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SOLUBILITY OF TOLUENE IN BOVINE LIVER GLUTAMATE DEHYDROGENASE SOLUTIONS AND ENHANCEMENT OF ENZYME ASSOCIATION

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SUMMARY

1. Linear self-association of bovine liver glutamate dehydrogenase (L-glutamate NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3) is considerably enhanced by saturation with toluene of buffered enzyme solutions (0.2 M sodium phosphate buffer (pH 7), 10^{-4} M EDTA). The association was followed by light scattering studies and can be described by a single association constant, K, equal to 52, 28.8 and 21.7 ml/mg at 10, 20 and 25°, respectively (the value of K at 25° in the absence of toluene is 2 ml/mg); benzene, which is more soluble in aqueous solutions than toluene, enhances glutamate dehydrogenase association even more.

2. Solubility studies of toluene in buffered glutamate dehydrogenase solutions, saturated with respect to toluene, may be interpreted to indicate that 8–13 moles of toluene are preferentially bound per 53 500 g of enzyme; below saturation conditions considerably less toluene is preferentially bound to the enzyme. The biological activity of glutamate dehydrogenase is not affected by toluene binding. The binding increases with decreasing temperature and increasing degree of association. It was found that toluene solubility is not increased by glutamate dehydrogenase in buffered solution containing $5 \cdot 10^{-3}$ M GTP, $5 \cdot 10^{-3}$ M NADH; in the presence of these effectors both biological activity and association of the enzyme are much reduced.

INTRODUCTION

Recently we have observed[1,2] that in aqueous buffered solution saturated with toluene, association of bovine glutamate dehydrogenase (L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3) is considerably enhanced. The rodlike associates formed are indistinguishable from those obtained in the absence of toluene, and may be reversibly dissociated subsequent to toluene removal.

In the present communication we show by light scattering studies that the association at various temperatures, in the presence of toluene, can still be described by one single constant, about one order of magnitude larger than in the absence of toluene. The association increases with decreasing temperature. Benzene, which is

somewhat more soluble in aqueous solutions than toluene, leads to higher degrees of association.

Solubility studies by the use of both gas phase chromatography analysis and the use of labeled toluene were undertaken in solutions saturated with respect to toluene and in solutions equilibrated with toluene–tridecane mixtures. In the presence of enzyme the solubility of toluene is greater than in the buffer alone; toluene solubility though does not increase in enzyme solutions containing both $5 \cdot 10^{-3}$ M GTP and $5 \cdot 10^{-3}$ M NADH. Addition of GTP and NADH at this concentration to buffered enzyme solutions saturated with toluene results in almost complete dissociation to the oligomeric hexamer.

Finally we have checked enzyme activity in the presence of toluene and find that no change in activity is observed within the error of the experimental determination.

EXPERIMENTAL

Solutions of glutamate dehydrogenase were prepared in phosphate buffer (0.2 M sodium phosphate buffer (pH 7), 10^{-4} M EDTA). For details of preparation of enzyme solutions and a description of the light scattering technique see EISENBERG AND TOMKINS[3]. Refractive index increments for wavelengths $\lambda = 436$ nm and $\lambda = 546$ nm used in the light scattering experiments have been reported by REISLER *et al.*[4]. The procedure of saturation of enzyme solutions with toluene was described by REISLER AND EISENBERG[2].

Solubility of toluene in glutamate dehydrogenase solutions saturated with respect to toluene was studied by two experimental methods. In the first procedure (A) the concentration of toluene in buffer and buffered enzyme solutions was determined by gas chromatography. In the second procedure (B) buffered enzyme solutions were equilibrated through a dialysis membrane with a buffer saturated with labeled radioactive toluene, the concentration of which was then determined by scintillation counting.

In Method A the layering and mixing technique of MOHAMMADZADEH *et al.*[5] was used for saturating the buffer and enzyme solutions with pure toluene. In other experiments buffer and enzyme solutions were equilibrated with toluene–tridecane mixtures of various compositions. Tridecane is immiscible with water and was used to measure distribution of toluene below its saturation concentration in the aqueous system. Enzyme concentrations were between 5 to 10 mg/ml and the phosphate buffer was as above. The chromatographic analysis was performed in columns packed with Chromosorb acid-washed 60/80 mesh, coated with 10% Carbowax 20 M, in Varian Aerograph Series 1200 and Hewlett Packard Model 5750 (F and M) instruments, equipped with flame ionization detectors. The toluene peak was calibrated by diluting known amounts of toluene with acetone and injecting different quantities of each solution into the gas chromatograph.

In Method B enzyme solutions, in buffer as above, were dialyzed against buffer solutions saturated with radioactive toluene. A small excess layer of radioactive toluene was maintained in the enzyme-free solutions. [*Me*-^{14}C]Toluene, 13.4 mC/mmole, was purchased from the Radiochemical Centre, Amersham, England. Samples of equilibrated buffer and buffer–enzyme solutions were counted in dioxane in a

Packard Model 3320 scintillation spectrometer. In both methods aqueous solutions carefully separated from supernatant organic phase were quickly chilled in stoppered flasks in ice buckets, to prevent toluene evaporation. The results presented are average values from a large number of experiments and the standard deviation is indicated with the results.

The biological activity of enzyme solutions at different enzyme concentrations, both in the presence and in the absence of toluene was followed spectrophotometrically by observing the kinetics of NADH or NADPH formation at $\lambda = 340$ nm (ref. 6). Activity assays were performed at pH 7 in phosphate buffer; at higher enzyme concentration NAD^+ was replaced by $NADP^+$ and whenever necessary NH_4Cl was added to slow down the reaction. Enzyme solutions were prepared in duplicates; one of the duplicate samples was layered with toluene. Both solutions were kept in the cold room for two days and were assayed at the same time. The accuracy of activity measurements is estimated to be $\pm 5\%$. Each experiment was repeated 2–3 times.

RESULTS AND DISCUSSION

Association

In a system in chemical equilibrium in which particles associate in dependence on concentration it is possible to determine from light scattering the reciprocal of an apparent quantity M_w,

$$\frac{1}{M_w} \equiv \frac{1}{M_w{}^0} + 2A_2c \tag{4}$$

from which the true molecular weight, $M_w{}^0$, can only be evaluated if the second virial coefficient, A_2, is known, or assumed to be known. We have discussed this problem in previous communications[4,7]. In the presence of toluene a rather fortunate circumstance arises in view of the fact that high molecular weights are obtained at low concentrations of enzyme. Thus, over a considerable range of molecular weights it is possible to neglect the second term on the right hand side of Eqn. 1 with respect to the first term and identify the apparent value M_w with the true value $M_w{}^0$.*

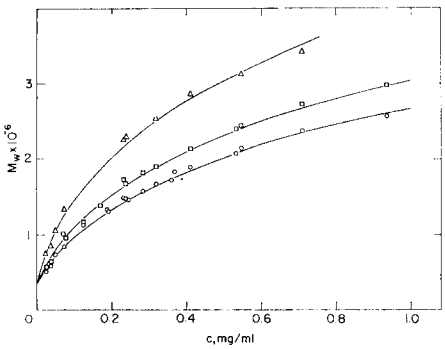
In Fig. 1 the variation of M_w with c (in solutions saturated with respect to toluene) is shown at three temperatures. In the absence of toluene the dependence of M_w on temperature is only very slight.

We have shown previously[4] that the indefinite associations of glutamate dehydrogenase in phosphate buffer, which yield open linear structures, may be represented by a stacking process with a single association constant K. For such a process

$$\left[\frac{M_w{}^0}{M_1}\right]^2 = 1 + 4Kc \tag{2}$$

where M_1 is the molecular weight (316 000) of the enzyme hexameric oligomer[3]. The molecular weight distribution in this type of polymerization is known[8] as the "most probable" distribution and the number average $M_n{}^0$ and z-average molecular weights

* At c approx. $0.3 \cdot 10^{-3}$ g/ml for instance, M_w approx. $1.5 \cdot 10^6$ at 25°; with A_2 approx. 10^{-5} mole · ml · g^{-2} (*cf.* REISLER *et al.*[4]; footnote 4) the second term of the right hand side of Eqn. 2, is estimated to be one percent only of the first term; with increasing concentration and increase in molecular weight this term assumes a more significant role.



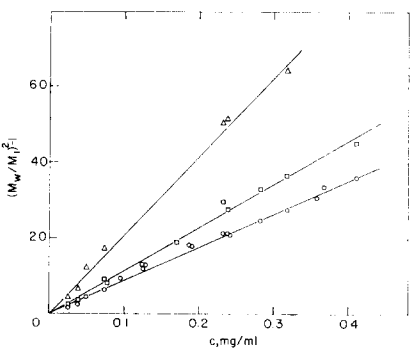


Fig. 1. Weight-average molecular weight M_w *versus* concentration in phosphate buffer saturated with toluene; ○, 25°; □, 20°; △, 10°. Curves, calculated; see text.

Fig. 2. Plots of $[(M_w/M_1)^2-1)]$ *versus* c; ○, 25°; □, 20°; △, 10°.

M_z^0 are related to M_w^0 by $2M_n^0 = M_w^0 + M_1$ and $2M_z^0 = 3M_w^0 - M_1^2/M_w^0$. These averages have been determined in the ultracentrifuge for the enzyme in phosphate buffer and the results strongly support the above kinetic scheme. In the presence of toluene the same kinetic scheme applies. Fig. 2 shows that, at low enzyme concentrations, $(M_w/M_1)^2 - 1$ is linear in c; deviations from linearity appear at values $(M_w/M_1)^2 - 1$ considerably higher than in phosphate buffer only (*cf.* Fig. 7 of

TABLE I

EQUILIBRIUM DISTRIBUTION OF TOLUENE IN PHOSPHATE BUFFER AND BUFFERED ENZYME SOLUTIONS, BY GAS PHASE CHROMATOGRAPHY ANALYSIS (METHOD A) AND BY RADIOACTIVE TOLUENE COUNTING (METHOD B)

Method	*Molar ratio toluene/ tridecane*	*Enzyme concentration (mg/ml)*	*Temp.*	*Toluene concentration (mg/100 ml) of*		*Moles of toluene excess per 53 500 g protein*
				Buffer	*Enzyme solution*	
(a) Solutions in equilibrium with pure toluene						
B		–	24°	62.1*		
A		5.8	24°	44.6 ± 2.0	52.8 ± 1.8	8.4
B		5.9	24°	47.2 ± 1.0	56.3 ± 1.5	9.4
A		6.6	24°	45.7 ± 1.8	56.1 ± 1.5	9.3
A		10.8	24°	45.1 ± 2.0	64.8 ± 2.0	10.8
A		4.7	2°	50.2 ± 2.0	60.4 ± 2.0	12.9
B		8.4	2°	52.0 ± 2.0	69.3 ± 1.0	12.2
A		4.7**	24°	38.0 ± 2.0	38.0 ± 2.0	0
B		5.9**	24°	41.1 ± 1.5	40.3 ± 1.0	0
(b) Solutions in equilibrium with toluene–tridecane mixtures						
B	0.46	10.5	24°	38.0 ± 2.0	41.0 ± 1.8	1.7
B	0.92	10.5	24°	39.0 ± 1.7	42.4 ± 1.0	1.9
B	1.53	10.5	24°	40.4 ± 1.5	46.3 ± 1.6	3.3
B	2.30	11.6	24°	42.4 ± 1.5	52.7 ± 1.8	5.2
B	4.59	10.5	24°	45.7 ± 1.9	60.5 ± 2.0	8.4

* Solubility in pure water.
** With $5 \cdot 10^{-3}$ M GTP and $5 \cdot 10^{-3}$ M NADH.

REISLER *et al.*[4]). From the slopes in Fig. 2 we calculate $K = 52$ ml/mg at 10°, 28.8 ml/mg at 20° and 21.7 ml/mg at 25°, about one order of magnitude larger than in phosphate buffer only (in which K equals 2 ml/mg at 20°).**

Benzene, which is more soluble in aqueous solutions than toluene (1.07 g benzene per l *versus* 0.46 g toluene per l in phosphate buffer, *cf.* MOHAMMADZADEH *et al.*[5]) enhances self-association of glutamate dehydrogenase even more than the latter. Thus, in enzyme solutions saturated with benzene, at 25°, at protein concentrations of 0.11 and 0.38 mg/ml, M_w is $1.88 \cdot 10^6$ and $2.56 \cdot 10^6$, respectively.

Solubility of toluene and biological activity

The results of the solubility studies under saturation conditions are presented in Table Ia. Data in Column 5 show that the solubility of toluene in the phosphate buffer employed is lower than in pure water. The solubility increases slightly with decreasing temperature but decreases upon addition of $5 \cdot 10^{-3}$ M GTP and $5 \cdot 10^{-3}$ M NADH. Satisfactory agreement is obtained between Methods A and B (*cf.* EXPERIMENTAL). Column 6 presents solubilities of toluene buffered enzyme solutions. Examination of the last column reveals that under saturation conditions about 8–13 moles of toluene are preferentially bound per enzyme subunit of 53 500 g. Decreasing the temperature to 2° increases toluene binding, which parallels the increase in molecular weight with decreasing temperature (Fig. 1). Examination of the last two rows of Table Ia shows that in the presence of $5 \cdot 10^{-3}$ M GTP and $5 \cdot 10^{-3}$ M NADH no toluene, within experimental error, preferentially binds to glutamate dehydrogenase. In buffer and enzyme solutions equilibrated with toluene–tridecane mixtures (Table Ib) considerably less toluene is preferentially bound per enzyme subunit (last column).

Enzyme activity measurements were performed over a 100-fold range of enzyme concentrations. Simultaneous assays, both in the presence and the absence of toluene allowed us to compare the biological activity of enzyme solutions at different states

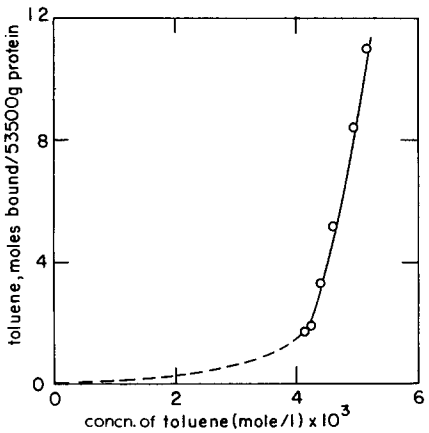


Fig. 3. Binding of toluene to glutamate dehydrogenase solutions in phosphate buffer, at 24°. The highest concentration point corresponds to solutions saturated with pure toluene. The dotted curve is based on the fact that to zero toluene concentration corresponds zero binding.

** Equilibrium sedimentation measurements (R. JOSEPHS, unpublished) provide additional evidence for the "most probable" molecular weight distribution in the presence of toluene.

of association. At the lower concentration assayed (approx. $3 \cdot 10^{-3}$ mg/ml) the enzyme is completely dissociated into the oligomeric form even in the presence of toluene. At the highest concentration assayed (approx. 0.3 mg/ml) M_w of the enzyme solution layered with toluene is about $1.5 \cdot 10^6$, whereas in the toluene free solution at the same enzyme concentration M_w is approx. $0.6 \cdot 10^6$. In all cases tested, over the entire concentration range of enzyme assayed the activity of the solution layered with toluene was, within experimental error ($\pm 5\%$), identical with that of the solution containing no toluene. Thus in spite of pronounced differences in the association level of the enzyme in the two solutions, biological activity was not affected.

Representation of the binding data in conventional Scatchard plots does not lead to a meaningful assignment of the number of binding sites per enzyme subunit. A similar result was found by MOHAMMADZADEH *et al.*[5] for the binding of benzene to β-lactoglobulin, although normal Scatchard plots were obtained by them in other protein-hydrocarbon systems.

A plot (Fig. 3) of the amount of toluene bound to glutamate dehydrogenase *versus* free toluene in aqueous buffer solutions indicates the cooperative nature[9] of the binding isotherm. The results are not precise enough and cannot easily be extended over a wide enough range of ligand concentration to permit quantitative interpretation of the data. Two forms of glutamate dehydrogenase oligomer, with distinct ligand binding affinity, and different capacity to associate, have been previously postulated[19] The cooperative nature of toluene binding can therefore derive from subunit interaction or enzyme association schemes.

With addition of GTP and NADH to buffered enzyme solutions the capacity of the enzyme oligomer to associate as well as its major biological activity are reversibly inhibited. Other effectors, such as ADP and NADH for instance, increase both activity and the capacity to associate. In the present case, though, these two properties are not coupled: if a buffered enzyme solution is carefully saturated with toluene the biological activity is not enhanced although the molecular weight greatly increases. Addition of $5 \cdot 10^{-3}$ M GTP and $5 \cdot 10^{-3}$ M NADH to an enzyme solution saturated with toluene, deactivates the enzyme, leads to dissociation to the oligomer form and inhibits toluene binding; the enzyme to which coenzyme and GTP are bound apparently undergoes a reversible structural change which blocks binding of aromatic hydrocarbon. It is not known to what extent the influence of toluene on glutamate dehydrogenase association is related to the fact that the enzyme may find itself in a related lipid (hydrophobic) environment in the mitochondrion.

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