2}}{\gamma_E \cdot \gamma_{S_1}} \\ K_2 &= \frac{(k_{-2} + k_{+3})}{k_{+2}} \cdot \frac{\gamma_{ES_1S_2}}{\gamma_{ES_1} \cdot \gamma_{S_2}} . \end{aligned} \quad (10)$$

If either C_{S_1} or C_{S_2} is large, Eqn (9) reduces to a form similar to that for a one-substrate reaction (Eqn (6)] and it is possible to obtain a Michaelis-Menten constant (K_2 and K_1, respectively) for the other substrate. According to the above relationships, when a polymer is added to the medium, the Michaelis-Menten constants for the two substrates should be modified by the factors $\gamma_{ES_1S_2}/(\gamma_E \cdot \gamma_{S_1})$ and $\gamma_{ES_1S_2}/(\gamma_{ES_1} \cdot \gamma_{S_2})$ in uncomplicated cases.

Competitive Inhibition

When a purely competitive inhibitor is added to an enzyme and its substrate the reactions can be described by

$$\begin{aligned} E + S &\rightleftharpoons ES \rightarrow E + P \\ E + I &\rightleftharpoons EI \end{aligned} \quad (11)$$

The Michaelis-Menten constant, K_m, is defined in Eqn 5 and the inhibitor constant, K_i, by

$$K_i = \frac{\gamma_E \cdot C_E \cdot \gamma_I \cdot C_I}{\gamma_{EI} \cdot C_{EI}} . \quad (12)$$

The Lineweaver-Burk equation that can be deduced is:

$$\frac{1}{v} = K_m \cdot \frac{\gamma_{ES}}{\gamma_E \cdot \gamma_S} \cdot \frac{1}{V} \left(1 + \frac{C_I}{K_i} \cdot \frac{\gamma_E \cdot \gamma_I}{\gamma_{EI}} \right) \cdot \frac{1}{C_S} + \frac{1}{V} . \quad (13)$$

Thus change in activities will modify K_i by the factor $\gamma_{EI}/(\gamma_E \cdot \gamma_I)$.

MATERIALS AND METHODS

Enzymes Substrates and Polymers

Streptococcal hyaluronate lyase was from the preparation used earlier [16]. The specific activity [16] of this preparation was 18 units/mg of protein. Lactate dehydrogenase from beef heart (Type III; No. L-2625; 10 mg/ml) and twice crystallized bovine trypsin (Type III; No. T-8253) were purchased from Sigma. Sodium hyaluronate was prepared from rooster comb by a procedure based on Scott's work [17, 18]. Human serum albumin (Lot No. RD056) was a gift from KABI Stockholm. α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide HCl (No. B-4875), L(+)-lactic acid (Grade L-I; No. L-1750), pyruvic acid (sodium salt; Type II; No. P-2256), NAD (Grade III; No. D 5755) and NADH (Grade III; No. D 7380) were all purchased from Sigma. Polyethylene glycol with a molecular weight of 3000—3700 (PEG 4000) was obtained from KEBO, Stockholm, and dextran with a molecular weight of 40000 (Dextran T 40; Lot No. 2514) from Pharmacia, Uppsala. All other chemicals used were of reagent grade.

Determination of Enzyme Activities

All measurements were performed in a Beckman DU-2 Spectrophotometer at a temperature of 37 °C and in a final solution volume of 3 ml. All samples were temperature equilibrated before the start of the experiments. Throughout the investigation the initial reaction rate was measured.

Polymer solutions were made by dissolving the required amount in the stated buffer. The pH of the resulting solution was checked and adjusted if necessary. In subsequent dilutions the solutions were weighed rather than pipetted and the weights converted to volumes using the known densities of the solutions. This procedure was to avoid pipetting errors due to high viscosities.

All regression lines in the Lineweaver-Burk plots have been calculated by the least-square method.

Hyaluronate Lyase. All experiments with this enzyme were performed in polyethylene glycol solutions rather than dextran solutions as dextran proved to be a competitive inhibitor. The buffer used was 0.1 M acetate buffer pH 5.6. In one series of experiments the concentration of hyaluronic acid as determined by the carbazol reaction [19] was 1 mg/ml and that of polyethylene glycol varied between 0 and 0.125 g/ml. In a second series the concentration of hyaluronic acid varied between 0.03 and 1 mg/ml and that of polyethylene glycol was 0.05 g/ml. The enzyme concentration was always 0.0167 units per ml. The reaction was followed by measuring the increase in absorbance at 225 nm [20].

Lactate Dehydrogenase. A series of experiments was performed to determine the effect of variations in the concentration of dextran on the enzyme activity. The reactions were carried out in 0.1 M phosphate buffer pH 7.3 containing 0.1 M sodium lactate, 1.59 mM NAD^+ and 1.33 μg/ml of lactate dehydrogenase. The dextran concentration was varied between 0 and 0.198 g/ml.

Apparent K_m values for lactate, pyruvate and NAD^+ were determined in the absence or presence of approximately 0.2 g/ml of dextran (0.194—0.234 g/ml in different series). K_m for lactate was determined in 0.1 M glycine buffer pH 9.5 containing 1.65 mM NAD^+, 0.2 μg/ml of enzyme and 0.001—0.01 M sodium lactate. K_m for NAD^+ was determined in the same buffer containing 0.02 M lactate, 0.133 μg/ml of enzyme and 27—135 μM NAD^+. K_m for pyruvate was determined in 0.1 M phosphate buffer pH 7.3 containing 70 μM NADH, 0.04 μg/ml of enzyme and 15—100 μM sodium pyruvate.

The pH dependence of lactate dehydrogenase was determined in the absence of dextran and in the presence of 0.198 g/ml of the polysaccharide. Phosphate buffer 0.1 M and pH 6.0, 7.0 and 8.0, and glycine buffer, 0.1 M and pH 9.0 and 10.0, were used. The concentration of lactate was 0.1 M; NAD^+ 2.62 mM; and enzyme 0.33 μg/ml.

The reaction rates were determined from the increase or decrease in absorbance at 340 nm and these values were recalculated into μmoles of substrate converted per minute [21]. The concentrations of NAD^+ and NADH in the solutions used above were all checked by spectrophotometry at 260 nm.

Trypsin. The trypsin activity was determined from the cleavage of benzoyl-arginine-*p*-nitroanilide using a molar absorption for *p*-nitroaniline of 8800 at 410 nm [22]. This activity could be competitively inhibited by the addition of human serum albumin. K_m for the substrate was determined in three media, *i.e.* in the absence of dextran and in the presence of 0.086 and 0.257 g/ml of dextran. In each series the measurements were made both with and without 14.3 mg/ml of serum albumin. 0.05 M veronal buffer pH 8.7 was used as solvent. The concentration of the substrate varied from 0.15—0.52 mM. Trypsin was added to give a final concentration of 4.5 μg/ml from a stock solution containing 450 μg/ml in 0.001 M HCl.

The pH optimum of trypsin was determined in the absence and presence of 0.216 g/ml of dextran. The medium was 0.1 M veronal buffer (pH 7—10), the concentration of the substrate 0.6 mM and the concentration of trypsin 4.5 μg/ml.

RESULTS AND DISCUSSION

Hyaluronate Lyase

The enzyme catalyzes the reaction described in Fig. 1 [23]. The unsaturated product formed enables the reaction to be studied by spectrophotometry. Disaccharides are formed quantitatively and the re-

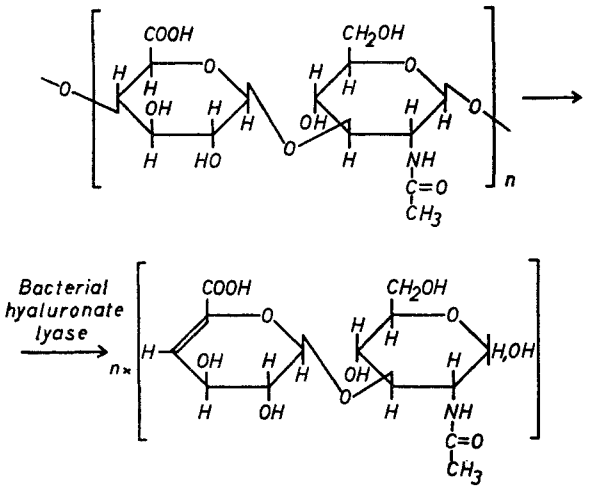

Fig. 1. *The reaction catalyzed by hyaluronate lyase*

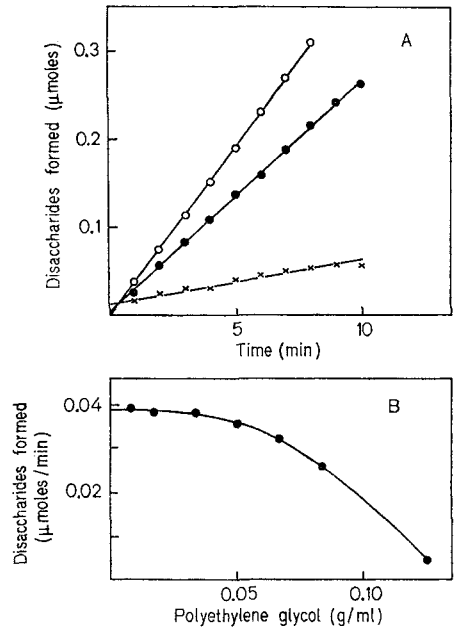

Fig. 2. *The effect of polyethylene glycol on the enzyme activity of hyaluronate lyase.* The experiments were performed with 1 mg/ml of hyaluronic acid and 0.0167 units of enzyme/ml. (A) The amount of product formed is a linear function of time and can be used to calculate the initial reaction rate. Present in the medium were: (○), no polymer; (●), 0.083 g/ml and (×) 0.125 g/ml of polyethylene glycol. (B) The initial reaction rate as a function of polyethylene glycol concentration

action can be regarded as irreversible. It is thus not possible to study the effect of a polymer on the final equilibrium [Eqn (3)]. The reaction is a one-substrate reaction and should be described by Eqn (7).

The enzyme activity at high substrate concentration decreases after addition of polyethylene glycol

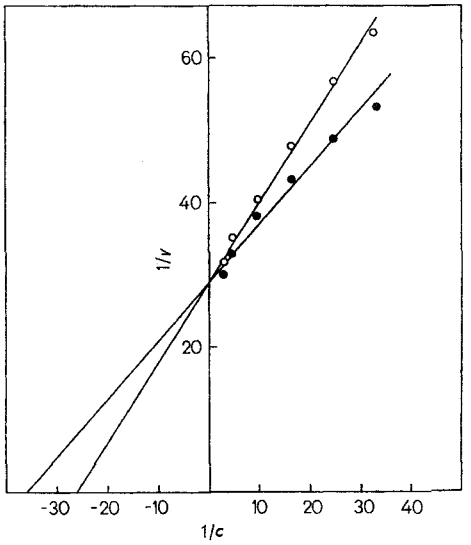

Fig. 3. *Lineweaver-Burk plot for hyaluronate lyase in the absence of polymer (○) and in the presence of 0.05 g/ml of polyethylene glycol (●).* Initial velocity, v, is measured in μmoles/min, concentration, c, is in mg/ml

as shown in Fig. 2. As mentioned in the introduction this can be due to a sieving effect. The decrease at a polymer concentration of 0.05 g/ml was, however, insignificant and the apparent K_m value for hyaluronate was therefore determined at this concentration (Fig. 3). The qualitative effect of polyethylene glycol on the appararent K_m value was that predicted by theory, *i.e.* an effect opposite to that of a competitive inhibitor. The K_m in buffer was 38.5 μg/ml and in polyethylene glycol 28 μg/ml.

The factor $\gamma_{ES}/(\gamma_E \cdot \gamma_S)$ in Eqn (7) calculated from the experimental data is 0.73. The value of γ_S is probably very large owing to the exclusion in the polymer solution of the large hyaluronic acid molecule (mol.wt. $\approx 10^6$; radius of gyration $\approx$ 200 nm [24]). On the other hand, the size of the polysaccharide molecule will not change appreciably on forming a complex with hyaluronate lyase and the factor γ_{ES} should be only slightly larger than γ_S. The exclusion of the enzyme is difficult to estimate owing to lack of knowledge of its molecular size.

If one assumes that polyethylene glycol is three times as efficient as dextran in excluding proteins [25], its exclusion properties at a concentration of 0.05 g/ml should be very similar to those of dextran of 0.1 to 0.15 g/ml. The partition of compounds between dextran gels and buffer (gel chromatography) has been studied by numerous authors [4, 8, 9]. as mentioned above. The effect of dextran on the chemical activity of a compound seems to be the same whether the dextran is present in solution or as a gel [5, 26]. By the relation given by Laurent and Killander [4], it is possible to calculate the partition coefficient, K_{av}

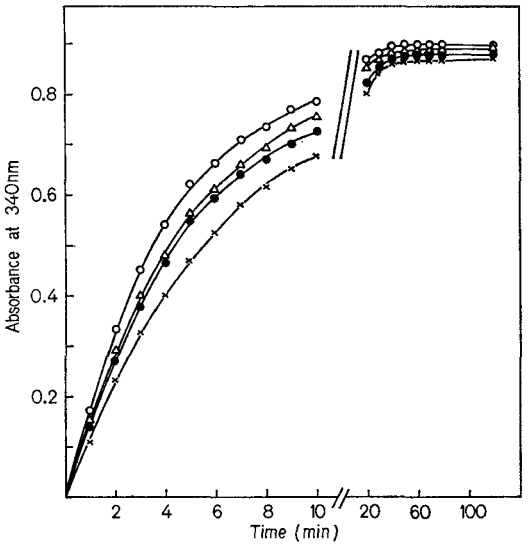

Fig. 4. *The lactate dehydrogenase catalyzed reaction between lactate and NAD+ followed spectrophotometrically in different dextran media.* Initial experimental conditions: pH 7.3, 1.33 μg/ml of enzyme, 0.1 M lactate, 1.59 mM NAD+. Dextran concn: ○, no dextran; △, 0.076 g/ml; ●, 0.138 g/ml; and ×, 0.198 g/ml. The equilibrium reached corresponds to an NADH/NAD+ ratio of 0.097 (0.0952 to 0.0987)

(= $1/\gamma$), for a molecule of any size when partioned between a dextran gel of known concentration and a buffer. An enzyme with a molecular weight of 30000 has an approximate K_{av} (= $1/\gamma_E$) of 0.25 on a Sephadex G-75 gel, which contains approximately 0.125 g/ml of dextran [4]. It is therefore reasonable to expect a value of $\gamma_{ES}/(\gamma_S \cdot \gamma_E) > 0.25$. Although a direct comparison between experiment and theory cannot be made in the present case, the shift in K_m is of the order of magnitude expected.

The pH-optimum of the enzyme in polyethylene glycol could not be determined as the protein precipitated over a wide pH-range.

Lactate Dehydrogenase

Lactate dehydrogenase catalyzes the reaction:

$$\text{Lactate} + \text{NAD}^+ \rightleftharpoons \text{Pyruvate} + \text{NADH} + \text{H}^+$$

The reaction reaches a finite equilibrium, which may be described by Eqn (3). Fig. 4 demonstrates that approximately the same equilibrium state is reached in a high concentration of dextran as in a pure buffer. The factor $(\gamma_X \cdot \gamma_Y)/(\gamma_A \cdot \gamma_B)$ is thus close to 1.0. For the case where the molecular sizes of the reactants and products are similar and providing only exclusion affects the chemical activity in the polymer medium, this result will be expected.

Fig. 4 also reveals that dextran affects the initial reaction rate to a much larger extent than the final equilibrium. When the reaction rate determined at a specifid polymere concentration is plotted against

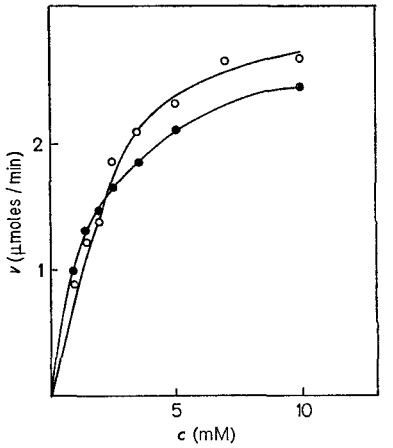

Fig. 5. *The initial reaction rate of the lactate dehydrogenase catalysed oxidation of lactate as a function of lactate concentration.* ○, in glycine buffer pH 9.5; ●, in buffer + 0.234 g/ml of dextran. The reaction mixtures contained: 0.2 μg/ml of enzyme and 1.65 mM NAD+

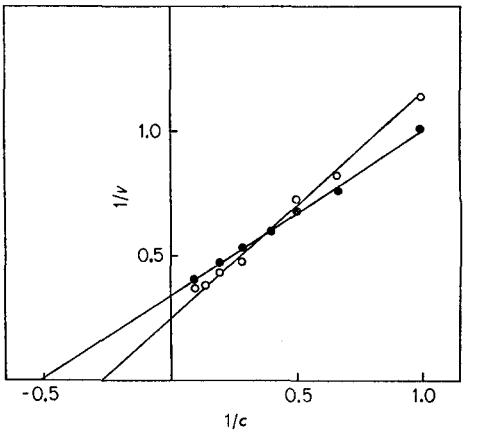

Fig. 6. *Lineweaver-Burk diagram of experimental data presented in Fig. 5.* ○, Experiments in glycine buffer pH 9.5; ●, in buffer + 0.234 g/ml of dextran. Units of v and c are as in Fig. 5

lactate concentration, a complex pattern is obtained (Fig. 5). At high substrate concentrations, where the reaction rate is independent of substrate concentration (or chemical activity of the substrate), the presence of dextran retards the reaction. This finding should probably be ascribed to the decrease in mobility of reactants, which must occur in a three-dimensional polymer network. At low substrate concentrations, however, the reaction rate is higher in the polymer solution than in the buffer. This can be interpreted in terms of an increased chemical potential of the substrate which is of a magnitude that it conceals the effects of sieving. When the data are plotted in a Lineweaver-Burk plot, one can extrapolate to a lower apparent K_m value in the polymer medium than in the buffer (Fig. 6).

Table 1. *Determination of the effect of dextran on* K_m *for lactate dehydrogenase*
The results are expressed as the ratio between the apparent K_m obtained in dextran and the K_m obtained in buffer

Exptl series	Dextran	Substrate for which K_m was determined		
		Lactate	NAD^+	Pyruvate
	g/ml			
1	0.234	0.60	0.67	0.52
2	0.194	0.58[a]	0.64	0.46
3	0.195	0.54	0.55	0.68
4	0.195	0.48	0.61	0.50

[a] In this experiment the dextran concentration was 0.212 g/ml.

The lactate dehydrogenase reaction can be regarded as a two-substrate reaction in which a ternary complex is formed by ordered addition [27]. The reaction should follow Eqn (9) [28]. Hence the ratios between the K_m value obtained in polymer medium and that obtained in buffer are given by $\gamma_{ES_1S_2}/(\gamma_E \cdot \gamma_{S_1})$ and $\gamma_{ES_1S_2}/(\gamma_{ES_1} \cdot \gamma_{S_2})$, respectively.

K_m was estimated for lactate, pyruvate and NAD^+ in dextran of approximately 20% concentration. K_m for NADH was not determined as it is known that high concentrations of pyruvate inhibit the enzyme [28]. The above mentioned ratios were determined and the results are tabulated in Table 1. Although there is a large spread among the values, they all lie in the range 0.5—0.7.

The shifts in K_m are of the magnitude expected from pure exclusion. One can assume in this case that the enzyme-substrate complexes are of the same size as the free enzyme, *i.e.* $\gamma_{ES_1S_2} = \gamma_{ES_1} = \gamma_E$. If the lactate and pyruvate molecules have radii of 0.3 nm and NAD^+ a radius of 0.6 nm (the values of the radii have been assumed to be approximately the same as those of sugars and alcohols of the same molecular weights [33]). the relationship derived by Laurent and Killander [4] gives a K_{av} value for pyruvate and lactate of 0.76 in a 23% dextran gel and a value for NAD^+ of 0.62.

The experimental uncertainty in the determinations does not allow a more detailed analysis of the results in terms of effects on individual rate constants.

Lactate dehydrogenase consists of a tetramer which may dissociate at low enzyme concentrations and this could affect the enzyme activity. As dextran, through the exclusion mechanism should shift the monomer-tetramer equilibrium towards the formation of tetramer, caution should be applied in the interpretation of the results. Hybridization experiments on the enzyme using comparable enzyme concentrations favour, however, the view that the enzyme exists as a tetramer under the present ionic conditions [29].

The effect of dextran on the lactate dehydrogenase reaction was also tested at various pH values (Fig. 7).

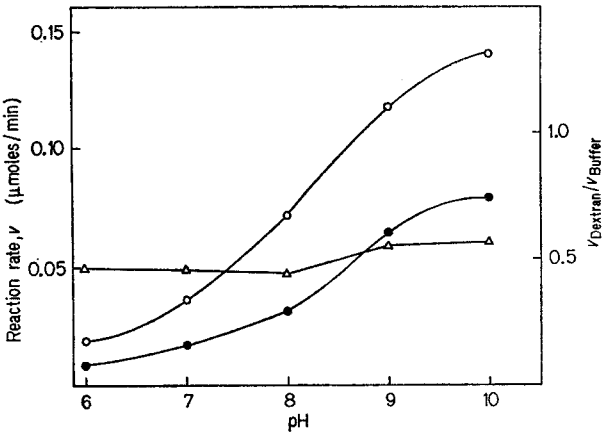

Fig. 7. *The pH dependence of the lactate dehydrogenase -catalyzed oxidation of lactate in buffer (○) and in buffer +0.198 g/ml of dextran (●).* The ratio between the reaction rate in dextran and that in buffer (△) is approximately constant in spite of a ten-fold difference in the absolute reaction rates at the extreme pH values. The reaction mixtures contained 0.33 μg/ml of enzyme, 2.62 mM NAD^+ and 0.1 M lactate

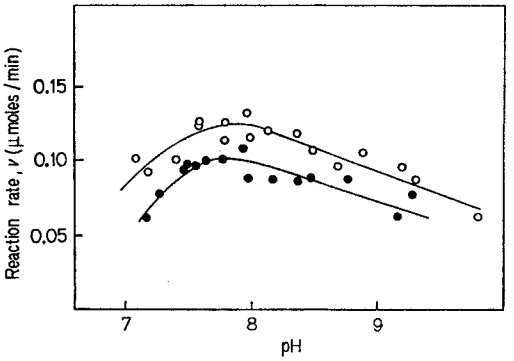

Fig. 8. *The pH optimum of the trypsin catalyzed cleavage of benzoyl-arginine-p-nitroanilide in veronal buffer (○) and 0.216 g/ml of dextran in veronal buffer (●).* The reaction mixtures contained 4.5 μg/ml of trypsin and 0.6 mM of substrate

This experiment was performed because it has been reported that the pH optima of enzymes change after coupling to gel matrices. The relative effect of the polymer seems, however, to be independent of the pH as expected on the basis of pure steric exclusion.

Trypsin

Trypsin releases *p*-nitroaniline from benzoyl DL-arginine *p*-nitroanilide. The former compound is yellow and the initial reaction can readily be followed spectrophotometrically [22]. The reaction has a relatively broad pH optimum (Fig. 8). Dextran in high concentration retarded the reaction rate but this effect was seemingly independent of pH (Fig. 8).

The solubility of the substrate is relatively low which prevents experiments at high substrate con-

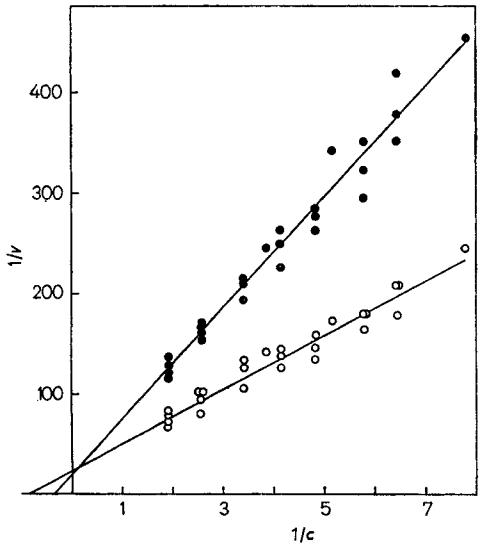

Fig. 9. *Lineweaver-Burk diagram of the trypsin catalyzed cleavage of benzoyl-arginine-p-nitroanilide in buffer (○) and after addition of 14.3 mg/ml of serum albumin which acts as a competitive inhibitor (●).* Each line is made up of points from four separate experimental series, the data for which are shown in Table 2. The reaction mixtures contained 4.5 μg/ml of trypsin and held pH 8.7. Reaction rate, v, is in μmoles/min and concentration, c, mM

Table 2. *The reproducibility of the calculation of* K_m *and* V *from Lineweaver-Burk diagrams for the trypsin catalyzed cleavage of benzoyl-arginine-*p*-nitroanilide*

The concentration is expressed in mM and reaction rate in μmoles/min. For experimental condition see Materials and Methods and Fig. 9

Exptl series	Intercept on ordinate	Intercept on abscissa	Slope of line
In buffer:			
1	36.4	1.19	30.6
2	24.1	0.82	29.4
3	35.6	1.07	33.3
4	27.7	0.83	33.3
Mean	31.0	0.98	31.7
Mean deviation	± 5.1 (± 16.3%)	± 0.15 (± 15.3%)	± 1.7 (± 5.2%)
In 14.3 mg/ml of albumin:			
1	14.3	0.203	70.8
2	28.0	0.477	58.8
3	30.4	0.473	64.2
4	31.1	0.447	69.6
Mean	26.0	0.400	65.9
Mean deviation	± 5.8 (± 22.5%)	± 0.099 (± 24.6%)	± 4.4 (± 6.6%)

centrations. This introduces an uncertainty in the determination of K_m and V by a Lineweaver-Burk diagram (Fig. 9). In order to test the reproducibility of these determinations several series of assays were performed, in buffer alone as well as in the presence of 14.3 mg/ml of serum albumin. In each series $1/V$ and $1/K_m$ were determined from the intercepts on the ordinate and abscissa, respectively, and the ratio K_m/V determined from the slope of the line (Table 2). Although it is clear that the error in the determination of both K_m and V from the intercepts is large, the reproducibility of the slope is good. Thus, in the following only the slopes of the Lineweaver-Burk plots have been introduced into the calculations.

When the reaction was performed in two different dextran media (0.086 and 0.257 g/ml) (Fig. 10), it was possible to extrapolate to lower K_m values than in buffer, in agreement with the earlier experiments with hyaluronate lyase and lactate dehydrogenase. Owing to the large experimental errors, no certain conclusions could, however, be drawn from this fact. It is, however, interesting to note (Fig. 10) that at the lower dextran concentration, the reaction rates were generally higher than in buffer alone indicating that the chemical activity of the substrate is enhanced by exclusion. At the higher dextran concentrations the reaction rates were lower, which would suggest the dominance of a sieving mechanism.

Figs. 11 and 12 demonstrate the inhibiting effect of albumin on trypsin in the presence of dextran. The

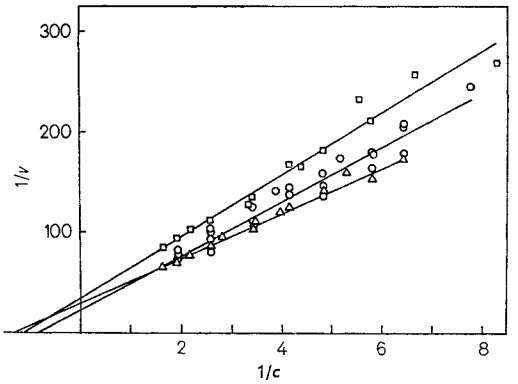

Fig. 10. *Lineweaver-Burk diagram of the trypsin catalyzed cleavage of benzoyl-arginine-*p*-nitroanilide in buffer (○); in 0.086 g/ml of dextran (△); and in 0.257 g/ml of dextran (□).* The reaction mixtures contained 4.5 μg/ml of trypsin and held pH 8.7. Units of v and c as in Fig. 9

figures should be compared with Fig. 9, where the experiments were performed in pure buffer. At 0.086 g/ml of dextran, no difficulty was experienced in obtaining the slope of the Lineweaver-Burk diagrams but at 0.257 g/ml and after addition of albumin, two lines have been drawn with different slopes. One line represents all experimental points but gives a negative intercept on the ordinate. The other is drawn through the points which give the lowest value of the slope. This line gives approximately the expected intercepts on the axes.

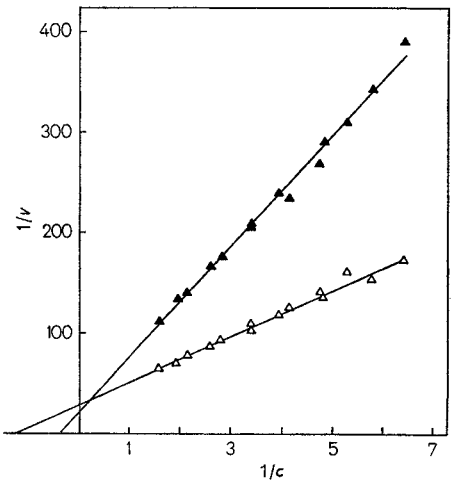

Fig. 11. *Lineweaver-Burk diagrams of the trypsin catalyzed cleavage of benzoyl-arginine-*p*-nitroanilide in 0.086 g/ml of dextran.* The experiments have been performed without (△) and with 14.3 mg/ml of serum albumin (▲) in the medium. The reaction mixtures contained 4.5 μg/ml of trypsin and held pH 8.7. Units as in Fig. 9

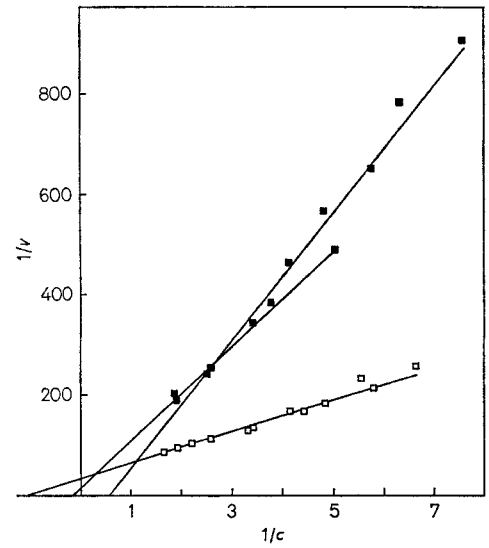

Fig. 12. *Lineweaver-Burk diagrams of the trypsin catalyzed cleavage of benzoyl-arginine-*p*-nitroanilide in 0.257 g/ml of dextran.* The experiments have been performed without (□) and with 14.3 mg/ml of serum albumin (■) in the medium. The reaction mixtures contained 4.5 μg/ml of trypsin and held pH 8.7. Units as in Fig. 9

The slope of a Lineweaver-Burk diagram in a system containing only enzyme and substrate is equal to K_m/V. When a competitive inhibitor is added it should be $(K_m/V)\ (1 + C_I/K_i)$. The ratio of the slopes of the two lines in Fig. 9 therefore gives the factor $(1 + C_I/K_i)$ which is equal to 2.08. The slope of the Lineweaver-Burk diagram after addition of polymer but without inhibitor is according to Eqn. (7), $(K_m/V)(\gamma_{ES}/\gamma_E\gamma_S)$. When an inhibitor is added it becomes, according to Eqn. (13), $(K_m/V) \cdot (\gamma_{ES}/\gamma_E \cdot \gamma_S) \cdot (1 + (C_I/K_i)\ (\gamma_E \cdot \gamma_I/\gamma_{EI}))$. The ratio of the slopes of the lines in Figs. 11 and 12 should therefore give the term $(1 + (C_I/K_i)(\gamma_E \cdot \gamma_I/\gamma_{EI}))$. The ratio in 0.086 g/ml of dextran is 2.43 and in 0.257 g/ml 3.14 or 4.13 (depending upon which line is chosen). From these data the values of $(\gamma_E \cdot \gamma_I/\gamma_{EI})$ could be calculated and they are tabulated in Table 3.

Table 3. *Influence of dextran on the trypsin inhibiting effect of serum albumin*

Comparison between theoretical and experimental values of the factor $(\gamma_E \cdot \gamma_I/\gamma_{EI})$. K_{av} was calculated according to Laurent and Killander [4]. $(\gamma_E \cdot \gamma_I)/\gamma_{EI}$ (calculated) was obtained from the K_{av} values for trypsin, serum albumin and trypsin-albumin complex. The radius of trypsin was taken as 1.94 nm, serum albumin 3.55 nm [4] and that of the trypsin-albumin complex estimated as 4.0 nm

Dextran	K_{av} (calculated) for:			$(\gamma_E \cdot \gamma_I)/\gamma_{EI}$	
	Trypsin	Serum albumin	Trypsin-albumin complex	Calc.	Exptl
g/ml					
0.085	0.51	0.17	0.116	1.32	1.32
0.257	0.112	0.0034	0.00097	2.4	2—3

The K_{av} values for trypsin, serum albumin and the trypsin-albumin complex, calculated by the relationship of Laurent and Killander [4], have been tabulated in Table 3. In the latter case, the assumption, was made that the complex has a radius of 4 nm which cannot be far from the correct value. The K_{av} values have been used to calculate the factor $(\gamma_E \cdot \gamma_I/\gamma_{EI})$ and very good agreement with the experimental values was obtained.

CONCLUDING REMARKS

The general agreement between theory and experimental data obtained here indicates that the present approach to enzyme reactions in polymer media can provide a foundation for further work. Relatively simple enzyme reactions were chosen to facilitate interpretation of the results. More complicated behaviour may be found with for example, allosteric enzymes where the binding of a regulating substance also has to be taken into account. Also the association of enzyme subunits should be affected by a polymer and this could affect enzyme activities.

The effects of neutral polymers even at quite high concentrations on the enzyme reactions studied were moderate. Although the polymer must have raised the chemical activities of the enzymes, substrates, inhibitor and complexes significantly, it did not disturb the equilibria to a great extent. This is clearly demonstrated in Table 3 which shows, for example, that at a dextran concentration of 0.257 g/ml the

chemical activity of serum albumin is increased 300-fold ($\gamma_I = 1/K_{av}$) while its activity as an inhibitor only increases 2–3 times. The increases in chemical activities of the enzymes, substrates and inhibitor are actually balanced by similar increases in chemical activities of the enzyme-substrate and enzyme-inhibitor complexes.

The only parameter studied systematically in this work is the effect of addition of a polymer on the chemical activities of the reactants ("exclusion"). Another parameter of equal importance is the sieve effect, which causes a decrease in collision frequency. The sieving mechanism will decrease the diffusion rates of both enzymes, substrates and inhibitors according to their sizes. A quantitative discussion of this effect has to be postponed until more is known about translational and rotational diffusion of the reactants in polymer solutions. There may certainly also be other effects encountered when enzymes are introduced into polymer systems, *e.g.* effects on the protein conformation, which can change reaction rates.

Coupling of enzymes to gels and membranes is now commonly performed for various purposes [30]. In such cases it would be valuable to predict how the enzymic properties change. It should, however, be stressed that both in natural and synthetic membranes the enzymes are generally immobilized by bonds to the membrane matrix. This has a drastic effect on the enzyme activity [31] which has not been encountered in the present studies. Furthermore, when such a matrix is equilibrated in a buffer the substrate concentration in the matrix will decrease due to the exclusion and the activity of the substrate will be the same as that in the surrounding medium. The inclusion of enzymes in gels has also resulted in changes in their pH optima owing to the slow diffusion of substrates and products between the gel and the surrounding medium [31,32]. In the present experiments, the pH dependence was not influenced by the presence of polymers.

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REFERENCES

1. Ogston, A. G., *Arch. Biochem. Biophys.* Suppl. 1 (1962) 39.
2. Laurent, T. C., and Ogston, A. G., *Biochem. J.* 89 (1963) 249.
3. Edmond, E., and Ogston, A. G., *Biochem. J.* 109 (1968) 569.
4. Laurent, T. C., and Killander, J., *J. Chromatog.* 14 (1964) 317.
5. Laurent, T. C., in *The Chemical Physiology of Mucopolysaccharides*, edited by G. Quintarelli, Little, Brown & Co., Boston 1968, p. 153.
6. Ogston, A. G., and Phelps, C. F., *Biochem. J.* 78 (1961) 827.
7. Laurent, T. C., *Biochem. J.* 93 (1964) 106.
8. Determann, H., *Gelchromatography*, Springer, Berlin, Heidelberg, New York 1967.
9. Fischer, L., *An Introduction to Gel Chromatography*. Elsevier, Amsterdam 1969.
10. Laurent, T. C., Björk, I., Pietruszkiewicz, A., and Persson, H., *Biochim. Biophys. Acta*, 78 (1963) 351.
11. Laurent, T. C., *Fed. Proc.* 25 (1966) 1128.
12. Giddings, J. C., *J. Phys. Chem.* 74 (1970) 1368.
13. Alberty, R. A., *Advan. Enzymol.* 17 (1956) 1.
14. Florkin, M., and Stotz, E. H. (Eds.), *Compr. Biochem.* 12 (1964).
15. Lineweaver, H., and Burk, D., *J. Amer. Chem. Soc.* 56 (1934) 658.
16. Laurent, T. C., Bárány, E., Carlsson, B., and Tidare, E., *Anal. Biochem.* 31 (1969) 133.
17. Scott, J. E., *Methods Biochem. Anal.* 8 (1960) 145.
18. Laurent, T. C., Ryan, M., and Pietruszkiewicz, A., *Biochim. Biophys. Acta*, 42 (1960) 476.
19. Dische, Z., *J. Biol. Chem.* 167 (1947) 189.
20. Greiling, H., *Hoppe Seyler's Z. Physiol. Chem.* 309 (1957) 239.
21. Horecker, B. L., and Kornberg, A., *J. Biol. Chem.* 175 (1948) 385.
22. Erlanger, B. F., Kokowsky, N., and Cohen, W., *Arch. Biochem. Biophys.* 95 (1961) 271.
23. Linker, A., Meyer, K., and Hoffman, P., *J. Biol. Chem.* 219 (1956) 13.
24. Laurent, T. C., and Gergely, J., *J. Biol. Chem.* 212 (1955) 325.
25. Iverius, P. H., and Laurent, T. C., *Biochim. Biophys. Acta*, 133 (1967) 371.
26. Ogston, A. G., and Silpananta, P., *Biochem. J.* 116 (1970) 171.
27. Takanaka, Y., and Schwert, G. W., *J. Biol. Chem.* 223 (1956) 157.
28. Hakala, M. T., Glaid, A. J., and Schwert, G. W., *J. Biol. Chem.* 221 (1956) 191.
29. Markert, C. L., and Massaro, E. J., *Science (Washington)*, 162 (1968) 695.
30. Silman, I. H., and Katchalski, E., *Ann. Rev. Biochem.* 35 (1966) 873.
31. Axén, R., Myrin, P.-Å., and Jansson, J. C., *Biopolymers*, 9 (1970) 401.
32. Goldman, R., Silman, I. H., Caplan, S. R., Kedem, O., and Katachalski, E., *Science (Washington)*, 150 (1965) 758.
33. Goodson, J. M., Distefano, V., and Smith, J. C., *J. Chromatog.* 54 (1971). 43.

T. C. Laurent
Medicinsk-Kemiska Institutionen, Uppsala Universitet
Box 551, S-751 22 Uppsala 1, Sweden