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"Catálisis, estabilidad y termoestabilidad enzimática en relación al diagrama de fases de un sistema de micelas invertidas."

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Resumen de la tesis titulada:

"Catálisis, Estabilidad y Termoestabilidad Enzimática en Relación al Diagrama de Fases de un Sistema de Micelas Invertidas"

que presenta Daniel Alejandro Fernández Velasco para obtener el grado de: Maestro en Investigación Biomédica Básica.

El agua es un componente esencial en la estructura, dinámica y función de las proteínas. Sin embargo, las técnicas experimentales que permiten determinar las interacciones agua:proteína, se encuentran en desarrollo. En la presente tesis, se estudia la respuesta funcional de una enzima ante cambios en la cantidad de agua que la rodea.

El sistema experimental consta de un solvente orgánico apolar (tolueno o propilbenceno), surfactante (Triton X-100 y/o fosfolípidos) y cantidades variables de agua. En la primera parte del trabajo, se determinaron las estructuras presentes en soluciones formadas a diferentes relaciones de los componentes arriba señalados.

Los resultados obtenidos mediante titulaciones en la interfase y dispersión de luz dinámica indican que a concentraciones de agua menores a ~0.8 %, las moléculas de agua hidratan la cabeza polar de los surfactantes. En la región de baja agua, el surfactante se encuentra en estado monomérico.

Al aumentar el contenido de agua de las soluciones analizadas, tiene lugar la formación de estructuras de un tamaño de 1000 Å, estas estructuras son termodinámicamente inestables;

esto es, el estado de agregación de los componentes del sistema se modifica con el tiempo.

Concentraciones mayores de agua producen micelas invertidas de menos de 150 Å de radio. Estas están formadas por una capa esférica de moléculas de surfactante cuya parte apolar se encuentra orientada hacia el solvente orgánico y cuya parte polar se orienta hacia el interior de la esfera, rodeando un volumen de agua reducido. La formación de estas estructuras tiene lugar a valores entre 8 y 27 moléculas de agua por molécula de Triton X-100. Cuando la cantidad de agua presente en el sistema rebasa el límite superior (27), la solución se vuelve inestable y tiene lugar la formación de dos fases.

En la segunda parte de esta tesis, se determinaron la actividad catalítica, estabilidad y termoestabilidad de la enzima hexocinasa introducida en las soluciones antes descritas. Los datos de actividad muestran que al aumentar el contenido de agua del sistema, la actividad catalítica aumenta. Sin embargo, los niveles obtenidos representan un máximo de 30% con respecto a la actividad enzimática detectada en medios completamente acuosos. Aparentemente, la baja actividad se debe a que el tamaño de la hexocinasa es mayor al volumen acuoso del interior de las micelas invertidas sin proteína.

Trabajos anteriores del laboratorio, muestran que las enzimas introducidas en sistemas con fosfolípidos presentan una alta termoestabilidad en el rango de baja agua del sistema. Los datos obtenidos en el presente trabajo, indican que la hexocinasa

también presenta una termoestabilidad elevada en la zona de baja agua del sistema. Experimentos de dispersión de luz dinámica, mostraron que la termoestabilidad observada se debe a la formación de micelas invertidas ajustadas al tamaño de la proteína en el interior de ellas, la cantidad de agua presente se restringe a una o dos monocapas de agua.

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ANTECEDENTES

Existen tres intereses básicos alrededor del estudio de enzimas en solventes orgánicos:

El primer enfoque, de orientación fisiológica, tiene como objetivo la comprensión de la actividad enzimática en medios que simulen la compartamentalización del agua en el interior del citoplasma (1). Los antecedentes de este enfoque se remontan a la incorporación de enzimas membranales en micelas invertidas de fosfolípidos en solventes orgánicos apolares (2). De esta manera, se desarrollaron una serie de trabajos, cuyo principal interés es reproducir in vitro las reacciones características del metabolismo. Con este fin, se limita la cantidad de agua del sistema, compartamentalizando enzimas y substratos en micelas invertidas. Entre los resultados más interesantes en este campo tenemos la incorporación y germinación de esporas en medios formados por fosfolípidos y medios apolares (3), ensayos de actividad que implican acoplamiento entre dos enzimas (4-6), asi como la actividad catalítica de complejos multienzimáticos como la cadena respiratoria bacteriana en sistemas de micelas invertidas (7).

Por otra parte, existen varios grupos interesados en rediseñar la catálisis acuosa para su utilización en solventes orgánicos. Este enfoque tiene sus orígenes en el estudio de la catálisis y termoestabilidad enzimática en proteínas suspendidas directamente en solventes orgánicos (8). Entre los logros más recientes en esta área, se encuentran la conversión enzimática de substratos hidrofóbicos (9), el cambio en la especificidad enzimática por

modificación de solvente (10), cambios en los requerimentos para la catálisis enzimática (6), la modificación de la constante de equilibrio para diversas reacciones (11) y el diseño por mutagénesis dirigida de enzimas estables y catalíticamente activas en solventes orgánicos (12).

Por último, nuestro grupo pretende determinar el papel del agua en el plegamiento de proteínas (13), la modificación por agua de los cofactores requeridos para la catálisis (6), asi como el papel del solvente en las interacciones proteína-proteína que hacen posible la catálisis (14). Esto es, entender el papel de la hidratación en el nexo estructura-función en las proteínas (15). En nuestro caso, para variar la cantidad de agua en contacto con la proteína se ha utilizado dos sistemas de micelas invertidas (5,13). Los antecedentes de nuestro enfoque se encuentran en los trabajos realizados en nuestro laboratorio sobre actividad enzimática en relación a la cantidad de agua en contacto con la enzima, contribución del agua en la termostabilidad enzimática (16,17), el uso de desnaturalizantes como herramienta para promover la interreación entre la proteína y el solvente (5), y la renaturalización enzimática (13).

OBJETIVOS

El presente trabajo es un estudio de las características fisicoquímicas de un sistema de micelas invertidas. Esto es con el objeto de entender el papel de la estructuración del medio y el agua en la expresión de la actividad y termoestabilidad enzimática. Este trabajo describe la caracterización fisicoquímica de sistemas formados por fosfolípidos y Triton X-100 teniendo como solvente tolueno o propilbenceno. Posteriormente se describen los factores del medio que influyen en la expresión de la catálisis y termoestabilidad de la hexocinasa.

A continuación se presenta una breve introducción sobre la relación entre las características de los sistemas de micelas invertidas y el funcionamiento enzimático. En seguida, se muestran los dos trabajos que se hicieron. En estos se analiza y discute la relación entre la estructura del sistema y la actividad y termoestabilidad de la hexocinasa.

INTRODUCCION

I. CARACTERISTICAS GENERALES DE LOS SISTEMAS DE MICELAS INVERTIDAS.

El término micela invertida se refiere a un región específica en el diagrama de fases triangular para mezclas ternarias formadas por un solvente orgánico apolar, un surfactante y agua. En el equilibrio esta región está formada por pequeñas esferas de agua (20-100 Å de radio) rodeadas por una capa de surfactante orientado por su parte polar a la superficie del agua. La parte hidrofóbica del surfactante está en contacto directo con el solvente apolar (15, 18-20). Este último componente es el de mayor concentración en la solución y forma el continuo en el que se encuentran suspendidas las micelas invertidas.

Diversos factores determinan la formación de este tipo de estructuras. Los más importantes son:

1.1-CONCENTRACIONES RELATIVAS DE LOS COMPONENTES.

En el apartado anterior se menciona que las micelas invertidas se ecuentran en regiones estrechas del diagrama ternario de la soluciones formadas por tres componentes. En otras palabras, la formación de micelas invertidas está limitada a un intervalo estrecho de concentraciones de agua (19) que en general son inferiores al 10 %. En estos sistemas, la cantidad de surfactante oscila entre el 1 y el 40 %, mientras que el solvente orgánico es el componente de mayor concentración (18-20). Es importante aclarar que no existen modelos con los cuales se pueda predecir, para un

sistema desconocido, la relación de concentraciones a las que se forman micelas invertidas. Por lo tanto, es necesario determinar experimentalmente la región del sistema en la que existen micelas. En términos generales, es posible caracterizar las dimensiones de la micela utilizando el parámetro Wo, definido como:

$$Wo = \frac{[H_2O]}{[\text{Surfactante}]}$$

Experimentos de dispersión de luz dinámica (21), resonancia magnética nuclear (22) y dispersión de neutrones (23) o rayos X de bajo ángulo (2) muestran que en general, al aumentar el número de moléculas de agua por molécula de surfactante (Wo), aumenta el tamaño micelar (18). De esta manera cuando se incrementa el contenido de agua manteniendo constante la concentración de surfactante, las micelas aumentan de tamaño. De manera opuesta, al mantener fija la cantidad de agua y aumentar la concentración del surfactante, aumenta el número de micelas y disminuye su tamaño. Por último, las micelas mantienen su tamaño y aumentan en concentración cuando la relación molar del agua y surfactante es igual al Wo del sistema de micelas invertidas (18). El razonamiento anterior, se ha utilizado para describir el incremento en el tamaño micelar al aumentar el contenido de agua en el sistema AOT/isooctano/agua, y solo es correcto para sistemas en los que el surfactante se localiza exclusivamente en la pared de la micela. Cuando el surfactante se particiona al solvente orgánico, el esquema anterior no es aplicable (19).

1.2- CARACTERISTICAS FISICOQUIMICAS DE LOS COMPONENTES:

La región del diagrama de fases en la que se obtienen micelas invertidas depende de las características fisicoquímicas de los componentes del sistema. De esta manera, los valores mínimo y máximo de Wo a los cuales se forman micelas, asi como las características de las mismas (número de agregación, propiedades del agua intramicelar y estabilidad a diferentes temperaturas, por ejemplo), dependen de las propiedades específicas del solvente orgánico y surfactante utilizados. Las características de las micelas formadas son producto de la interrelación determinada por la estructura molecular de los componentes de la solución y las interacciones no covalentes (puentes de hidrógeno, interacciones dipolo-dipolo y dipolo-dipolo inducido) presentes entre los diferentes componentes del sistema (20).

Solvente Orgánico:

Las propiedades del solvente en solución determinan el tipo de micela que se forma. Esto se debe a que la disposición espacial de los grupos químicos del solvente determina las interacciones solvofóbicas dominantes en la interfase formada por el solvente orgánico y el extremo apolar del surfactante (19). En general, al aumentar la apolaridad del solvente, disminuye el número de agregación del surfactante (número de moléculas de surfactante que forman una micela) (24). Así mismo, el grado de hidratación del surfactante disminuye al aumentar la apolaridad del medio (19). En algunos casos, el solvente orgánico penetra en la región hidrofóbica de la pared micelar (25), en este caso, la estructura del solvente, modifica el tamaño de la micela dependiendo del grado de penetración del mismo.

Surfactante:

Otro determinante en la formación de micelas invertidas, es la presencia de un surfactante. Estas moléculas amfifílicas separan los compartimentos acuoso y orgánico del sistema. Es por esto, que la estructura y propiedades químicas de estas moléculas modulan y determinan el equilibrio entre ambas fases (26). Tanto las identidades moleculares de las regiones polar y no polar de la molécula, asi como las interacciones específicas de estas dos regiones con el agua y el solvente orgánico determinan el grado de hidratación del surfactante y la solubilidad del surfactante en el medio orgánico y por lo tanto, el número de agregación en la micela.

En algunos casos, la formación de micelas invertidas requiere la presencia de un cosurfactante, generalmente un alcohol de cadena larga $CH_3-(CH_2-)_n-CH_2OH$ (donde $n \geq 5$). Al incorporar alcoholes con diferentes valores de n, es posible variar la polaridad de la pared de la micela y en esta forma facilitar la conversión de substratos hidrofóbicos (27).

Agua:

La cavidad interior de las micelas invertidas está formada por moléculas de agua. Las propiedades de este componente dependen fuertemente del tamaño de la micela (22). Los datos obtenidos por medio de la resonancia magnética nuclear muestran que al incrementar el Wo (y por tanto el radio micelar) en el sistema AOT/isooctano/agua, el corrimiento químico de los protones del agua (δ_{H2O}) difiere del observado en soluciones acuosas. A medida que el tamaño de la micela aumenta, el δ_{H2O} alcanza el valor obtenido en

medios acuosos (22,28). El aumento en el tamaño micelar como función del Wo, asi como las propiedades del agua al variar las condiciones del sistema, dependen en gran medida de las interacciones agua/surfactante, asi como de la distribución de estos dos componentes en el seno del sistema (19). Por lo tanto, el valor de Wo al cual se observan modificaciones en las características del agua, depende de los componentes utilizados.

1.3- TEMPERATURA

Cambios en la temperatura modifican la región del diagrama de fases en la cual es posible obtener micelas invertidas. Generalmente, los sistemas ternarios se caracterizan a presión atmosférica y temperatura ambiente. Sin embargo, el estudio del comportamiento del sistema al variar la temperatura ofrece una mayor versatilidad cuando se estudian fenómenos dependientes de temperatura tales como la desnaturalización de proteínas (16,17) y el efecto hidrofóbico *(vide infra)*. La estabilidad estructural de las micelas invertidas al aumentar la temperatura depende del tamaño de la micela y de la naturaleza química de la fase orgánica. En términos generales, al aumentar el Wo disminuye la temperatura a la cual existe alteración de fases, y en consecuencia se modifica la estructura de las micelas invertidas (19). Al aumentar la temperatura, disminuye el efecto hidrofóbico, por lo cual disminuye la solubilidad del surfactante en el solvente orgánico (19). Este aumento en la concentración efectiva del surfactante, modifica la región del diagrama de ternario en la cual se forman micelas invertidas (19).

Por otra parte, la integridad estructural de las micelas

invertidas al aumentar la temperatura es necesaria para estudiar la velocidad de la difusión intramicelar como función de la temperatura y de esta manera determinar los parámetros de activación de la cinética de intercambio($\Delta H^{\ddagger}$ y $\Delta S^{\ddagger}$) (29). A partir de estos valores, es posible determinar la barrera energética que limita la velocidad de choque e intercambio de contenidos intramicelares. La dinámica de este intercambio determina el tamaño de la micela, además de promover o limitar las interacciones agua/proteína, proteína/proteína y substrato/proteína.

II. INCORPORACION DE PROTEINAS

Existe poca información sobre los parámetros cinéticos y termodinámicos que determinan el encarcelamiento de biopolímeros en soluciones de micelas invertidas. Sin embargo, en el caso de proteínas solubles, se ha encontrado que bajo condiciones óptimas no existen impedimentos termodinámicos para que la proteína se localice en el microambiente acuoso de las micelas. El primer modelo de solubilización de proteínas en micelas invertidas se desarrolló en base a consideraciones geométricas (30). Los modelos posteriores incorporan tamaño y distribución de cargas en la proteína (31), interacciones electrostáticas (32) y minimización de la energía libre del sistema al solubilizar la proteína (33).

Se debe señalar que no existe un modelo general que explique todos los resultados experimentales. Las predicciones teóricas se limitan a una proteína específica dentro de un sistema determinado (α-quimotripsina en AOT/isooctano/agua, por ejemplo (23)). Sin embargo, mientras mayor sea el volumen acuoso del interior de la micela en relación al volumen de la proteína, menor será la

perturbación estructural de la micela llena y viceversa.

III. CARACTERISTICAS FUNCIONALES DE ENZIMAS INCORPORADAS EN MICELAS INVERTIDAS

Las propiedades estructurales y catalíticas de las proteínas incorporadas en micelas invertidas dependen del tamaño de la micela huesped y de las propiedades del agua intramicelar. Al aumentar el tamaño micelar y el Wo, las propiedades del agua intramicelar (δ_{H2O}, capacidad calorífica y actividad acuosa) se modifican hasta presentar los valores obtenidos en agua pura (1,15,18-20, 34,35). Esto es de interés ya que una de las características de las enzimas incorporadas en micelas invertidas es la dependencia de la actividad catalítica con respecto al contenido de agua del sistema. En el sistema AOT/Isooctano/agua, a Wo cercanos a 5, la Vmax es varias veces menor a la obtenida en medios completamente acuosos. Al incrementar el contenido de agua del sistema, la catálisis aumenta. Existen algunos trabajos que señalan que la actividad resultante puede ser similar, y aun superior a la que se observa en medios completamente acuosos (36). En algunos casos, se ha determinado que el máximo nivel de actividad se obtiene a Wo cercanos a 10 (19). Empero, esta no es una regla general (vide infra), sino el reflejo de las características particulares del sistema estudiado. La variación de la actividad catalítica en función de la relación molar agua/surfactante (Wo) refleja la cantidad de agua necesaria para los movimientos catalíticos inherentes a la catálisis. Sin embargo, no existe un modelo general para calcular la distribución del agua en las micelas al introducir proteína (23,30,32,33). Por lo tanto, es difícil

determinar la cantidad de agua en contacto con la proteína necesaria para la catálisis. Como primera aproximación, es posible asociar comportamientos enzimáticos específicos (catálisis y estabilidad, por ejemplo), a diferentes contenidos de agua del sistema.

La determinación de alteraciones estructurales en las enzimas solubilizadas en micelas invertidas depende del contenido de agua del sistema. De esta manera, cuando el contenido de agua del sistema es elevado y la catálisis es similar a la obtenida en medios completamente acuosos, la estructura secundaria de la proteína incorporada en micelas es similar a la encontrada en solución (19). Por otra parte, en condiciones de baja agua (y por lo tanto, baja catálisis), La fosforescencia de triptofanos (37), indica modificaciones en la microviscosidad del interior de la proteína. Esta reducción en la flexibilidad de la enzima, podría explicar el porque en estas condiciones las enzimas muestran una actividad baja.

Actividad y Termoestabilidad Enzimática en sistemas formados por Fosfolípidos, Triton X-100 y agua en Tolueno.

La mayor parte de los resultados experimentales expuestos anteriormente se refieren al sistema AOT/Isooctano/Agua. De particular importancia para el presente trabajo, es la caracterización del sistema formado por fosfolípidos y Triton X-100 en tolueno. A continuación, se describen los resultados que permiten relacionar en este sistema, el contenido de agua con la catálisis y estabilidad enzimática.

En el sistema formado por 15 % Tritón X-100, 85 % tolueno, y 10 mg fosfolípidos por mL de tolueno y cantidades variables de agua (TPT), una gran variedad de enzimas presentan una actividad catalítica reducida con respecto a la que se obtiene en medios completamente acuosos (15). La adición de agentes que promueven las interacciones solvente-proteína como la urea y sales de guanidinio elevan la actividad (5). El efecto activador de la guanidina en este sistema es dependiente de las interacciones interproteícas durante la catálisis. Es por esto que pequeñas diferencias estructurales entre isoenzimas de la deshidrogenas láctica originan diferentes patrones de activación por sales de guanidina (16). Por otra parte, en la región de baja agua (menos de 0.8 % v/v) en el sistema TPT, la ATPasa mitocondrial es capaz de hidrolizar ATP a temperaturas que en agua causan inactivación total (17).

Los experimentos de actividad enzimática en el TPT indican que las interacciones agua-proteína necesarias para la catálisis se encuentran restringidas en todo el intervalo de concentraciones de agua previo a la separación de fases (0.6-7.2 %). Por otra parte, la termoestabilidad y baja catálisis presentes en la región de baja agua del sistema sugieren que al disminuir la cantidad de agua en el sistema, las restricciones en la movilidad enzimática son aun mayores (15). Resulta entonces necesario determinar las características estructurales de este sistema para determinar de que manera el contenido de agua modifica los factores estructurales que determinan la disminución de la actividad enzimática y el aumento en la termoestabilidad.

A continuación se presenta la caracterización estructural de sistemas formados por Tritón X-100 ± fosfolípidos en tolueno o

propilbenceno. Posteriormente se determina la actividad, estabilidad y termoestabilidad de la hexocinasa en diferentes regiones de estos sistemas. Por último se intenta definir el papel de la hidratación de la proteína en sus características funcionales.

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REVERSE MICELLE CREATION AND WATER INDUCED ENZYME FUNCTIONAL RESPONSE.

1.- Dynamic Light Scattering and Phase Titration of Reverse Micelles Systems.

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Abstract

Reverse micelles systems formed in either toluene or propylbenzene with Triton X-100 and water were characterized by phase boundary titrations and dynamic light scattering. As function of water:surfactant molecular ratios, four regions with distinct structural features were encountered. Titration of the system with water revealed a first region of transparency. In this region, obtained up to a 1:1 water:Triton X-100 ratio, Light scattering measurements indicated that the addition of water hydrated Triton X 100 monomers. As water content was increased, a turbid region quoted by Water:Triton X-100 ratios of 1 to 7.6 for toluene and 1 to 4.2 for propylbenzene was obtained. This thermodynamically unstable region is formed by large size, polydisperse structures. Transparent systems containing small size (27-150 Å), thermodynamically stable reverse micelles, were encountered when subsequent water additions lead to water:Triton X-100 ratios between 7.6 and 26.8 for toluene and 4.2 to 15.1 for

propylbenzene. In this region, micellar size increased with water content. Further water additions induced phase separation. A similar titration of the aforementionated systems in the presence of phospholipids, revealed in the first region of transparency up to 10 molecules of water per phospholipid molecule. As compared to Triton X-100 systems, Phospholipid-Triton X-100 mixed systems presented a displacement of the second region of transparency towards higher contents.

Micellar size obtained from the phase diagrams just described, were used in the following manuscript to explore how enzyme catalysis and thermostability was affected by the microenvironment of the guest micelle.

INTRODUCTION

It has been documented that enzymes may be entrapped in the water pool of reverse micelles formed with synthetic detergents with or without co-surfactants. Once entrapped in reverse micelles, enzymes may carry out catalysis through a process that is affected by the amount of water, the type and quantity of surfactant, and the nature of the organic solvent[1,2]. A general feature of reverse micelles is that increasing amounts of water in the system increase the water core of reverse micelles[3]. This is most likely related to the enzyme activity, since it is known that the rate of catalysis of enzymes entrapped in reverse micelles increases as the size of the water pool is increased. At optimal water concentrations activity may reach the values observed in 100% aqueous systems[4-7].

In systems formed with phospholipids, toluene and water[8,9], or those which in addition contain Triton X-100[10], the same general phenomena were observed. However, in this system enzymes exhibit a catalytic activity that is much lower, and a thermostability that is much higher than that observed in totally aqueous systems, or under optimal water concentrations in other types of reverse micelles[9-11].

As knowledge of factors that affect enzyme activity and stability is of importance for basic and practical reasons, we have characterized the system formed with Triton X-100 with or without phospholipids in two organic solvents of different polarity. The results described in this manuscript were used to gain insight into the factors that affect the stability and catalysis of yeast

hexokinase when introduced into these systems (see following manuscript).

MATERIALS AND METHODS

Chemicals: The following were obtained from the indicated sources: propylbenzene from Aldrich, toluene from Merck. Triton X-100, phospholipids and Tris (Hydroxymethyl aminomethane) (Tris) from Sigma; Type IIS soy bean phospholipids were purified according to Kagawa and Racker[13].

Water Solubility Diagrams and Phase Boundary Titrations. Phase diagrams and phase boundary titrations were determined as described by Laane et al[12]. with some modifications. The protocol was as follows: a series of tubes containing 3.4 mL of organic solvent (either toluene or propylbenzene) and different amounts of Triton X-100 were prepared. These optically transparent solutions were titrated with an aqueous solution of 20 mM Tris-HCl pH 7.4 to the point at which turbidity and transparency appeared. The end of titration was reached when further additions of aqueous buffer produced turbidity and phase separation. After each addition of aqueous buffer, the tubes were vigorously stirred for one min and allowed to stand for 3-5 min. At this time turbidity or transparency was assayed by eye.

Wo_t (ratio of water molecules per molecule of Triton X-100), was calculated from the data obtained from titrations of mixtures that contained different concentrations of Triton X-100. A linear regression analysis of the experimental points at the phase transitions was made. The inverse of the slope expressed in molar

terms defines Wo_t at the transition point. Wo_t in the presence of phospholipids was determined by the same methodology in solutions with constant organic solvent:phospholipid ratios.

The same experimental approach was used to determine the ratio of water to phospholipid molecules (Wo_p), except that in these experiments, the organic solvent:Triton X-100 ratio was maintained constant. The titrations were carried out at 25 °C.

Dynamic Light Scattering: Particle size at various points of the phase diagrams were determined by dynamic light scattering (DLS). Essentialy the diffusion coefficent of the scattering particles was determined[14]. The diffusion coefficient D was expressed as function of the hydrodynamic radius R of the scattering particle by Stokes-Einstein's equation.

$$D = \frac{kT}{6\pi\eta_0 R} \qquad (1)$$

where k is Boltzman's constant, T absolute temperature and η_o the viscosity of the solvent. Shear viscosity for different organic solvent/Triton X-100 solutions was determined at 25 °C with an Uhbelode capillary viscosimeter (Fig. 1). Shear viscosity values for all Triton X-100 concentrations were obtained by interpolation of the data in Figure 1 and thereafter used in R calculations. The DLS apparatus used has been described before[15]. An Argon-ion laser (λ= 488 nm) of 100 mW (LEXEL model 75) was used as light source. The laser beam with a diameter of 100 microns was focused in the sample cell. Scattered light was collected at 90° and focused into

a photon counting photo-multiplier tube (ITT model FW-130). The output went to a high bandwidth digital pre-amplifier. The signal was finally processed by a digital correlator (Langley-Ford model 1096). DLS data were fitted using the first two cumulants in a cumulant's expansion[16] of the time autocorrelation function:

$$g_1(t) = e^{-\Gamma t - \frac{1}{2}\mu_2 t^2}$$

where g_1 is the heterodyne time correlation function, Γ and μ_2 are the first and second cumulants. The first cumulant Γ is related to the diffusion coefficient D through the relationship $\Gamma = q^2 D$, where q is the magnitude of the scattering vector given by

$$q = \frac{4\pi n_0}{\lambda_0} \sin\frac{\theta}{2}$$

Θ being the scattering angle, and n_0, and λ_0 the solvent effective index of refraction and incident wavelength, respectively. The second cumulant μ_2 is related to the width of the particle size distribution function, according to the equation[17]

$$\sigma = \frac{q^2 kT\sqrt{\mu_2}}{6\pi\eta_0\Gamma^2}$$

where σ is the standard deviation (square root of variance) of particle size distribution.

The medium refractive index (n_0) was calculated as the weighted volume fraction of the refractive index of the components[18]

$$n = n_T \phi_T + n_b \phi_b$$

where n_0, n_T and n_b are the refractive indexes of solution, Triton X-100 and organic solvent respectively. Φ_T and Φ_b are the volume fractions of the components of the solution. The refractive indexes of toluene and propylbenzene based systems for all concentrations of Triton X-100 were taken as 1.4962 and 1.4911 respectively. These values were obtained from the mean of the calculated n values at the lowest and highest concentrations of Triton X-100 used. The error introduced by this assumption is approximately 0.01%.

Relative concentration of scattering particles was calculated according to Rayleigh equation[14]:

$$I_d = I_0 K c M \rho(\theta)$$

where I_d is the total scattered intensity, I_0 is the incident intesity, c is mass concentration and $\rho(\Theta)$ is a structural factor.

Dynamic Light Scattering experiments were carried out at room temperature. All solutions were filtered through 0.5 μM Millipore filters. Each addition of a water solution was followed by 2-3 min of magnetic stirring. The autocorrelation function was obtained from data accumulated for at least 90 sec. Measurements remain stable under these conditions.

RESULTS

DLS and phase titrations with water of systems formed in toluene or propylbenzene and Triton X-100 with or without phospholipids were carried out in the low water corner of the tertiary phase diagram (Fig. 2). When phospholipids were added, either the ratio of organic solvent:phospholipids, or the ratio of organic solvent:Triton X-100 was maintained constant. Thus the systems can be treated as pseudotertiary.

Phase Boundaries as a Function of Triton X-100 Concentration.

Titrations of solutions with different concentrations of Triton X-100 in toluene with progressive amounts of water revealed sharp boundaries between four different regions (Fig 3), the water:Triton X-100 ratios at which the changes took place are shown in Table I. The first region was transparent (T1), and was observed up to $Wo_t \leq 1$, most likely it involved the hydration of the terminal hydroxyl group of one molecule of Triton X-100 by one molecule of water (see below data obtained by dynamic light scattering). As Wo_t was increased from 1 to 8, a turbid solution (R1) was obtained. In this region, the exposure of water to the organic solvent was reduced via the formation of large size aggregates (vide infra). At ratios of 8 and 27 molecules of water per molecule of Triton X-100, a drastic transition took place and led to the appearence of a second region of transparency (T2). In this region, water incorporation was maximized through formation of reverse micelles. Additions of water to Wo_t higher than 27 rendered the system turbid and phase separation took place.

Data with propylbenzene as solvent, (Table I and Fig. 4) revealed phase boundary transitions similar to those described for

revealed phase boundary transitions similar to those described for toluene. The Wo_t values of the first boundary were almost the same in both solvents. This would be expected, since it corresponds to hydration of the polar hydroxyl group of Triton X-100. However in the rest of the phase diagram, transitions in propylbenzene took place at Wo_t values lower than in toluene. Thus, modification in the interactions of the solvent with the apolar region of the surfactant gave rise to differences in hydration of the surfactant polar heads.

The inclusion of phospholipids either in the toluene or the propylbenzene based systems affected, but slightly the $\underline{Wo_t}$ values at the phase boundaries of the T1 region (Table I). However, phospholipids displaced the boundaries of the T2 region towards higher water contents. In this respect it is noted that as the ratio of Triton X-100 to phospholipid molecules was increased, T2 boundaries converged asymptotically to the values observed in the absence of phospholipids. This indicates a concentration dependent effect of phospholipids on Triton X-100 hydration.

Phase Boundaries as a Function of Phospholipid Concentration.

Water titrations of solutions with different concentrations of phospholipids in toluene or propylbenzene that contained a constant concentration of Triton X-100 were carried out (Fig. 5). With toluene as solvent, the T1 region was obtained at values of 11 molecules of water per molecule of phospholipid ($Wo_p \leq 11$). With propylbenzene as solvent, a transparent solution was observed up to a ratio of 9 molecules of water per phospholipid ($Wo_p \leq 9$). Both values are in agreement with the reported values for phospholipid hydration in benzene as obtained by ^{1}H-NMR and infrared analysis

[19,20]. In these latter studies it was found that the first hydration shell of phospholipids was formed by 9-11 water molecules.

The Wo_p values at the second transparent region depended strongly on Triton X-100 concentration (not shown). However at all concentrations of Triton X-100 assayed, the inclusion of phospholipids into the system caused a shift in the phase boundaries. This indicates that in a mixture of Triton X- 100 and phospholipids, both components build up the micellar wall in the T2 region.

Characterization of the System by Dynamic Light Scattering.

In the absence of added water, DLS analysis of solutions of Triton X-100 in toluene or propylbenzene up to a concentration of 50 % (v/v) showed no autocorrelation function. The absence of defined structures indicates solvation of individual Triton X-100 molecules by the organic solvent. When water was progressively introduced and in accordance with results on phase boundary transitions (Fig. 3 and 4), DLS revealed four well defined regions. In the T1 region, independently of the solvent, Triton X-100 or phospholipid concentration, no scattering particles were observed. As surfactant monomers are below the limit of detection by DLS, the findings imply that in the T1 region, surfactant molecules are in a monomeric state. The relevance of this observation will be discussed in the following manuscript.

In the R1 region, DLS detected an intense signal of scattering particles of the order of 1000 nm. It was not possible to do a precise characterization of these large size polydisperse structures due to multiple scattering effects. These turbid regions remained unchanged (to DLS) for 5 minutes, but at longer times

phase separation took place.

At the edge of the R1/T2 boundaries, increases in water concentration produced a sharp decrease in particle size. At this stage (T2 region) formation of reverse micelles took place. After a minimum value was reached further water increments brought about a progressive rise in the radii of reverse micelles (Figs. 6 and 7). With both, toluene (Fig. 6) and propylbenzene (Fig. 7), the region of water concentration in which reverse micelles existed increased as the concentration of Triton X-100 increased.

With respect to the dimensions of the micelles, DLS experiments showed that micellar size depended on the solvent (compare Fig. 6 and 7 with toluene and propylbenzene respectively), and the presence of phospholipids (see A and B in Figs. 6 and 7). Indeed depending on the composition of the system, the hydrodynamic radii of the micelles ranged from 27 to 150 Å.

From the data it is clear that the nature of the solvent exerted an strong effect on the dimensions of the micelles. Notwithstanding the presence of phospholipids, with propylbenzene, particle size was significantly smaller than in the more polar toluene. (compare Figs. 6 and 7) i.e. at equal Wo_t values, reverse micelles in propylbenzene were approximately 20 Å smaller. The difference may be due to a lower aggregation number, or a tighter packing of Triton X-100 molecules in propylbenzene.

The dimensions of the minimal water core of reverse micelles in the T2 regions are shown in Table II. Particularly at the light of the results described in the following manuscript, it is noteworthy that in propylbenzene, the internal water pool is several times smaller than in toluene.

Relative scattered intensity in the T2 region, increased with water content (Fig. 8). From these data and the dimensions of the particles, the concentration of reverse micelles was calculated (Fig. 8 inset) As water was gradually increased, there was an initial increase in reverse micelle concentration and a decrease in particle size. This reflects formation of an increasing number of reverse micelles upon breakage of remaining R1 large structures. After the minimum particle size and the maximum micellar concentration were obtained, further water additions near the T2/R2 boundary, lead to an increase in micellar size and a decrease in micellar concentration.

With respect to the contribution of phospholipids to micellar size, it was found that reverse micelle dimensions were nearly the same with or without phospholipids (Compare A and B in Figs. 6 and 7). However, as a consequence of the inclusion of phospholipids to the micellar wall, higher amounts of water were needed to reach the T2 region. Furthermore, in the presence of phospholipids, the range of water concentrations in which reverse micelles were observed was larger than in their absence.

DISCUSSION

In the titrations with water, the first transparent region corresponds to hydration of the polar hydroxyl group of Triton X-100, and in the presence of phospholipids to hydration of their phosphate moiety. As in this region no particles were detected, it is concluded that aggregation of surfactant molecules did not take place. Instead, it is likely that a transient bicontinuous

multilayer of Triton X - 100 molecules was formed as an increasing number of surfactant molecules became hydrated. Further increases in water concentration led to aggregation of surfactant monomers into a thermodynamically unstable net of large size polydisperse structures. After a certain threshold was achieved (Table 1), higher water concentrations brought about breakage of these structures and formation of thermodynamically stable reverse micelles of well defined dimensions. At this stage (T2 region), the higher thermodinamically stable surface:volume ratios were attained. As calculated from the size and relative scattered intensities throughout the T2 region, it is likely that water incorporation in this region took place via an increase of the inner water core of the micelle. At the end of the T2 region, further water additions led to macroscopic heterogeneity and phase separation.

The radii of reverse micelles in the T2 region of the toluene based systems (50 Å) were similar to those of micelles of Triton X-100 in aqueous solution (48 Å)[21]. In the all aqueous system the inclusion of one molecule of sphingomyelin per four molecules of Triton x 100 produced an increase of 8 Å in micellar radii[21]. In the toluene and propylbenzene systems with Triton X-100, the addition of phospholipids changed the amount of water required for formation of reverse micelle, but the micellar radii remained unchanged (Fig. 6 and 7). It is pointed out that in toluene and propylbenzene systems, the ratio of Triton X-100 to phospholipid molecules was 25 i.e. six times lower than that used in experiments in all aqueous media (4)[21].

It has been described that no simple relation exists between

micellar size and Wo when the surfactant molecules are not totally located at the oil/water interface[22]. As no structures were found in the absence of water or in the T1 region, it is concluded that in this section of the phase diagram, the whole population of Triton X-100 molecules was in a soluble state. As water concentration was raised the equilibrium between Triton X - 100 monomers and aggregates shifted towards the latter. In the T2 region Triton X -100 molecules gave rise to reverse micelles. In this region increasing water concentration led to an increase in micellar size reflecting a progressively higher surfactant hydration (Wo_t) and in consequence an increase in the dimensions of the internal water pool.

The strong contribution of the polarity and the spatial structure of the organic solvent on the dimensions of reverse micelle systems become evident if the data of Zhu, Feng and Schelly[23] are compared to those described here. By controlled partial pressure-vapor pressure osmometry and quasi-elastic light scattering studies of a cyclohexane/Triton X-100/water system, these authors found [23] a maximal Wo value of 5.5 before phase separation took place. In the toluene and propylbenzene based systems, the values were 26.8 and 15.1 respectively. There are also differences in the dimensions of the structures formed in cyclohexane and those formed in toluene and propylbenzene. In the conditions of Zhu Feng and Schelly, the diameter of the particles varied between 133 and 610 Å. Moreover, in the absence of added water, the authors detected structures with a diameter of 213 Å, maximal size being observed at a Wo of 1. In toluene and propylbenzene, no structures were observed at this level of

hydration. Using the log P value (log of the partition of a given solvent in octanol and water), as index of solvent polarity[24,25], the value for cyclohexane (3.4) is intermediate between that of toluene (2.8) and propylbenzene (3.8). Apparently the polarity of the solvent by itself would not seem to account for the differences observed. Hence it is likely that geometrical differences between the ring of the cyclohexane molecule and the benzene ring of toluene and propylbenzene determine differences in micellar size via changes in solvent penetration into the surfactant core of the reverse micelle. In fact, Zhu, Feng and Schelly[23] ascribed the aggregation behavior of Triton X-100 in cyclohexane to changes in solvent penetration i.e. cyclohexane molecules penetrate into the micellar core producing large size structures. Therefore, in the case of toluene and propylbenzene, a reduced penetration of the solvent into the micellar core, would allow a closer packing of Triton X-100 molecules, producing reverse micelles with a relative smaller radii.

The effect of the various structures that appear throughout the water solubility diagrams in toluene and propylbenzene on the activity and stability of enzymes in such systems is described in the following manuscript.

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FIGURE LEGENDS

Figure 1. Dependence of Shear Viscosity on Triton X-100 Concentration in Toluene (●-●) and Propylbenzene (o-o).

Figure 2. Definition of Range of Composition investigated. At 25°C. The boundary depicted as I is the upper limit of Triton X-100 concentrations investigated, whereas the R2 boundary shows the water concentrations at which phase separation takes place.

Figure 3. Triton X-100/Water Pseudosecondary Phase Diagram of Toluene based Systems. 3.4 mL of toluene with (●-●) or without (o-o) 10 mg phospholipids/mL organic solvent and the indicated amounts of Triton X-100 were titrated with a Tris HCl pH 7.4 water solution until a transparent/turbid transition appeared. The titration was continued until further aqueous additions gave phase separation.

Figure 4. Triton X-100/Water Pseudosecondary Phase Diagram of Propylbenzene based Systems. 3.4 mL of propylbenzene with (●-●) or without (o-o) 10 mg phospholipids/mL organic solvent and the indicated amounts of Triton X-100 were titrated as described in Figure 3

Figure 5. Phospholipids/Water Pseudosecondary Phase Diagrams of the Toluene and Propylbenzene Systems at the First Transparent Region. Boundaries depict the composition of the system at the point at which a phase transition appeared. The inverse of phase boundaries slopes, expressed in molar quantities defines the specific Wo_p at each transition. Wo_p values in the presence of the indicated amounts of Triton X-100, are 10.8, 15.1 and 16.2 in toluene (●-●), and 8.5 (o-o) in propylbenzene based systems.

Figure 6. Hydrodynamic Radii Profile obtained by DLS Analysis of the T2 region of the Triton X-100/Water Toluene based Systems in the Absence (A) and Presence (B) of Phospholipids. Solvent, surfactant(s), and water concentrations, are as described in Figure 3. Triton X-100 concentrations in (A) were 17 %(■-■), 19 % (□- □), 20.9 % (●-●), 22.7 (○-○), 24.4 (▲-▲) and 26 %(△-△). In the presence of phospholipids (B), the 15 % (■-■), 17 %(□-□), 19 % (●-●), 20.9 % (○ -○), 22.7 % (▲ -▲) and 26 % (△ -△) Triton X-100 concentrations in toluene are shown.

Figure 7. Hydrodynamic Radii Profile obtained by DLS Analysis of the T2 region of the Triton X-100/Water Propylbenzene based Systems in the Absence (A) and Presence (B) of Phospholipids. Solvent, surfactant(s), and water concentrations are as indicated in Figure 4. Triton X-100 concentrations in (A) were 10.5 % (■-■), 12.8 % (□-□), 15 % (●-●), 17 % (○-○), 19 % (▲-▲) and 22.7 % (△-△) In the presence of phospholipids, the 15 % (■-■), 17 %(□-□), 19 % (●-●), 20.9 % (○-○), 22.7 % (▲-▲) and 26 % (△-△) Triton X-100 concentrations in propylbenzene are shown.

Figure 8. Increase in Relative Scattered Light Intensity as a Function of Water Content in the T2 Region of the 22.7 % (v/v) Triton X-100 Toluene System. Water concentrations corresponds to the conditions of Fig. 3. The inset shows micellar size (●-●) vs. relative concentration (□-□) (The latter was calculated as described under Methods)

TABLE I: Wo_t values at phase transitions.

Boundary	T1/R1	R1/T2	T2/R2
Toluene	0.6	7.6	26.8
Propylbenzene	0.5	4.2	15.1
Toluene + Phospholipids	0.9	*	*
Propylbenzene + Phospholipids	1.1	*	*

T1/R1 presented values were calculated from the systems without or with 10 mg phospholipids/mL organic solvent. *(R1/T2 and T2/R2 bounadies in the presence of phospholipids depend on the relative concentrations of Triton X-100 and phospholipids.

TABLE II: Calculated inner water core of T2 region reverse micelles.

Solvent	Toluene	Propylbenzene
Water core (Å)	55.2	8
+Phospholipids		
Water core (Å)	30.1	12

Inner water core radii were calculated from minimum hydrodynamic radii in the T2 region and Triton X-100 length(27 Å)[26].

Figure 1

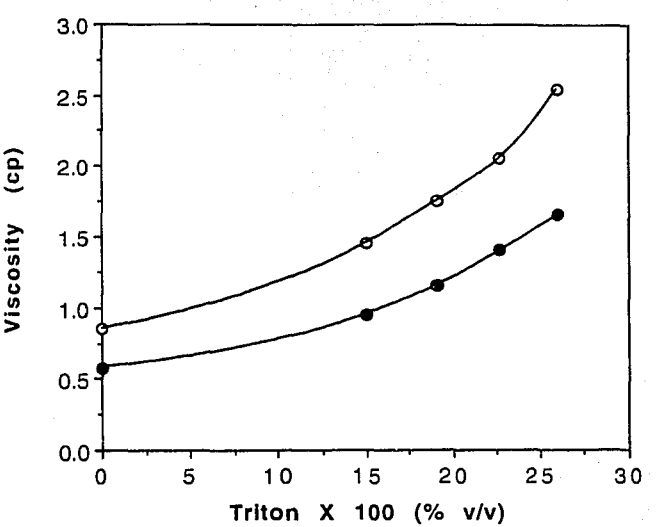
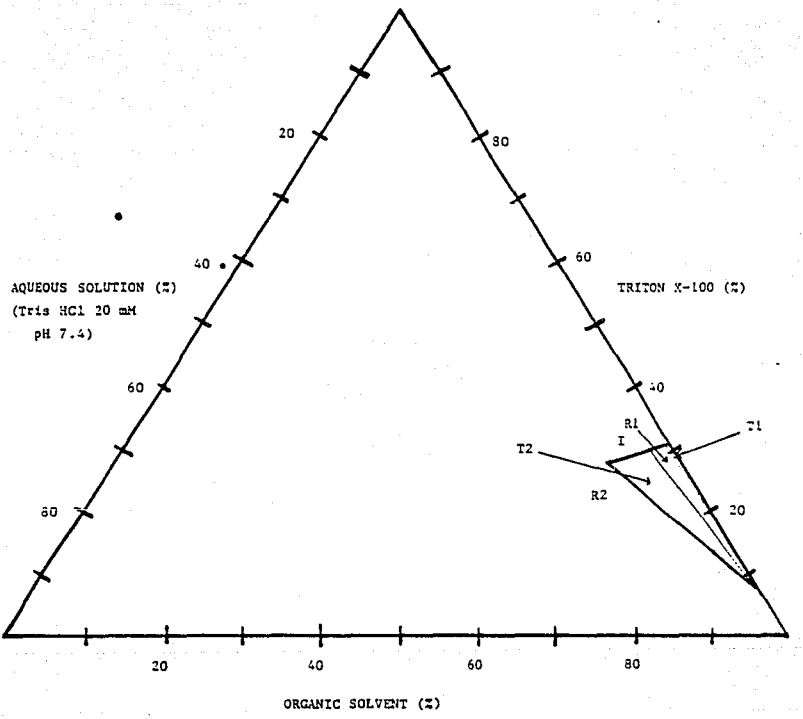


Figure 2


AQUEOUS SOLUTION (%)
(Tris HCl 20 mM
pH 7.4)
TRITON X-100 (%)
ORGANIC SOLVENT (%)
20
40
60
80
R1
T1
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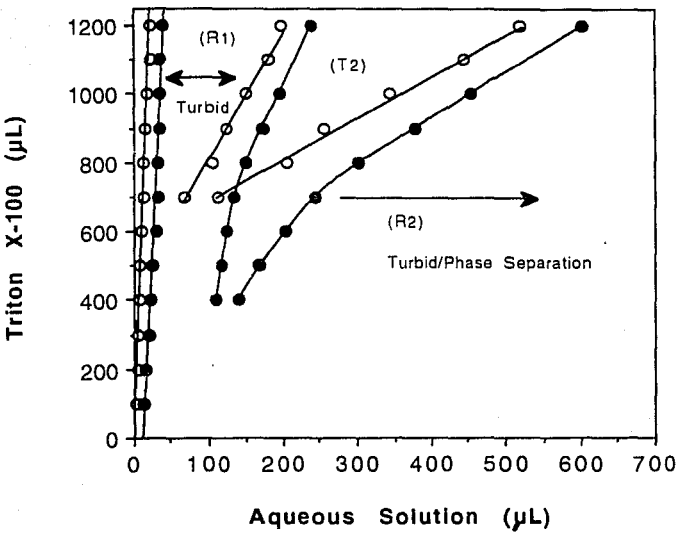
R2

Figure 3


Triton X-100 (µL)
1200
1000
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200
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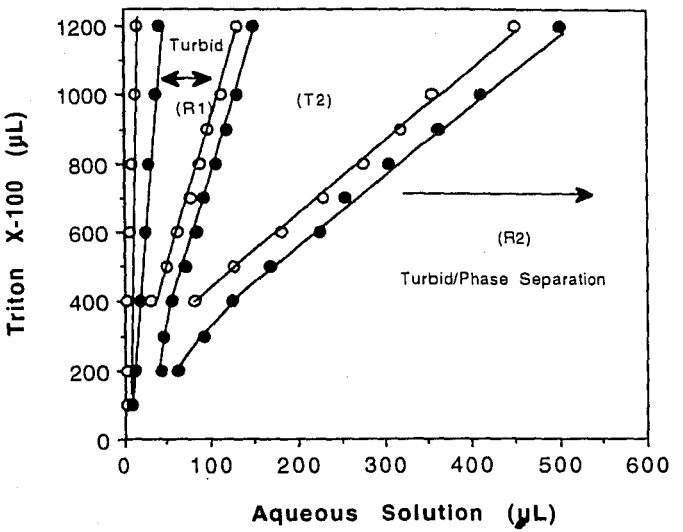
Aqueous Solution (µL)
(R1)
Turbid
(T2)
(R2)
Turbid/Phase Separation

Figure 4


Triton X-100 (µL)
1200
1000
800
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0
Turbid
(R1)
(T2)
(R2)
Turbid/Phase Separation
0
100
200
300
400
500
600
Aqueous Solution (µL)

Figure 5

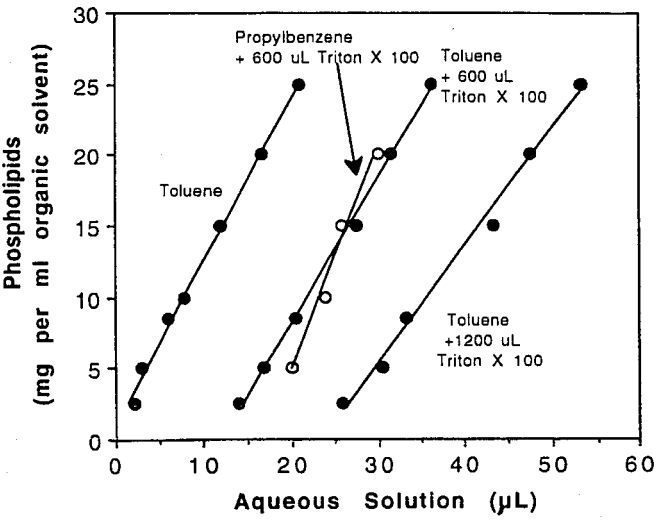

Phospholipids
(mg per ml organic solvent)
Aqueous Solution (µL)
Propylbenzene
+ 600 uL Triton X 100
Toluene
Toluene
+ 600 uL
Triton X 100
Toluene
+1200 uL
Triton X 100
0
5
10
15
20
25
30
0
10
20
30
40
50
60

Figure 6

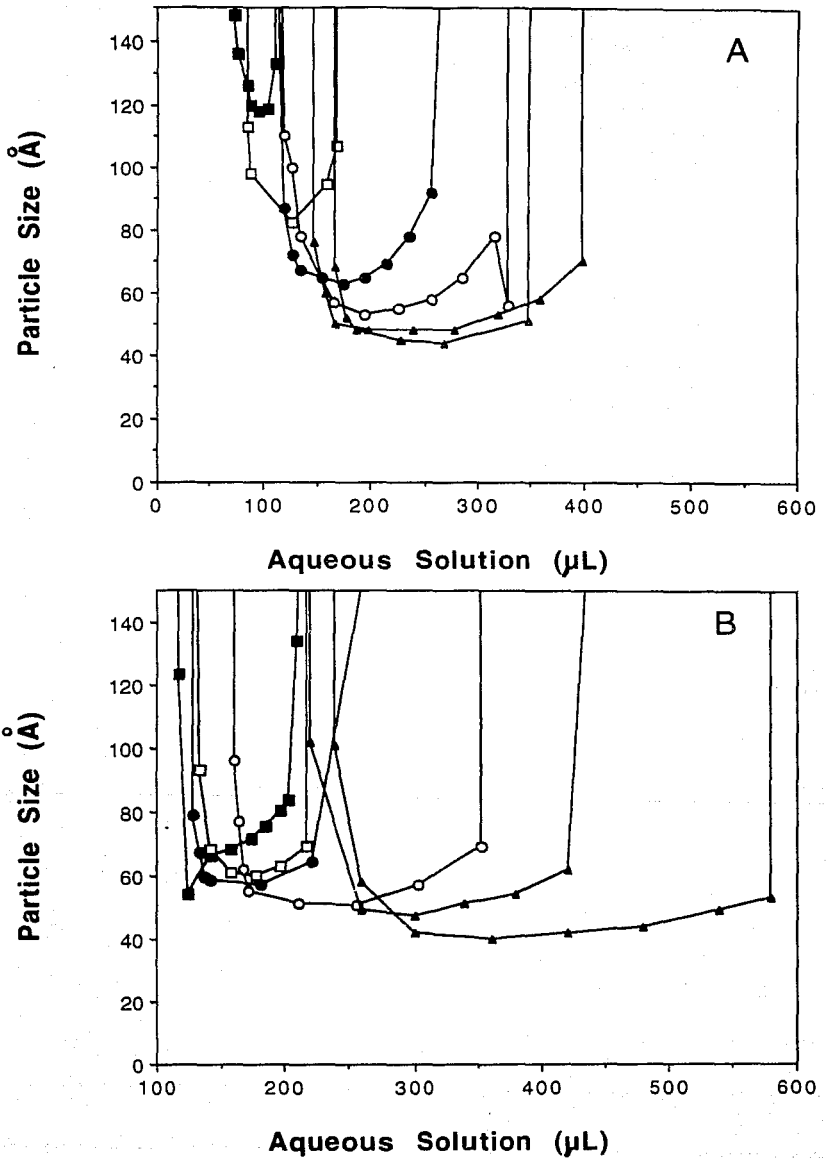

A
B
Particle Size (Å)
Aqueous Solution (µL)
0
20
40
60
80
100
120
140
100
200
300
400
500
600

Figure 7

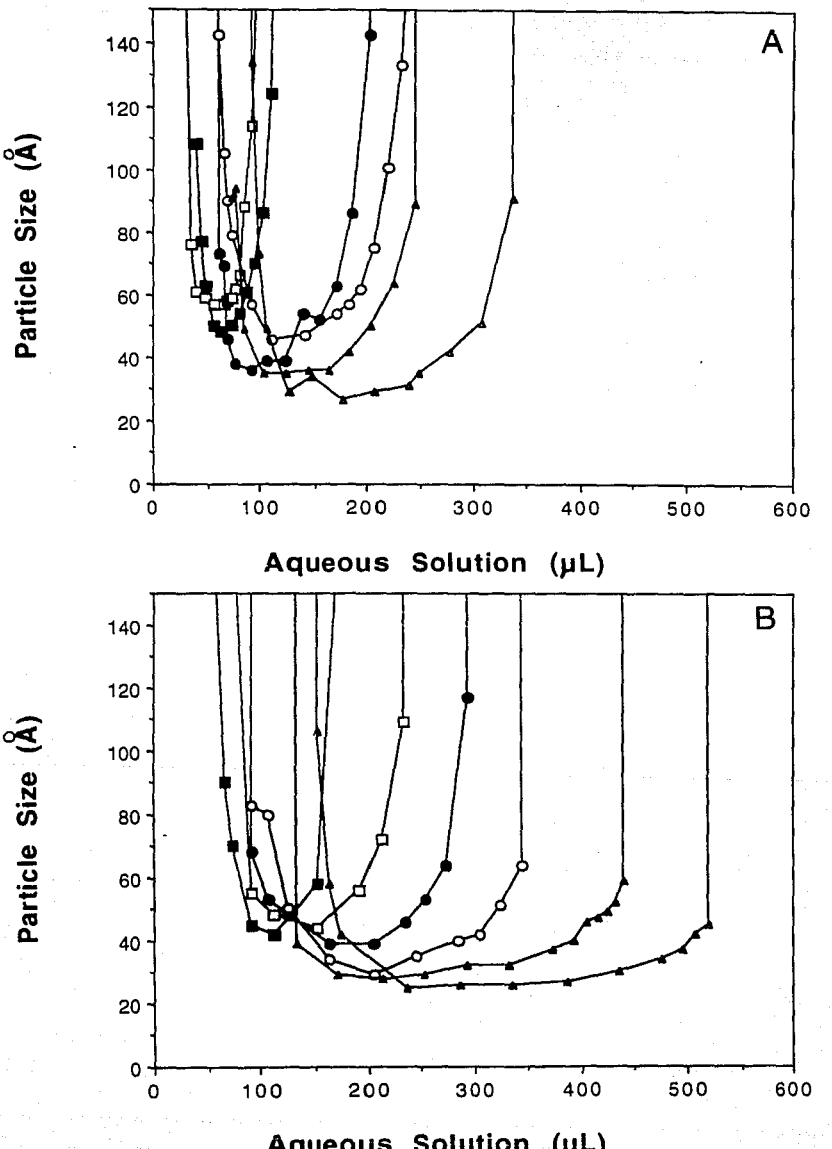
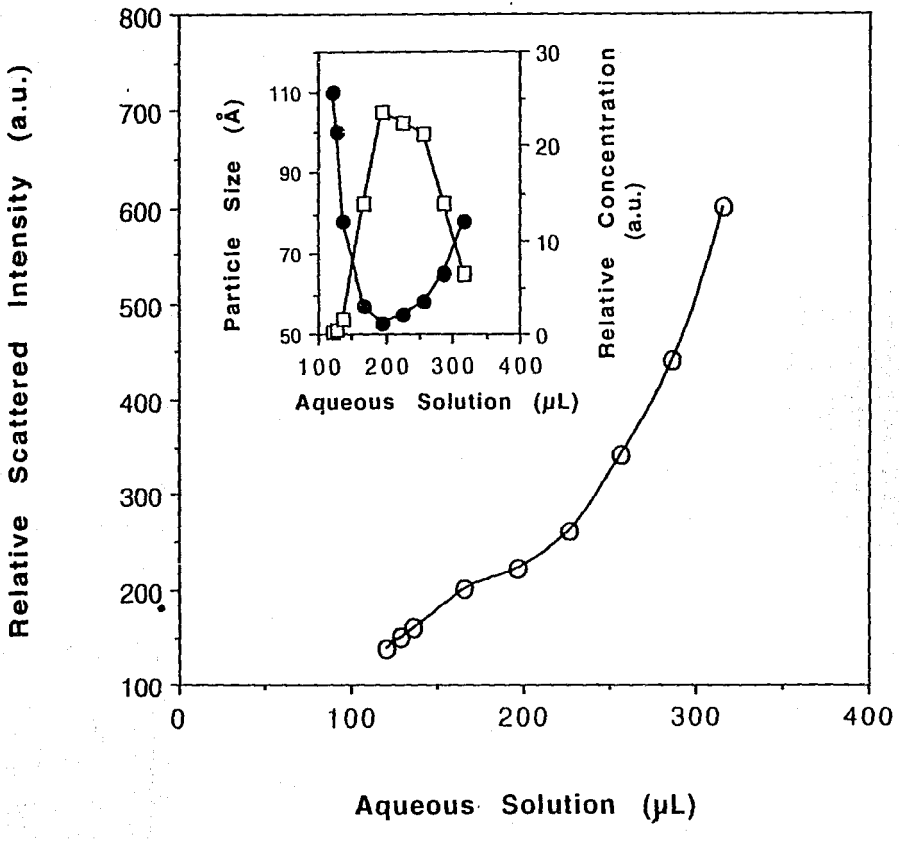

A
B
Particle Size (Å)
Aqueous Solution (µL)
0
20
40
60
80
100
120
140
0
100
200
300
400
500
600

Figure 8


Relative Scattered Intensity (a.u.)
800
700
600
500
400
300
200
100
0
100
200
300
400
Aqueous Solution (μL)
Particle Size (Å)
110
90
70
50
Relative Concentration (a.u.)
30
20
10
0
100
200
300
400
Aqueous Solution (μL)

REVERSE MICELLE CREATION AND WATER INDUCED ENZYME FUNCTIONAL RESPONSE.

2.- Phase behavior in Enzyme Catalysis and Thermostability.

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ABSTRACT

Catalysis, stability and thermostability of yeast hexokinase were determined in the microenvironments of the two organic solvent/Triton X-100/phospholipids systems characterized in the previous manuscript. In accordance to micellar size determined in the absence of enzyme, It was found, that although enzyme catalysis increases with water content, the maximum obtained values in the toluene and propylbenzene systems represent 30 and 1.6 % respectively of the activity in all aqueous media. Catalytic activity correlated with micellar size as determined in the T2 region in the absence of protein (see preceding manuscript). A comparison of the dimensions of hexokinase and those of reverse micelles at relatively high water concentrations (T2 region) suggests that in this region, hexokinase entrappment increases the inner volume of the micelle.

High enzyme thermostability was only observed in the first transparent region (T1) of the system that contained phospholipids. In this region, hexokinase induced the formation of reverse micelles from dispersed surfactant monomers. The striking similarity in the dimensions of hexokinase entrapped in reverse micelles as determined by dynamic light scattering measurements in the T1 region with those of hexokinase as obtained from X ray diffraction studies of the enzyme in a crystaline environment suggest that high thermostability, and low catalytic rates result from restrictions in mobility imposed by a low water environment.

INTRODUCTION

Water is an essential component in protein structure, function and dynamics [1,3]. However the experimental techniques that allow direct measurement of water-protein interactions have not been fully developed [4]. An experimental approach that allows the study of water-protein interactions with different, but low amounts of water is to entrap proteins in the interior of reverse micelles[5]. In these systems it is possible to vary the size of the micelle, and hence the amount of water in contact with the protein. The catalytic activity of enzymes entrapped in reverse micelles generally increases as the amount of water is increased[6,7]. The higher activity, obtained with increasing amounts of water has been ascribed to a higher flexibility of the protein molecule, and/or shifts in the properties of intra-micellar water towards those of bulk water[5].

It has also been reported that at low levels of water, enzymes dispersed in organic solvents[8], or entrapped in reverse micelles formed with phospholipids (with or without Triton X-100) exhibit a high thermostability[9,10]. In the latter conditions enzymes may carry out catalysis at temperatures that in all water media cause rapid denaturation[11]. Thus it has been proposed that in a low water environment, enzymes are relatively rigid and that this accounts for their low catalytic rates and high thermostability[5].

In the preceding manuscript we characterized by phase boundary titrations and dynamic light scattering the Triton X-100 and phospholipid based systems. In this work, we studied the relation that may exist between enzyme activity and thermostability with the structure of the micelles that harbor the enzyme hexokinase.

This enzyme (E.C. 2.7.1.1) catalyses the reaction:

D-Glucose + ATP → Glucose 6-phosphate + ADP

The dimensions of the enzyme in its crystalline state are known[12].

MATERIALS AND METHODS

Solutions of yeast hexokinase (Type F-300 baker's yeast Sigma) were prepared by dissolving the lyophilized, ammonium-sulfate-free commercial preparation in 10 mM Tris-HCl pH 7.4 . In all water media, the specific activity was around 240 umol min^{-1} mg^{-1} as assayed as described in reference .

The activity of hexokinase entrapped in reverse micelles hexokinase was assayed at 25°C. In the protocol[13], a water solution that contained 40 mM Tris-HCl pH 7.4, 10 mM ATP, 3 mM $MgCl_2$, and 25 mM glucose was transferred to a system formed by Triton X-100, with or without phospholipids. The bulk of the solvent was either toluene or propylbenzene; variations in system composition are indicated under Results. The reaction was started by the injection of hexokinase followed by stirring and incubation. The systems, either transparent or turbid did not exhibit phase separation within the experimental times. The total amount of water in the system varied between 0.2 and 12.0 % (v/v). At various incubation times, the reaction was arrested by mixing 0.4 mL of the reverse micelle system with 1 mL of 0.5 M perchloric acid. To this mixture 1 ml of water saturated isobutanol/benzene (1:1) was added and vigorously stirred. The mixture was centrifuged to achieve phase separation. Afterwards the organic phase was eliminated. In the water phase glucose 6-phosphate was determined. An aliquot of 0.9

mL of the water phase was neutralized with K_2CO_3, and mantained for 2 hours in an ice bath. Potassium perchlorate was eliminated by centrifugation. The amount of glucose-6-phosphate was determined in 1 mL of aqueous solution that contained 50 mM phosphate Tris, 0.22 mM NADP and 0.1 mg bovine serum albumin. The pH of this solution was adjusted between 6.8 and 8.0 . After recording absorbance at 340 nm, approximately 1 unit of glucose-6-phosphate dehydrogenase was added. The amount of NADPH produced was determined by its absorbance at 340 nm[14]. From absorbance values, the amount of glucose 6 phosphate formed in the reverse micelles was calculated[13]. Blanks without hexokinase were included in all experiments.

Control experiments in all regions of the phase diagrams were made in order to determine the recovery of glucose 6 phosphate. In all cases, and up to 200 nmoles of glucose 6 phosphate, recovery from the organic solvent system into the water phase was close to 100 %.

Stability and thermostability of hexokinase in the organic solvent systems was determined from the activity of hexokinase that remained after various treatments. The enzyme as a water solution in 10 mM Tris-HCl pH 7.4 was transferred to the organic solvent systems at 25 °C. Afterwards the system was incubated for the times and at the temperatures indicated under results. Afterwards the samples were cooled to a temperature of 25 °C. Thereafter, a water solution that contained the components for assay of activity (see above) was introduced; in these assays the water concentration was always 6 %. The reaction was arrested and glucose 6 phosphate was determined as described above.

The methodology of the dynamic light scattering experiments was

described in the preceding manuscript. However, in 100 % water as well as in the first transparent region (T1), the two different relaxation times observed in the correlation function were modeled with the heterodyne correlation function $g_1(t)$ defined as:

$$g_1(t) = A_1 e^{-\Gamma_1 t} + A_2 e^{-\Gamma_2 t}$$

where A_1 and A_2 are the relative populations of small and large particles, and Γ_1 is related to the hydrodynamic radius of the particles R_1 by the equation

$$\Gamma_1 = \frac{q^2 kT}{6\pi\eta_0 R_1}$$

(see the accompanying manuscript for details)

RESULTS

Hexokinase activity and micellar size determined in the absence of enzyme.

Catalytic activity of a wide variety of enzymes has been determined at fixed ratios of toluene to Triton X-100 (85:15, v/v)[13]. Here the activity of hexokinase as a function of water and

surfactant concentration in the organic solvent system (Figs. 1 & 2) was determined throughout the entire transparent-turbid-transparent regions of the four phase diagrams determined in the absence of protein in the preceding work. As water was gradually increased, three regions of activity could be observed. Up to 0.8 % water (T1 zone), activity was low and difficult to assay accurately. As water concentration was raised in the R1 region, activity increased steadily. In the third region corresponding to the T2 zone, increase in activity with water content were lower than in the R1 region. Activity depended on the amount of water in the system, but also on the concentration of Triton X-100. As the concentration of the latter increased, the pattern of activity shifted to higher water concentrations (Fig. 1 & 2). As shown in the accompanying manuscript, there is also a similar shift in the boundaries between transparent and turbid regions in the protein free systems as the concentration of Triton X-100 is varied.

Throughout the entire water range, hexokinase activity in every organic solvent system studied was several-fold lower than in all water media (250 $\mu mol^{-1}\ mg^{-1}$). However, activity was much lower in systems formed with propylbenzene than in those formed with toluene (Compare data in Figs. 1 and 2). In this respect, it is relevant to note that protein free reverse micelles in the T2 region with propylbenzene, have a minimum inner water radii at least 15 Å smaller than in the corresponding region of the toluene system (see Table II previous manuscript). Apparently there is a relation between T2 region micellar size in the absence of protein, and the expression of enzyme activity (vide infra)

Effect of Phospholipids on the Activity of Hexokinase in Organic Solvent Systems.

In accordance with previous data on the activity of the mitochondrial ATPase entrapped in reverse micelles in toluene[11], it was found that the inclusion of phospholipids did not affect hexokinase activity (compare A & B in Figs. 1 & 2). In the propylbenzene systems (Fig. 2), the inclusion of phospholipids produced an almost two-fold increase in activity. Nevertheless, it is noticed that the maximum values obtained in this solvent were below 1.7 % of the activity detected in all aqueous mixtures.

Stability of Hexokinase in Organic Solvent Systems

At 25 °C the catalytic activity of hexokinase transferred to organic solvents systems formed without phospholipids and low amounts of water (T1 region), decayed in 30 min. to less than 10 % of the original activity. In contrast, hexokinase transferred to systems that contained phospholipids did not lose activity in a four hour incubation period. Hence, in the first transparent region phospholipid molecules create a microenvironment for hexokinase that prevents its inactivation.

The effect of phospholipids on the stability of hexokinase in organic solvents at high temperatures systems was also determined. In systems that contained phospholipids in either toluene (Fig. 3A) or propylbenzene (Fig. 3B) and less than 0.8 % water (v/v), the enzyme was highly stable. Less than 10 % of the activity was lost in 10 minutes at 60 °C (Fig. 3). In all aqueous mixtures, 98 % of the activity was lost in 5 min. of incubation at 50 °C (not shown).

It is noted that in the presence of phospholipids, the T1 region remains transparent at temperatures as high as 90 °C. On the other hand, R1 and T2 regions exhibit drastic phase separation in the 30-40 °C temperature range. Thus hexokinase exhibits thermostability at low water contents and when entrapped in temperature stable systems.

Structure of the Organic Solvent System with 0.2 % Water with and without Hexokinase.

In order to ascertain the reasons for the high thermostability of hexokinase in the phospholipid system with low water contents, and the reduced stability of the enzyme in the same region of the systems without phospholipids, it was necessary to determine the characteristics of the structure that harbors the enzyme at water concentrations of less than 0.8 % . As shown in the preceding manuscript, no structures could be detected by DLS in solutions of phospholipids and Triton X-100 in either toluene or propylbenzene. Thus it became of importance to ascertain the structures (if any) that formed when hexokinase was introduced into the system.

Analysis by dynamic light scattering of an aqueous solution of hexokinase revealed bodies of two different radii (Fig. 4). First channels sensed a population with an average hydrodynamic radius of 58.5 ±4 Å. Most likely this population represents the native hexokinase as the unit cell dimensions of the dimeric enzyme as determined by X-ray diffraction are 144.2 x 87.4 x 99.4 Å[12]. From the last channels, another population with an approximate average radius of 1000 Å was also detected. This probably corresponds to protein aggregates. When the same amount of hexokinase was introduced in the first transparent region of the toluene-Triton X

- 100 system without phospholipids (a condition in which the enzyme is not stable), only large size aggregates were observed (not shown). In contrast, two well-defined populations of particles were apparent when hexokinase was transferred to systems that contained phospholipids (Fig. 5). Double-exponential analysis of this data showed two populations; one corresponded to aggregates of a size similar to that observed in the absence of phospholipids, and another with a radii of 83.1 ±5.8 Å. The latter equals the dimensions of the protein determined in water plus a monolayer of surfactant molecules (25 Å) surrounding the enzyme.

It is noted that due to experimental limitations, DLS data were obtained at a protein concentration that was 1000 times higher than in the thermostability experiments. As protein aggregation is a concentration dependent process, most likely in the thermostability experiments aggregation was lower.

It is noteworthy that the dimensions of hexokinase in the micelle and hexokinase in crystals[12] are nearly the same. This suggests that under conditions in which the enzyme is thermostable there would be about one or two monolayers of water molecules per enzyme.

DISCUSSION

Catalysis and Thermostability.

Two main features differentiate the systems under study from other types of reverse micelles. Whereas in AOT reverse micelles, activity increases with water content towards the value obtained in 100 % aqueous media[6], in the Triton X-100/phospholipid systems, even under optimal conditions, the activity of all the enzymes that

have been tested is always several fold lower than in all aqueous media[5,13]. In addition, in phospholipid-containing systems, a high enzyme thermostability is observed at low water concentrations (T1 region)[10,11].

It has been proposed that with respect to water there is an inverse relation between the rates of enzyme catalysis and thermostability[5]. At water concentrations in which catalysis is low, the enzyme displays high thermostability. In the toluene or propylbenzene phospholipid/Triton X-100 systems, this relation holds. Nevertheless it should be noted that the effect of phospholipids on activity depends also on the organic solvent and cosurfactants introduced. Recently, Peng and Luisi[21] reported similar kinetic parameters in Isooctane/hexanol/lecithin and 100 % aqueous systems for Trypsin and α-Chymotrypsin catalysis.

A particular feature of phospholipid hydration[15], as compared to surfactants that have also been used to form reverse micelles (AOT[16] or CTAB), is that in phospholipids, up to 9-11 molecules of water form the first hydration shell; an additional 10-12 water molecules are required for formation of a second hydration shell[17,18]. NMR analysis of water protons in phospholipid, Triton X-100, toluene and water systems showed that water exists mainly as "bound" water (Shoshani L. et al ., unpublished results), particularly at concentrations in which enzymes exhibit low rates of catalysis and high thermostability. Therefore it is very likely that the bound character of water in the hydration shell of phospholipids[15] is related to the distinct properties that enzymes acquire in micelles of the phospholipid type.

Hexokinase entrapment in the T2 region.

Another relevant feature in the behavior of enzymes in reverse micelles concerns the dimensions of the host-micelle. From the present data it is clear that the mean value of the inner water core of T2 region reverse micelles formed in toluene or propylbenzene (28 and 8 Å respectively) is smaller than that of hexokinase (58.5 Å Fig. 4), yet this enzyme, as well as larger ones [11] can be readily transferred to the system. Therefore, in the T2 region, the internal core of hexokinase-containing reverse micelles should adjust to the size of the protein. The determination of the size of protein-filled and unfilled micelles is difficult to asses due to the observed aggregation of hexokinase in aqueous solutions (Fig. 4)

A similar increase in micellar radii upon protein uptake has been experimentaly determined in the AOT/Isooctane/water system[19,20]

It is noted that, even in the best charaterized reverse micelle system (AOT/isooctane/water) the complex effect of micellar size upon protein uptake is the current focus of theoretical and experimental efforts[22,23].

Hexokinase-Induced Reverse Micelle nucleation in the T1 region.

At water concentrations below 0.8 % (v/v), we failed to detect micelles. However when hexokinase was introduced, the system was stable and bodies with dimensions equivalent to those of the enzyme plus a monolayer of surfactant molecules were clearly apparent. In consequence it would appear that at low water levels (T1 region) solubilization of hexokinase takes place via aggregation of

monomeric surfactant molecules. These findings may not be surprising, since hydration of the polar groups in the surface of the protein should lead to orientation of the polar groups of the surfactant, yielding a thermodinamically favorable system. Nevertheless, it is stressed that in the phospholipid-containing systems, native dimeric hexokinase was encapsulated, whereas in their abscence, only inactive protein aggregates were incorporated.

Finally, it is noteworthy that the dimensions of hexokinase incorporated in reverse micelles that contain phospholipids and a low water content, and those of crystalline hexokinase are strikingly similar[12]. This sugests that upon formation of its own micelle, the enzyme acquires a crystalline-like state that hinders protein movements necessary to manifest the main features of the life cycle of an enzyme: catalysis and denaturation[24].

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FIGURE LEGENDS

Figure 1.- Activity of Hexokinase in Toluene-Triton X-100 Systems as a Function of Water Concentration. Activity of hexokinase (250 μmol $min^{-1}mg^{-1}$ in 100 % water) was assayed in the absence (A) or presence (B) of 10 mg phospholipids/mL toluene. Triton X-100 concentration (v/v) in (A) was 19 (o-o) and 22.7 % (●-●). or 15 (o-o) and 22.7 % (●-●) in (B). T2 region of transparency in the absence of protein (see accompanying manuscript) are shown in the lower part of the plot.

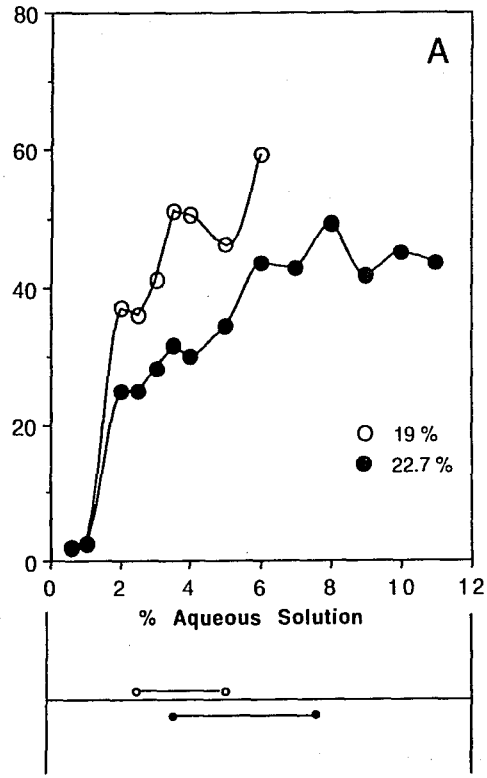
Figure 2.- Activity of Hexokinase in Propylbenzene Systems as a Function of Water Concentration. Triton X-100 concentration was 19 % (o-o) or 23 % (●-●) in propylbenzene (v/v). Hexokinase catalysis was assayed in the absence (A) or presence (B) of 10 mg phospholipids/mL toluene. T2 region of transparency in the absence of protein (see accompanying manuscript) are shown in the lower part of the plot.

Figure 3.- Thermostability of Hexokinase in Organic Solvent-Triton X-100 Systems that contained Phospholipid. Hexokinase was transferred to the organic solvent system formed in toluene (A) and propylbenzene (B) and incubated for 10 min at 60°C. The system contained 15 % (●-●), 19 % (o-o) or 23 % (■-■) Triton X-100 (v/v) and the indicated water contents. No thermostability (i.e. less than 15 % residual activity) was observed in the absence of phospholipids. In 100 % water, hexokinase was completely inactivated by a 10 min incubation at 50°C.

Figure 4.- Dynamic Light Scattering Analysis of Hexokinase in a Water Solution of 20 mM Tris HCl pH 7.4. The concentration of hexokinase was 1.5 mg mL^{-1}). The inset shows the average hydrodynamic radii calculated for each population by double exponential analysis.

Figure 5.- Dynamic Light Scattering Analysis of Hexokinase in the T1 region of 19 % (v/v) Triton X-100 in Toluene that contained 10 mg Phospholipis per mL and 0.2 % Water. The data were fitted by a cumulant expansion of the correlation function. The concentration of hexokinase was 0.6 mg mL^{-1} organic system. The inset shows the two populations sensed by a double exponential fit.

gure 1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40
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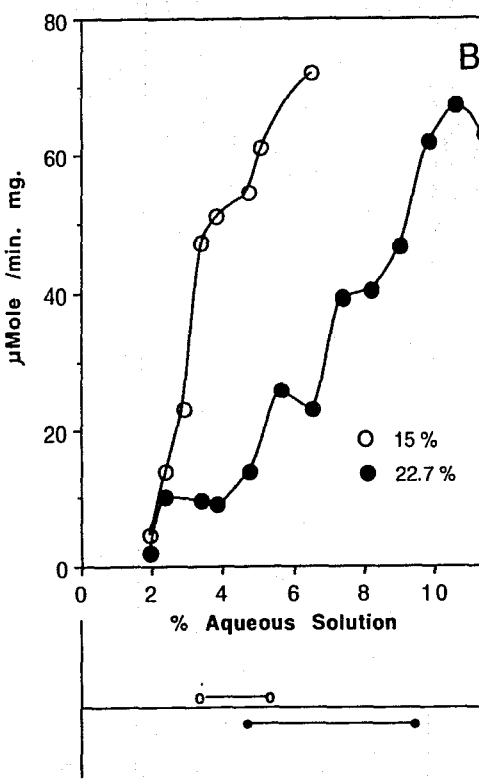
0
2
4
6
8
10
12
% Aqueous Solution
19 %
22.7 %


B
80
60
40
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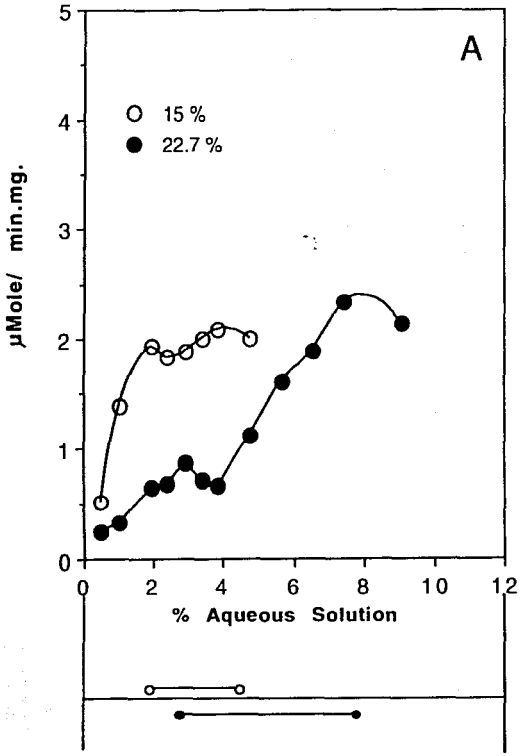
0
µMole /min. mg.
0
2
4
6
8
10
% Aqueous Solution
15 %
22.7 %

Figure 2


A
15 %
22.7 %
µMole/ min.mg.
% Aqueous Solution
0
1
2
3
4
5
0
2
4
6
8
10
12

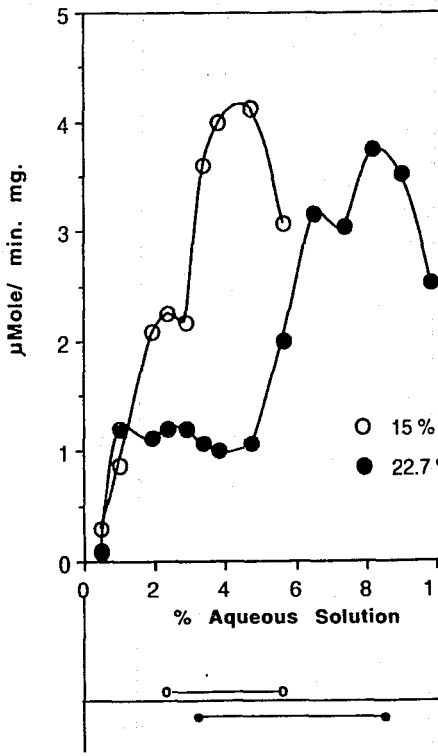
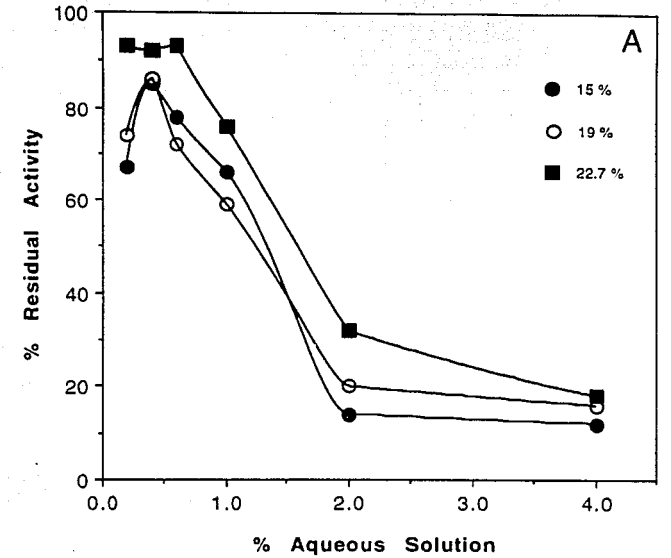

15 %
µMole/ min. mg.
% Aqueous Solution
0
1
2
3
4
5
0
2
4
6
8

Figure 3


A
15 %
19 %
22.7 %
% Residual Activity
% Aqueous Solution
0
20
40
60
80
100
0.0
1.0
2.0
3.0
4.0

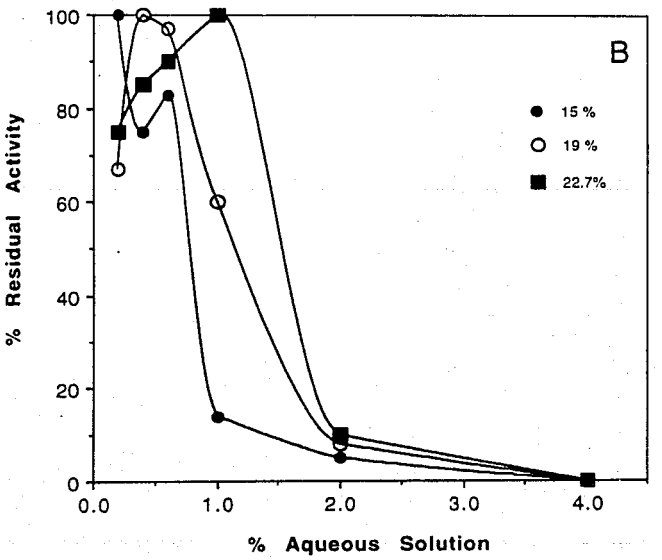
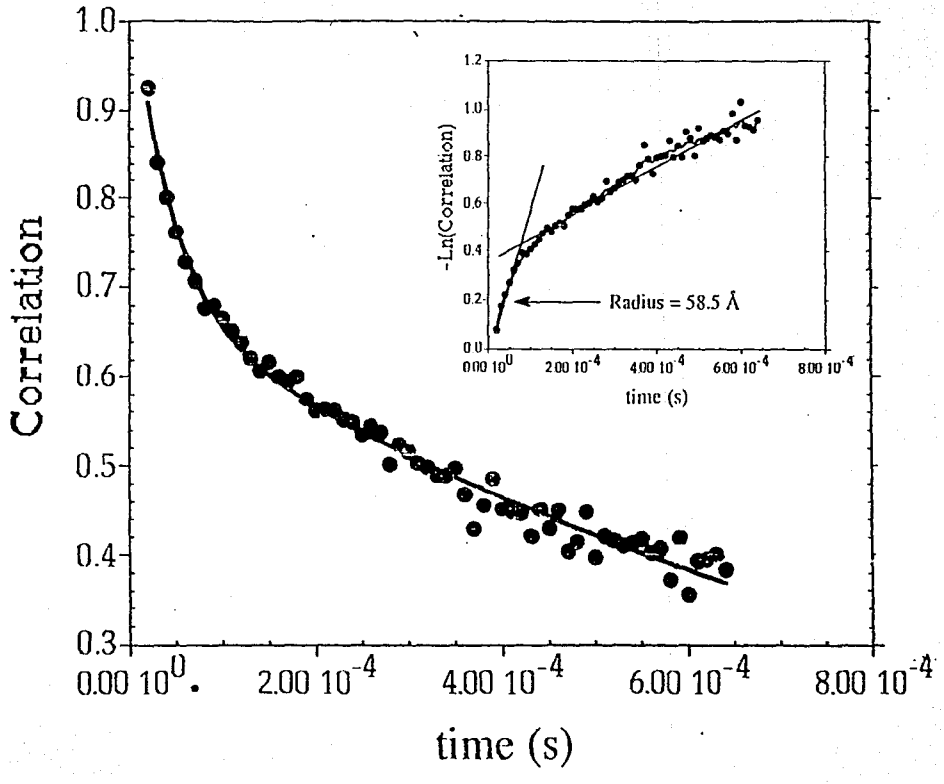

B
15 %
19 %
22.7%
% Residual Activity
% Aqueous Solution
0
20
40
60
80
100
0.0
1.0
2.0
3.0
4.0

Figure 4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0.7
0.6
0.5
0.4
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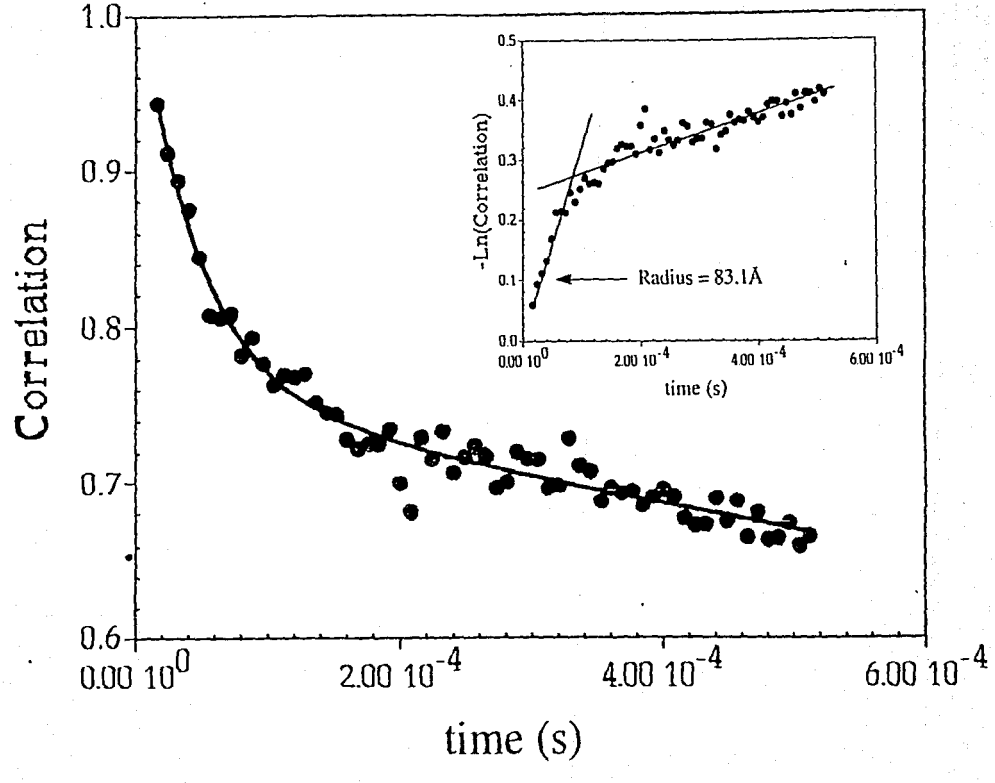
6.00 10^-4
8.00 10^-4
time (s)
-Ln(Correlation)
Radius = 58.5 Å
time (s)

Figure 5


Correlation
1.0
0.9
0.8
0.7
0.6
0.00 10^0
2.00 10^-4
4.00 10^-4
6.00 10^-4
time (s)
-Ln(Correlation)
0.5
0.4
0.3
0.2
0.1
0.0
Radius = 83.1Å
0.00 10^0
2.00 10^-4
4.00 10^-4
6.00 10^-4
time (s)

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Activity of heart and muscle lactate dehydrogenases in all-aqueous systems and in organic solvents with low amounts of water

Effect of guanidine chloride

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The effect of urea and guanidine hydrochloride (GdmCl) on the activity of lactate dehydrogenases from heart and muscle was studied in standard water mixtures and in reverse micelles formed with *n*-octane, hexanol, cetyltrimethylammonium bromide and water in a concentration that ranged over 2.5 – 6.0% (by vol.). In all water mixtures GdmCl (0.15 – 0.75 M) and urea (0.5 – 3.0 M) inhibited the activity of the enzymes at non-saturating pyruvate concentrations. At concentrations of pyruvate that proved inhibitory for enzyme activity due to the formation of a ternary enzyme-NAD-pyruvate complex, GdmCl and urea increased the activity of the enzymes. This increase correlated with a decrease of the ternary complex, as evidenced by its absorbance at 320 – 325 nm. In the low-water system it was found that: (a) at all concentrations of pyruvate tested (0.74 – 30 mM), GdmCl enhanced the activity of the heart enzyme to a similar extent; (b) in the muscle enzyme, GdmCl inhibited or increased the activity through a process that depended on the concentration of pyruvate and GdmCl; (c) under optimal conditions, the activation by GdmCl was about two times lower in the muscle than in the heart enzyme, although in all-water media the activity of the muscle enzyme was twice as high. The expression of lactate dehydrogenase activity in the low-water system was higher with the heart than with the muscle enzyme compared to their activities in all-water media (about 260 and 600 μmol min^{-1} mg^{-1} in the heart and muscle enzymes respectively). Apparently for catalysis, the water requirement in the heart enzyme is lower than in the muscle enzyme. It is likely that the different response of the two enzymes to solvent is due to their distinct structural features.

For a long time it has been known that guanidine salts and urea disrupt the three-dimensional structure of proteins [1]. However, there are reports that indicate that the activity of an important number of enzymes is diminished [2 – 5] or enhanced [6 – 12] by denaturants at concentrations that do not produce gross changes of protein structure. The mechanisms that account for these effects are not known with certainty, but it has been suggested that they take place through modifications of the catalytic site, or to an increase in the flexibility of the enzyme. In the preceding paper [13], it was shown that, in reverse micelle systems with a low water content, the activity of four out of five enzymes tested could be increased severalfold by denaturants. Here the activities of heart and muscle lactate dehydrogenases were studied in all-aqueous media and in a low-water system with and without denaturants. The studies were an attempt to ascertain if indeed, and how, the structural differences of the isoenzymes affect their functional response to solvent modifications. The experiments were made in all-water mixtures and in reverse micelles formed with cetyltrimethylammonium bromide, hexanol, and *n*-octane [14, 15].

Lactate dehydrogenase has the advantage that it is one of the most thoroughly studied enzymes. There are extensive studies of its kinetics [16 – 21], crystallographic data of the enzyme with and without substrates [16, 22 – 25], and information of the amino acid residues that affect the specificity and properties of the catalytic site [18 – 21, 26]. Also there is considerable knowledge of the structure of various isoenzymes [24, 27 – 31] and how this relates to their catalytic properties. Of relevance to this work is that the activity of lactate dehydrogenase is inhibited by a ternary complex of enzyme, NAD, and pyruvate. Direct evidence for this complex has been observed in lactate dehydrogenases from bovine heart [32], rabbit muscle [33 – 35], rat liver [36], chicken heart [32, 37], dogfish muscle [32], and pig heart [38].

It was found that in all-aqueous media, denaturants decrease the affinity of the enzyme for pyruvate and impair the formation of the abortive enzyme-pyruvate-NAD complex. In the low-water system, another action of water and denaturant

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Abbreviation. GdmCl, guanidine hydrochloride.

Enzyme. Lactate dehydrogenase (EC 1.1.1.27).

solutions became apparent when the activities of the two enzymes were compared.

MATERIALS AND METHODS

Lactate dehydrogenases from bovine heart, hog heart, and hog muscle were obtained from Boehringer. For all experiments the enzymes were dialyzed against 0.1 M potassium phosphate pH 7.4, 10 mM EDTA, and 1 mM dithiothreitol for 72 h in the cold. The buffer was changed three times. The activities of the heart and muscle enzyme at optimal substrate concentrations in several experiments were around 260 and 600 μmol min^{-1} mg^{-1} on average.

Enzyme activities

In most of the experiments in all-water mixtures, activity was followed by recording the changes in absorbance at 340 nm of a 1-ml mixture at 24°C. The components of the mixtures are detailed in Results. Measurements of activity at various temperatures were carried out in the reaction mixtures indicated; the reaction was stopped with 1 M KOH. Thereafter the remaining NADH was determined as described in the preceding paper [13].

The low-water system [13–15] employed was formed with cetyltrimethylammonium bromide, hexanol, *n*-octane, and water (3.0–6.0%, by vol.). The methodology employed was described in the preceding paper [13]. Reversed micelles were prepared by mixing 0.160 g cetyltrimethylammonium bromide, 1.98 ml octane and 0.22 ml hexanol with 0.05 ml phosphate buffer in water until a clear system was obtained. For the assay of enzyme activities, a water solution with the substrates and denaturants was transferred to the organic solvent system. Unless otherwise stated, the reaction was started by the subsequent addition of the enzyme, followed by vigorous stirring for 10 s. Thereafter recording of absorbance at 340 nm was started. The concentrations of the components are expressed in relation to the amount of water introduced. The effect of GdmCl on some of the characteristics of the system were described in the preceding paper [13] where it was shown that, at the concentrations used, there were only minor changes in the characteristics of the system.

Recovery of lactate dehydrogenase from the low-water system

Muscle lactate dehydrogenase could be recovered from the organic solvent by the following procedure. The enzyme was transferred to the cetyltrimethylammonium bromide/hexanol/*n*-octane as described above. A 50-μl aliquot of the mixture was layered over 1.0 ml 40 mM phosphate pH 7.4, 10 mM EDTA and 1 mM dithiothreitol in the centrifuge tubes of a 50 Ti rotor. The tubes were immediately centrifuged for 60 min at 40000 rpm. At the end of the run, the tubes were removed from the rotor and the upper layer discarded. Mixing of the lower aqueous phase and small upper layer was prevented by careful handling of the tubes. The water phase was used for assay of lactate dehydrogenase activity.

Determination of the enzyme-NAD-pyruvate complex

In all-aqueous media, formation of the ternary enzyme-NAD-pyruvate complex was followed by recording spectra over 300–400 nm in an SLM Aminco DW 2000 in a 1-ml cuvette thermostatted at 24°C. Spectra were obtained according to the protocols described by From [33] in 0.8 ml that contained 100 mM potassium phosphate pH 7.4, 10 mM EDTA, 1 mM dithiothreitol, 2 mM NAD^+, 30 mM pyruvate and the heart (0.574 mg) or muscle (0.477 mg) enzyme. Other details are described below. Attempts were made to measure formation of the complex in the low-water system; however this was not possible, since the highest amount of protein that could be transferred into the low-water system was about 50 μg/ml total mixture. This was an amount that proved too low to obtain reliable spectra.

GdmCl and freshly prepared urea solutions were adjusted to pH 7.4. Protein of the heart and muscle enzyme was determined by their absorbance at 280 nm, $A^{0.1\%}_{1\,cm}$ = 1.5 and 1.45, respectively [39, 40].

RESULTS

Effect of GdmCl and urea on lactate dehydrogenase activity in standard water mixtures

The effect of GdmCl and urea on the activity of heart (Fig. 1A) and muscle (Fig. 1B) lactate dehydrogenases was studied at various concentrations of pyruvate. At non-saturating pyruvate concentrations, the two compounds inhibited the activity; a Lineweaver-Burk plot showed that the inhibition was apparently of the competitive type (insets). In agreement with previous data [32–38, 41, 42], it was found that concentrations of pyruvate higher than those required to achieve maximal rates were inhibitory in both the heart and muscle enzymes; the inhibiting effect of pyruvate was more marked with the former (Fig. 1A and B). At inhibitory concentrations of pyruvate, GdmCl and urea increased the activity (Fig. 1A and B). At the highest pyruvate concentration assayed, the activities reached with the denaturants were lower than those obtained with optimal pyruvate concentrations. However, there was a pyruvate concentration at which GdmCl increased the activity of the enzymes above that of the control at optimal pyruvate concentrations.

The relation between the effect of GdmCl and urea on the K_m for pyruvate, and their effect on the rate of the reaction at inhibiting pyruvate concentrations with the heart and muscle enzymes, is shown in Table 1. GdmCl and urea at increasingly higher concentrations (0.15–0.75 M and 0.5–3 M, respectively) produced a progressive increase in the K_m for pyruvate; significant diminutions of the V_{max} values were observed in the muscle enzyme only with the highest concentrations of GdnHCl and urea studied. Table 1 also shows that, with an inhibiting concentration of pyruvate (30 mM), the aforementioned concentrations of denaturants brought about a progressive increase in the rate of the reaction. Hence there seems to be close relationship between the affinity of the enzyme for pyruvate and the enhancement of catalytic rates at inhibiting substrate concentrations. In the light of the activities of the isoenzymes observed in low-water systems (see below), it is of relevance that in all conditions the activity of the muscle enzyme was higher than that of heart.

It has been reported that the inhibitory effect of high pyruvate concentrations is due to the formation of an abortive enzyme-NAD-pyruvate ternary complex [32–34, 41, 42] and that the formation of this complex is favored at low temperatures [41, 42]. Thus the effect of different concentrations of GdmCl and urea on the activity of the heart enzyme was tested at various temperatures with a pyruvate concentration which at 24°C was non-inhibitory. GdmCl at 35°C, and urea at 35°C and 24°C did not modify the activity, but at lower

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The effect of GdmCl and urea on the activity of heart (Fig. 1 A) and muscle (Fig. 1 B) lactate dehydrogenases was studied at various concentrations of pyruvate. At non-saturating pyruvate concentrations, the two compounds inhibited the activity; a Lineweaver-Burk plot showed that the inhibition was apparently of the competitive type (insets). In agreement with previous data [32–38, 41, 42], it was found that concentrations of pyruvate higher than those required to achieve maximal rates were inhibitory in both the heart and muscle enzymes; the inhibiting effect of pyruvate was more marked with the former (Fig. 1 A and B). At inhibitory concentrations of pyruvate, GdmCl and urea increased the activity (Fig. 1 A and B). At the highest pyruvate concentration assayed, the activities reached with the denaturants were lower than those obtained with optimal pyruvate concentrations. However, there was a pyruvate concentration at which GdmCl increased the activity of the enzymes above that of the control at optimal pyruvate concentrations.

The relation between the effect of GdmCl and urea on the K_m for pyruvate, and their effect on the rate of the reaction at inhibiting pyruvate concentrations with the heart and muscle enzymes, is shown in Table 1. GdmCl and urea at increasingly higher concentrations (0.15–0.75 M and 0.5–3 M, respectively) produced a progressive increase in the K_m for pyruvate; significant diminutions of the V_{max} values were observed in the muscle enzyme only with the highest concentrations of GdnHCl and urea studied. Table 1 also shows that, with an inhibiting concentration of pyruvate (30 mM), the aforementioned concentrations of denaturants brought about a progressive increase in the rate of the reaction. Hence there seems to be close relationship between the affinity of the enzyme for pyruvate and the enhancement of catalytic rates at inhibiting substrate concentrations. In the light of the activities of the isoenzymes observed in low-water systems (see below), it is of relevance that in all conditions the activity of the muscle enzyme was higher than that of heart.

It has been reported that the inhibitory effect of high pyruvate concentrations is due to the formation of an abortive enzyme-NAD-pyruvate ternary complex [32–34, 41, 42] and that the formation of this complex is favored at low temperatures [41, 42]. Thus the effect of different concentrations of GdmCl and urea on the activity of the heart enzyme was tested at various temperatures with a pyruvate concentration which at 24°C was non-inhibitory. GdmCl at 35°C, and urea at 35°C and 24°C did not modify the activity, but at lower

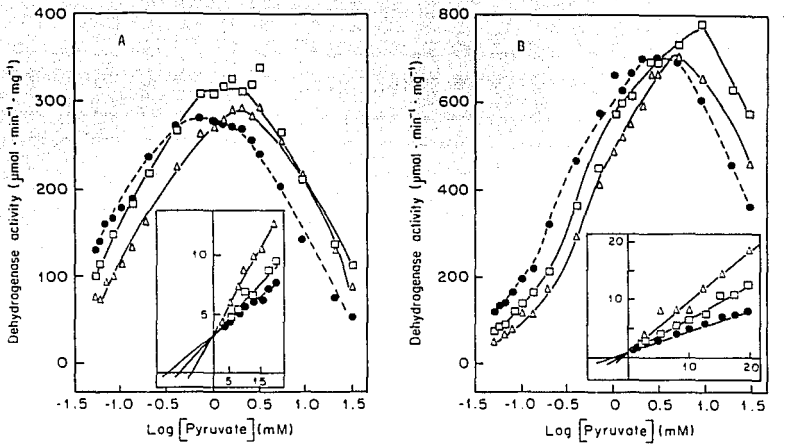

Fig. 1. Effect of GdmCl and urea on the activities of heart (A) and muscle (B) lactate dehydrogenases at various pyruvate concentrations. The incubation mixture contained 20 mM phosphate pH 7.4, 0.2 mM NADH and the indicated concentrations of pyruvate, (●——●) without and (▲——▲) with 0.25 M GdmCl or (△——△) with 1.25 M urea. The reaction was started with the enzyme (0.04 μg). Final volume 1.0 ml, temperature 24° C. The insets show the Lineweaver-Burk plots with non-saturating pyruvate concentrations.

Table 1. Effect of GdmCl and urea on the K_m for pyruvate and their effect on activity at high pyruvate concentrations in all-water media. The experimental conditions and the determination of K_m were as in Fig. 1 from Lineweaver-Burk plots. The values shown are the average of at least three experiments.

Addition	Concn	Heart enzyme			Muscle enzyme		
		K_m	V_{max}	activity with 30 mM pyruvate	K_m	V_{max}	activity with 30 mM pyruvate
	M	μM	μmol min^{-1} mg^{-1}		μM	μmol min^{-1} mg^{-1}	
	–	66	305	58	275	753	259
GdmCl	0.15	80	355	80	290	715	361
	0.25	130	380	115	420	768	466
	0.5	148	328	158	750	640	499
	0.75	500	312	196	1400	489	459
Urea	0.5	60	322	69	250	715	316
	1.25	155	328	100	520	742	336
	2.0	240	305	126	570	638	347
	3.0	560	230	135	2500	476	366

temperatures there was activation by both GdmCl and urea (Fig. 2); maximal activation was attained with 0.2–0.25 M GdmCl and 1.0–1.5 M urea. The activity progressively decreased above a given denaturant concentration but note that higher concentrations were required for half-maximal inactivation at the lower temperatures. As urea increased the activity of lactate dehydrogenase, it may be inferred that the increase in activity by GdmCl was not solely due to a salt effect.

The ternary enzyme-NAD-pyruvate complex has an absorption band in the region of 320–330 nm with a peak at 322 nm or 325 nm [33, 35, 37, 38, 43]. As shown in Fig. 3 (A and C), the addition of pyruvate to heart and muscle lactate dehydrogenases incubated with NAD induced within 15 s the formation of an absorbance band with a maximum at 319 nm. This was followed by a slower increase in absorbance that reached a constant value after about 4 min. The same results were obtained by adding NAD to enzymes incubated with pyruvate (data not shown). After 10 min, 0.25 M GdmCl was added; it produced a rapid decrease in absorbance (Fig. 3A and C) which did not change after 10 min of incubation. It is noted that with both enzymes, the absorption band was not

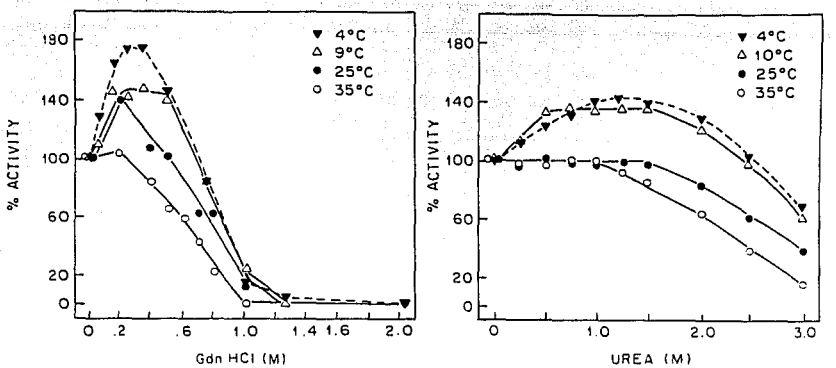

Fig. 2. Effect of GdmCl and urea on the activity of heart lactate dehydrogenase at various temperatures. The incubating conditions were as in Fig. 1, except that the mixtures contained 0.74 mM pyruvate and the indicated GdmCl and urea concentrations. The temperature was as indicated. 100% refers to the activity in the absence of denaturant, i.e. 412, 211, 44, and 26 μmol min^{-1} mg^{-1} at (——) 35 °C, (●——●) 25 °C, (△——△) 9 °C or 10 °C, and (▼- - -▼) 4 °C, respectively.

completely abolished by the addition of GdmCl: in four different experiments, a markedly constant decrease of about 30% was observed. The data of Fig. 3B and D show that the addition of GdmCl to the inhibited enzymes produced an almost immediate increase in activity; however, the activity was not increased to a maximal value. That is, GdmCl brought about only a partial reversal of the inhibition and a partial diminution of the ternary enzyme-NAD-pyruvate complex.

Activities of heart and muscle lactate dehydrogenases in low-water media and the effect of GdmCl

Similarly to many other enzymes [44–53], heart lactate dehydrogenase placed in a low-water environment exhibits low catalytic rates but, as shown in previous work [13, 53], in such conditions, the activity of the heart enzyme is markedly increased by urea and GdmCl. In order to explore the mechanism involved, the activities of heart and muscle lactate dehydrogenases were studied in the low-water system formed with cetyltrimethylammonium bromide, hexanol and *n*-octane [13–15]. In the preceding paper [13], it was shown that the characteristics of the system were not significantly modified by GdmCl. Moreover it is noted that heart and muscle lactate dehydrogenase were studied under identical conditions; hence any differences in activities should be due to the intrinsic kinetic and structural features of the enzymes.

In the organic solvent, the activity of heart and muscle lactate dehydrogenases increased as the amount of water was raised (Fig. 4). At the highest water concentration assayed, the activity of the heart enzyme was close to 70% of that detected in all-aqueous media, whereas that of muscle was around 20% (Fig. 4, inset). Thus it would appear that the water requirements for expression of activity were lower in the heart enzyme than in the muscle enzyme.

In a system that contained 3.0% water and pyruvate in a concentration range of 0.74–30 mM, 1.2 M GdmCl produced a severalfold increase in the activity of the heart enzyme (Fig. 5A). In the experimental conditions employed, the enzyme was rapidly saturated by substrate, hence the K_m, with or without GdmCl, could not be determined. Nevertheless the data indicate that GdmCl increased the activity in conditions in which substrate was not limiting.

The optimal concentration of GdmCl for activation was 1.25 M at low and high substrate concentrations (not shown). In addition, it is noted that when the experiment of Fig. 5A was repeated with the enzyme from hog heart, the results obtained (not shown) were quantitatively similar to those obtained with the enzyme from bovine heart.

In the enzyme from hog muscle the effect of GdmCl was markedly different (Fig. 5B). In the range of 0.74–30 mM pyruvate, 0.5 M GdmCl brought about activation, but the extent of activation diminished as the concentration of pyruvate was raised. On the other hand, 1.0 M GdmCl produced inhibition of the activity in the low pyruvate range, and activation at high pyruvate concentrations. With 30 mM pyruvate, the activity reached with the latter concentration of GdmCl was higher than with 0.5 M GdmCl.

In the low-water system the biphasic action of 1.0 M GdmCl in the muscle enzymes, at low and high pyruvate concentrations, was similar to that detected in all-water media (compare data of Table 1 and Fig. 5B). This suggests that in the low-water system GdmCl also affected the K_m of the enzyme for pyruvate. In fact the data of Fig. 5B show that in the presence of 1.0 M GdmCl, the activity increased with the concentration of pyruvate introduced (the K_m was around 4 mM); whereas in the absence of GdmCl, the activity was apparently saturated with 1 mM pyruvate.

It was also noted that with optimal GdmCl concentrations, the activity reached with the muscle enzyme was 2–2.5 times lower than with the heart enzyme (compare data of Fig. 5A and B), even though in all-water media the activity of the enzyme from muscle was twice as high as that from heart (Fig. 1 and Table 1).

It was considered possible that the lower activity of the muscle enzyme in the low-water system could be due to its

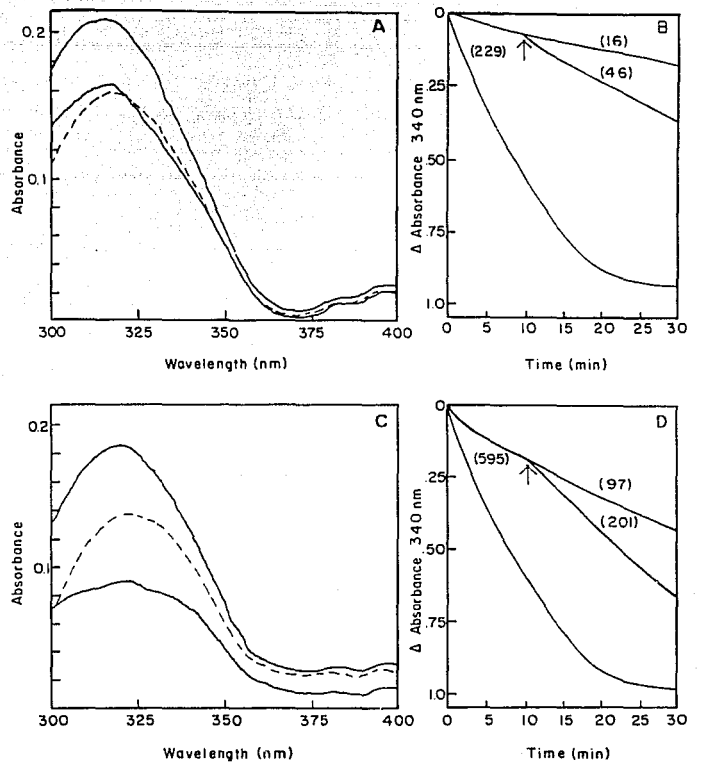

Fig. 3. Effect of GdmCl on the ternary enzyme-NAD-pyruvate complex and activity of heart and muscle lactate dehydrogenases. (A) Spectra obtained with heart enzyme; (C) spectra with the muscle enzyme. The incubation mixture was 100 mM phosphate pH 7.4, 10 mM EDTA and 1 mM dithiothreitol. 2 mM NAD and 0.57 mg heart of 0.48 mg muscle enzyme. Formation of the complex was started by the addition of 30 mM pyruvate. Final volume 0.8 ml. The solid curves were obtained by recording enzyme and NAD (reference) against enzyme, NAD and pyruvate minus water (reference) against pyruvate. This protocol followed that of From [33]. The lower solid curves are the spectra obtained 15 s after the addition of pyruvate, whereas the upper curves are the spectrum obtained 10 min after the addition of pyruvate. The dashed lines indicate the spectra obtained after the addition of 0.25 M GdmCl; these did not change in a 10-min interval. Almost identical spectra were obtained by starting the reaction with addition of NAD to enzyme plus pyruvate (not shown). (B) Activity of the heart enzyme with 0.74 mM pyruvate; (D) activity of the muscle enzyme with 3.0 mM pyruvate (the concentrations at which maximal activity was detected; see Fig. 1) and with 30 mM pyruvate. In the latter, the arrow indicates the addition of 0.25 M GdmCl. The numbers at the side of the traces indicate the rates of activity.

denaturation upon its transfer to the organic solvent system. This possibility was examined by transferring the enzyme to the organic solvent system under the usual conditions. After 60 min of incubation the enzyme was transferred back to water (see Methods). The recovery of enzyme activity was more than 90%. Unless the enzyme renatured upon its transfer to all-water media, the data indicate that the relatively low activity of the muscle enzyme in the low-water media was not due to denaturation during its transfer to the organic solvent system.

DISCUSSION

Effect of denaturants on the activity of lactate dehydrogenase in standard water systems

In conventional water systems, denaturants increased the activity of heart and muscle lactate dehydrogenases, but only at high pyruvate concentrations. The following observations indicate that this is due to a denaturant-induced decrease in the formation of the extensively documented enzyme-NAD-

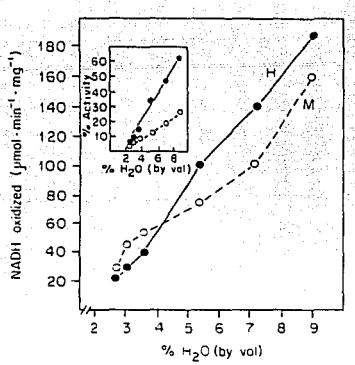

Fig. 4. Activity of heart and muscle lactate dehydrogenases in the cetyltrimethylammonium bromide/hexanol/octane system at various water concentrations. To the mixture of cetyltrimethylammonium bromide, *n*-octane, and hexanol, different volumes of water solutions were added so as to yield, in the water phase of the system, 100 mM phosphate pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 2.0 mM pyruvate, NADH at a constant amount (300 nmol ml total mixture) and the indicated water concentration (by vol.). The reaction was started by the addition of (●—●) 0.1 μg heart enzyme or (○—○) 0.06 μg muscle enzyme ml total mixture. After vigorous stirring, the mixture was transferred to a spectrophotometer cuvette and absorbance at 340 nm was recorded. The inset shows the data expressed as a percentage of the activity observed in all-water media in which 100% is the activity of the enzymes in all-water media. These were: 280 and 586 μmol min^{-1} mg^{-1} for heart and muscle, respectively.

pyruvate abortive complex [32–38, 41, 42]. (a) The enhancement of activity by denaturants took place only at inhibiting pyruvate concentrations. (b) Their favorable effect on catalysis was more evident in the low-temperature range. (c) The level of the enzyme-NAD-pyruvate complex that was reached upon incubation with NAD and pyruvate was diminished by GdmCl. Nevertheless, the present data also indicate that, at non-saturating pyruvate concentrations, denaturants inhibited in an apparently competitive form with pyruvate the activities of both the heart and the muscle enzymes.

The opposite effects of these denaturants on catalysis at high and low pyruvate concentrations may be rationalized as a common factor by assuming that the formation of the enzyme-NAD-pyruvate complex depends on the affinity of the enzyme for pyruvate. That is, a condition that lowers the affinity of the enzyme for pyruvate should result in both inhibition of activity at low pyruvate concentrations, and an increase in activity at pyruvate concentrations that lead to formation of the abortive complex. The inverse relation between the effect of denaturants on the K_m of the enzyme for pyruvate and their favorable effect on the activity at inhibiting pyruvate concentrations (Table 1) substantiates the possibility. Hence it is suggested that urea and GdmCl, at concentrations that do not produce denaturation, induce, directly or indirectly, a change in the active site of lactate dehydrogenase that results in a decrease in the affinity for pyruvate.

Activity of lactate dehydrogenases in the low-water system and the effect of denaturants

In the low-water system, in a wide range of pyruvate concentrations, GdmCl increased the activity of lactate dehydrogenase from bovine and hog hearts. Although the effect of GdmCl on the K_m of the enzyme could not be determined, the data indicate that GdmCl produced a severalfold increase in the velocity of the reaction under conditions in which substrate availability was not limiting. On the other hand, with the muscle enzyme, it was found that at saturating concentrations of pyruvate, activation by GdmCl was lower than in the heart enzyme, and that GdmCl produced an increase in the K_m for pyruvate. Therefore, in a limited water space, the heart and the muscle enzymes respond differently to equivalent concentrations of GdmCl. Moreover, differences were also found when the activity of the enzymes were measured at various water concentrations, i.e. considering the activities of the enzymes in all water media as 100%, the heart enzyme responded better to gradual increases in the amount of water (inset in Fig. 4).

Differences in the structure of heart and muscle lactate dehydrogenases

As the assays of the activities of the heart and the muscle lactate dehydrogenases were carried out under identical conditions, their different response to solvent should be due to their structural and kinetic differences. In fact, according to Eventoff et al. [24], the major structural differences between lactate dehydrogenases become apparent during catalysis. Although the heart and muscle enzymes have a high sequence similarity, there are differences in their substrate binding domains. At the catalytic site, the heart enzyme has a glutamine that may form an extra hydrogen bond with one of the phosphate residues of NAD. In the muscle enzyme this residue is substituted by an alanine [24]. In consequence, it was suggested that this amino acid substitution could account for the ability of the heart enzyme for form the ternary enzyme-NAD-pyruvate complex more readily [24]. In addition, there are two regions in which there are significant differences in amino acid composition [24, 29], i.e. the amino-terminal arm and the region that comprises residues 294(293) to 310(308). It has been proposed that these two regions build up the principal contacts between subunits [24].

The data in all-water media indicated that, in both enzymes, GdmCl diminished to nearly the same extent the level of the ternary enzyme-NAD-pyruvate complex and that, at similar levels of the complex, the activity of the muscle enzyme was higher than that of heart (see Figs 1 and 3). On these grounds, it is likely that, in the low-water system, the existence of the complex is not the factor that determines the higher activity of the heart enzyme at equivalent amounts of water or GdmCl solution. Hence, the possibility that subunit interactions are central to the expression of catalysis must be considered. Nevertheless, during catalysis, lactate dehydrogenases undergo extensive structural arrangements (see Fig. 22 in Holbrook et al. [14]) which indicates that, in a catalytic cycle, there are numerous points of interaction between the protein and solvent. With the data available, it is not possible to explain in molecular terms the different solvent sensitivity of the isoenzymes, but the data illustrate the importance of variations of protein structure in relation to protein–solvent interactions during catalysis.

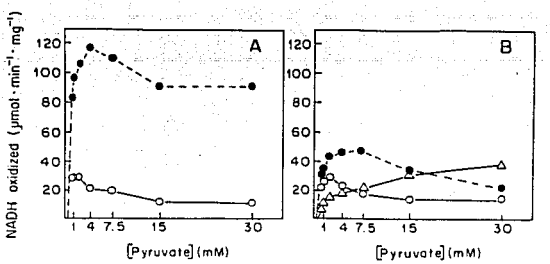

Fig. 5. Effect of GdmCl on the activities of heart (A) and muscle (B) lactate dehydrogenases in the cetyltrimethylammonium bromide/hexanol/octane system with 3.0% water and various pyruvate concentrations. The experiment was carried out as in Fig. 4, except that the system contained 3.0% (by vol.) water and the indicated concentrations of pyruvate. (A) The activity of heart lactate dehydrogenase was measured (○——○) without or (●——●) with 1.25 M GdmCl (final concentration in the water phase). (B) The activity of muscle lactate dehydrogenase was assayed (○——○) without or with (●——●) 0.5 M or (▽——▽) 1.0 M GdmCl.

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Enzyme activation by denaturants in organic solvent systems with a low water content

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The effect of urea and guanidine hydrochloride (GdmCl) on the activity of heart lactate dehydrogenase, glycerol-3-phosphate dehydrogenase, hexokinase, inorganic pyrophosphatase, and glyceraldehyde-3-phosphate dehydrogenase was studied in low-water systems. Most of the experiments were made in a system formed with toluene, phospholipids, Triton X-100, and water in a range that varied over 1.0–6.5% (by vol.) [Garza-Ramos, G., Darszon, A., Tuena de Gómez-Puyou, M. & Gómez-Puyou, A. (1990) *Biochemistry 29*, 751–757]. In such conditions at saturating substrate concentrations, the activity of the enzymes was more than 10 times lower than in all-water media. However the activity of the first four aforementioned enzymes was increased between 4 and 20 times by the denaturants. The most marked activating effect was found with lactate dehydrogenase; with 3.8% (by vol.) water maximal activation was observed with 1.5 M GdmCl (about 20-fold); 4 M urea activated, but to a lower extent. Activation by guanidine thiocyanate was lower than with GdmCl. The activating and inactivating effects of GdmCl on lactate dehydrogenase depended on the amount of water; as the amount of water was increased from 2.0% to 6.0% (by vol.), activation and inactivation took place with progressively lower GdmCl concentrations. When activity was measured as a function of the volume of 1.5 M GdmCl solution, a bell-shaped activation curve was observed. In a low-water system formed with *n*-octane, hexanol, cetyltrimethylammonium bromide and 3.0% water, a similar activation of lactate dehydrogenase by GdmCl and urea was observed. The water solubility diagrams were modified by GdmCl and urea, and this could reflect on enzyme activity. However, from a comparison of denaturant concentrations on the activity of the enzymes studied, it would seem that, independently of their effect on the characteristics of the low-water systems, denaturants bring about activation through their known mechanism of action on the protein. It is suggested that the effect of denaturants is due to the release of constraints in enzyme catalysis imposed by a low-water environment.

Experiments with enzymes in contact with low amounts of water have shown that catalysis takes place at low rates, and that these increase as the amount of water is increased [1–6]. The available data indicate that the conformational changes that are necessary for maximal catalysis require a given amount of water [1, 2, 4–6]. Thus, it seems that in addition to other factors, maximal catalytic rates also require optimal protein–solvent interactions. In this work it was asked if the low catalytic rates that enzymes exhibit in a low-water environment could be enhanced by modifications of the interactions between the protein and the surrounding media. The modifications were produced by agents that facilitate the exposure of protein groups into the solvent, i.e. guanidine salts and urea.

Although the mechanism by which these denaturants disrupt the native structure of proteins is not completely understood [7–11], their general effect is to increase the exposure of protein groups to the solvent, either by increasing the solubilization of amino acid residues [12–14], by binding to particular groups or structures [15, 16], or by changing the structure of water [17]. With respect to the effect of denaturants in enzyme catalysis, in the majority of the enzymes tested, urea and guanidine salts produce loss of activity at concentrations which bring about alterations of protein structure. Nevertheless, with some enzymes it has been found that at

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Abbreviations. GdmCl, guanidine hydrochloride; GdmSCN, guanidine thiocyanate; TPT, a mixture of 15% (by vol.) Triton X-100 in toluene and 8.5 mg soybean phospholipid/ml; Triton X-100, octylphenoxypoly(ethoxyethanol).

Enzymes. Lactate dehydrogenase (EC 1.1.1.27); glycerol-3-phosphate dehydrogenase (EC 1.1.1.8); hexokinase (EC 2.7.1.1); inorganic pyrophosphatase (EC 3.6.1.1); glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12).

concentrations lower than those that produce extensive changes of proteins, denaturants increase catalytic activity, usually by less than twofold [18–21] (but see [22, 23]). These results have been explained either by a denaturant-induced alteration of the catalytic site or through an effect of the denaturant on the flexibility of the enzyme.

Here it is described that in a low-water system, the activity of four enzymes from mammalian tissues or yeast cells, i.e lactate dehydrogenase, glycerol-3-phosphate dehydrogenase, hexokinase and soluble inorganic pyrophosphatase, was increased from 4 to 20 times by either guanidine salts or urea. With glyceraldehyde-3-phosphate dehydrogenase no activation was observed. The most marked activating effect of the denaturants took place with lactate dehydrogenase; therefore, this enzyme (for review and kinetics see [24–26]) was used to characterize the effect of the denaturants in low-water systems.

For the studies in low-water systems, enzymes were entrapped in the interior of reverse micelles [3, 27]. The latter were formed by introducing low amounts of water (1–6.5%, by vol.) into a mixture of detergents, amphiphilic molecules and an apolar organic solvent. Two variations of this type of system were used, i.e. one formed with toluene, phospholipids, and Triton X-100 [4], and another that was made up with *n*-octane, hexanol and cetyltrimethylammonium bromide [28–30]. The effect of denaturants on the characteristics of these system were determined. A brief report of the general effect of guanidine hydrochloride (GdmCl) on lactate dehydrogenase activity in the former low-water system has been published [31].

MATERIALS AND METHODS

Chemicals

The following were obtained from the indicated sources: lactate dehydrogenase (LDH-H4, bovine heart) from Boehringer; glycerol-3-phosphate dehydrogenase (type I rabbit muscle), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), hexokinase (type F-300, baker's yeast), inorganic pyrophosphatase (baker's yeast), Triton X-100, soybean phospholipids, and GdmCl from Sigma; guanidine thiocyanate (GdmSCN) from Boehringer; urea from Bio-Rad; cetyltrimethylammonium bromide salt from CalBiochem; toluene, *n*-octane and hexanol from Merck.

Experimental system

In most of the experiments with low amounts of water, the system referred to as TPT was used; it consisted of a mixture of toluene and Triton X-100 (85:15, by vol.) that contained 8.5 mg soybean phospholipids/ml. Some of the characteristics of the system have been described [4]. For the assay of the various enzymatic activities the following general procedure was used. To 1 ml TPT mixture, 2 µl enzyme solution was injected followed by vigorous stirring (0.5 min). The reaction was started by addition of an aqueous solution that contained the substrates in the indicated buffer with or without denaturants, followed by vigorous stirring. The volume of water introduced into the system was calculated to yield the desired percentage (by volume) of water. It was assumed that the components added, including the enzyme and the denaturants, distributed in the water introduced; thus all the concentrations of substrates and cofactors refer to the water phase of the system. The reaction was stopped with alkaline or acid water solutions as indicated below.

A low-water system formed with *n*-octane, hexanol, and cetyltrimethylammonium bromide [28–30] was also used for the measurement of lactate dehydrogenase activity. The typical protocol was as follows. To a mixture of 1.46 g cetyltrimethylammonium bromide and 2.0 ml hexanol, *n*-octane was added to a volume of 20 ml. This was followed by the addition of 317 µl 100 mM phosphate pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 4.2 mM pyruvate and 5.5 mM NADH; after this addition, the mixture was vigorously stirred until a clear system was attained. To 1 ml of the latter system 12.8 µl water with or without denaturants was added, followed by stirring. The reaction was started by the addition of 2.0 µl lactate dehydrogenase (0.15 mg/ml); after stirring, activity was measured by recording the disappearance of NADH at 340 nm. In this protocol the final water concentration was 3.0% (by vol.). In some experiments, denaturants were added after the enzyme and the substrates had been introduced into the system.

Lactate dehydrogenase

Prior to use the enzyme from bovine heart was dialyzed as described [31]. The $A^{0.1\%}_{1\,cm}$ value of 1.5 [32] at 280 nm was used for the calculation of protein concentration. In totally aqueous mixtures, the preparations used had a specific activity of 260 ± 30 µmol min^{-1} mg $protein^{-1}$ as assayed spectrophotometrically at 340 nm in a mixture that contained 100 mM phosphate pH 7.4, 0.74 mM pyruvate, and 0.2 mM NADH at a temperature of 24 °C. The activity of the enzyme in the TPT system was measured in mixtures that contained 20 mM phosphate pH 7.4, 30 mM pyruvate, 7.5 mM NADH and the indicated concentrations of the denaturant; generally 0.7 µg enzyme was introduced/ml TPT.

The reaction was stopped by mixing 0.4 ml of the TPT incubation mixture with 1 ml 1 M KOH. Subsequently, to achieve clear phase separation, 1 ml water-saturated isobutanol:benzene (1:1) was added. After stirring for 90 s, the mixture was centrifuged and the organic phase discarded. NADH was determined spectrophotometrically in the neutralized aqueous phase with lactate dehydrogenase and excess pyruvate.

Glycerol-3-phosphate dehydrogenase

Before use the ammonium sulfate suspension was centrifuged and the pellet dissolved in 40 mM triethanolamine pH 7.4 and 10 mM EDTA; afterwards the enzyme was desalted by the centrifuge column method [33]. The column was previously equilibrated with the same buffer. Protein was calculated from the absorbance at 280 nm, i.e. $A^{0.1\%}_{1\,cm} = 0.515$ [34]. In all water systems, the activity was assayed by recording the decrease in absorbance at 340 nm of a mixture that contained 270 mM triethanolamine pH 7.4, 9 mM EDTA, 0.9 mM dithiothreitol, 2.53 mM dihydroxyacetone phosphate, 0.13 mM NADH, and the desired concentrations of denaturants. The specific activity of the enzyme ranged over 80–89 µmol min^{-1} mg^{-1}. In the TPT system, this enzyme (1.8 µg/ml) was assayed in mixtures that contained (in the water phase) 40 mM triethanolamine pH 7.4, 10 mM EDTA, 1 mM dithiothreitol, 9 mM dihydroxyacetone phosphate and 7.5 mM NADH. At the desired times as in the lactate dehydrogenase assay, the reaction was stopped by mixing 0.4 ml TPT

mixture with 1 ml 1 M KOH and the remaining NADH was determined.

Glyceraldehyde-3-phosphate dehydrogenase

Before use the enzyme was passed through a centrifuge column that had been equilibrated with 20 mM pyrophosphate pH 8.5, 1 mM EDTA and 1 mM dithiothreitol. The assay in all water mixtures was as described elsewhere [35]. The reaction mixture contained 25 mM sodium pyrophosphate pH 8.5, 51 mM sodium arsenate, 0.24 mM NAD, 3.2 mM dithiothreitol, 1 mM glyceraldehyde 3-phosphate prepared from glyceraldehyde-3-phosphate diethyl acetal monobarium salt. Protein was calculated from the absorbance of the enzyme at 278 nm $A^{1\%}_{1\,cm} = 1.02$ [36]. In the TPT system, the reaction mixture contained (in the water phase) 13 mM pyrophosphate pH 8.5, 25 mM sodium arsenate, 7.5 mM NAD, 3 mM dithiothreitol, 8.5 mM glyceraldehyde 3-phosphate and approximately 7.5 µg enzyme ml TPT. The reaction was stopped as described for the lactate dehydrogenase assay and NADH determined accordingly.

Hexokinase

Solutions of the enzyme were prepared by solubilization of the lyophilized, ammonium-sulfate-free commercial preparation in 5 mM Tris HCl pH 7.4. The specific activity was around 250 µmol min^{-1} mg^{-1}. In all water media, the activity was assayed in mixtures that contained 40 mM Tris/HCl pH 7.4, 3 mM $MgCl_2$, 10 mM ATP and 25 mM glucose. The reaction was arrested with 0.5 M perchloric acid. The samples were neutralized with K_2CO_3. The samples were centrifuged to eliminate potasssium perchlorate. In the supernatant the amount of glucose 6-phosphate formed was determined in 1 ml of a mixture that contained 0.22 mM NADP, 0.1 mg bovine serum albumin, and approximately 1 unit glucose-6-phosphate dehydrogenase. In the TPT system, the reaction was assayed in a mixture that contained (in the water phase) 40 mM Tris HCl pH 7.4, 3 mM $MgCl_2$, 10 mM ATP, 25 mM glucose, the desired concentrations of the denaturants and 0.38 µg enzyme/ml TPT. The reaction was arrested by adding 0.4 ml of the TPT reaction mixture to 1 ml 0.5 M perchloric acid. This was followed by the addition of 1 ml isobutanol/benzene and, after vigorous stirring, the organic phase was eliminated (as in the experiments with lactate dehydrogenase). In the remaining water phase the amount of glucose 6-phosphate was determined as described above.

Pyrophosphatase

The ammonium-sulfate-free lyophilized powder was dissolved in 5 mM Tris/HCl pH 7.4 to a concentration of 0.6 mg/ml. In all water media, the activity was measured in 40 mM Tris/HCl pH 7.4, 3 mM $MgCl_2$, and 3 mM [γ-^{32}P]-pyrophosphate. The reaction was stopped with 6% trichloroacetic acid. The amount of [^{32}P]phosphate formed was determined as described before [4]. In the TPT system, the activity was measured in 40 mM Tris/HCl pH 7.4, 3 mM $MgCl_2$, 3 mM [^{32}P]pyrophosphate, and 0.2 µg enzyme/ml TPT. The reaction was stopped by mixing 0.4 ml of the TPT mixture with 1 ml 6% trichloroacetic acid. The amount of [^{32}P]phosphate formed was determined as described elsewhere [4].

General features of enzyme assays

It is pointed out that in the organic solvent systems, except for pyrophosphatase (in which a radioactive substrate was employed), the concentration of the substrates used was higher (in the water phase) than in all-aqueous media. The reason for this was that in a system that contained 15–60 µl water/ml total system, the net amount of substrate becomes critical. Thus in order to have measurable amounts of product formation or substrate disappearance for significant lengths of time, the concentration of the substrates had to be increased. It is noted that, except for inorganic pyrophosphatase, substrates were introduced at saturating concentrations; at twice or half the concentrations indicated, the activity did not show significant changes. Thus activity was measured at saturating substrate concentrations. The following paper [37] describes in more detail the activity and the effect of denaturants on heart and muscle lactate dehydrogenases at several substrate concentrations.

In all experiments, the rates of activity were calculated from plots of product formed or substrate that was consumed at various times of incubation. With all enzymes, except hexokinase, the rates were linear with time until about 50% of the substrate had been transformed. With hexokinase, the amount of glucose 6-phosphate formed in the first 5 min of incubation was the index for activity. In all experiments blanks were included; these contained all the components except the enzyme.

Many of the experiments were carried out with molar concentrations of urea and guanidine salts. At these concentrations, the amount of water in the system may be significantly affected; for instance, in a 10 M urea or 6 M GdmCl solution, about half the volume is accounted for by the denaturant [38]. Thus this factor had to be taken into account when enzyme activity was studied at different concentrations of denaturants, i.e. with 1.5 M GdmCl and 2.0 M urea the water content in the denaturant solution was approximately 12% lower.

Determination of the area in the phase diagram where reverse micelles were formed

This was determined as described by Hilhorst et al. [29] and Laane et al. [39] except that the aqueous phase contained 20 mM Tris/HCl pH 7.4, or 40 mM phosphate buffer. When the effect of denaturants on the phase diagram was determined, the water phase contained either buffer plus the indicated concentrations of GdmCl or urea.

Composition of the organic phase and the interphase

The effect of denaturants on the composition of the interphase and the continuous phase of the cetyltrimethylammonium bromide/hexanol/*n*-octane/water system was determined by the phase boundary method as described by Hilhorst et al. [29] and Laane et al. [39] in a system that contained 40 mM phosphate pH 7.4 with or without 1.25 M GdmCl. When *n*-octane was added to the transparent reverse micelle system, it became turbid due to solubilization of hexanol. The turbid suspension was titrated with hexanol until transparency was again achieved. The procedure was repeated five times. A plot of the amounts of *n*-octane vs hexanol yields a straight line; the intercept at the ordinate gives the amount of hexanol at the interphase and the water phase, whereas the slope gives the amount of hexanol in the continuous phase.

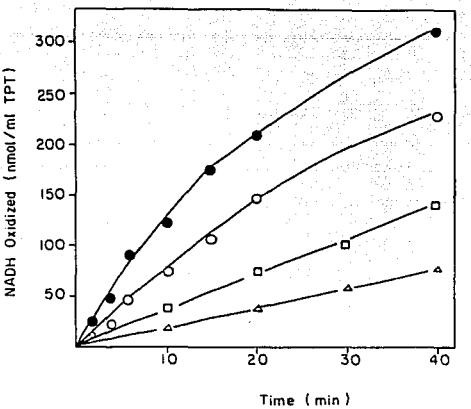

Fig. 1. Activity of lactate dehydrogenase in the TPT system at various enzyme concentrations. Different amounts of the enzyme in 2 μl buffer were transferred to 1 ml of the TPT system. Activity was started by adding a water solution that contained the substrates. In the final reaction system, the concentration of the components was 20 mM phosphate pH 7.4, 30 mM pyruvate, 7.5 mM NADH and 3.8% (by vol.) water. At different times, aliquots of the mixture were withdrawn to assay the amount of NADH remaining (see Materials and Methods). The following amounts of enzyme/ml TPT were used: (△——△) 0.384 μg; (□——□) 0.768 μg; (▽——▽) 1.4 μg; (●——●) 2.3 μg.

Partition of enzyme substrates between water and organic phases

To 1 ml of a water solution that contained 40 mM phosphate pH 7.4 and either 30 mM [^{14}C]pyruvate, 2.0 mM NADH, or 1 mM [^{3}H]ATP with or without 1.5 M GdmCl, 1 ml toluene or *n*-octane was added. The mixtures were vigorously stirred for 24 h at room temperature in closed test tubes. At that time the two phases were separated and the amount of pyruvate, NADH or ATP was quantitated in the water and organic phases.

RESULTS AND DISCUSSION

Enzymes at low levels of hydration [1, 2, 6], when placed in organic solvent that contains either surfactants [3, 27], or phospholipids with or without a detergent [4, 40, 41], or when suspended in organic solvents [5, 42], exhibit low catalytic rates that increase with the amount of water in the system. Once transferred to the TPT system with 3.8% (by vol.) water, the activity of lactate dehydrogenase was about 40 times lower than that observed in all-water media (260 μmol min^{-1} mg^{-1}). In the TPT system, lactate dehydrogenase activity was proportional to the amount of enzyme introduced (Fig. 1) and was almost linear with time until about 50% of the NADH introduced was exhausted.

Effect of urea, GdmCl and GdmSCN on lactate dehydrogenase activity in all-water media and in the TPT system

The effect of urea and guanidine salts on lactate dehydrogenase activity was assayed in standard aqueous mixtures and in the TPT system. In all-aqueous media, the activity increased as the concentration of GdmCl or GdmSCN were raised to 0.2 M or 0.075 M, respectively; at these concentrations the average activity was about 1.2 and 1.5 times higher than in the absence of guanidine (Fig. 2A). At higher concentrations the activity diminished and was abolished with 1.5 M GdmCl and 0.5 M GdmSCN. Up to 1.5 M, urea did not modify the activity, but at higher concentrations, the activity progressively diminished (Fig. 2A). The effect of 0.2 M GdmCl was reversible, i.e. lactate dehydrogenase incubated with 0.2 M GdmCl for 30 min, after a 100-fold dilution, exhibited an activity almost identical to that of an enzyme that had not been exposed to GdmCl (data not shown). The latter activity could again be increased by 0.2 M GdmCl. The mechanism of activation of lactate dehydrogenase by GdmCl and urea is discussed in the following paper [37].

In the TPT system with 3.8% (by vol.) water, the activity of lactate dehydrogenase was greatly increased by introducing urea, GdmCl or GdmSCN; however, there was an optimal denaturant concentration at which maximal activation was detected (Fig. 2B). At optimal concentrations of urea and GdmSCN (about 4.0 M and 3.0 M, respectively), the activities were about 4 and 9 times higher than in their absence. With GdmCl the increase in activity was more important: with 1.5 M to 2.0 M GdmCl, activation was nearly 20-fold [31] and reached a value that was around 40% of that detected in all-water media in the absence of GdmCl (compare data of Fig. 2A and B). It is noted that in the TPT system, KCl did not enhance the catalytic activity of lactate dehydrogenase (not shown); thus it would seem that the effect of GdmCl is not due to a salt effect.

Enzyme activity in relation to the characteristics of organic solvent systems

In ascertaining the mechanism by which denaturants produce an increase of enzyme activity in low-water systems, it was necessary to determine if these agents modify the characteristics of the system and, if such modifications exist, how they relate to the effect of denaturants on enzyme activity. With the same aim it was important to determine if denaturants also increased enzyme activity in a system different from TPT; the system chosen was that formed with cetyltrimethylammonium bromide, hexanol and *n*-octane [28–30]. Fig. 3 shows the solubility diagrams of water (20 mM Tris·HCl pH 7.4) and denaturant solutions in the two systems. The denaturant concentrations studied correspond to those at which near-maximal activation of lactate dehydrogenase was observed in the two systems (Fig. 2, and see below). It may be observed in the diagram of the TPT system that denaturants produced changes in the region of reverse micelles; in the cetyltrimethylammonium bromide system only the right boundary of the diagram was shifted.

Of particular interest in the TPT system are the phase changes that took place with 15% (by vol.) Triton X-100 as this was the concentration at which the experiments were carried out. Fig. 4 illustrates the region of reverse micelles (see bars) and activities with different amounts of water and a 1.5 M GdmCl solution. The activity increased with the

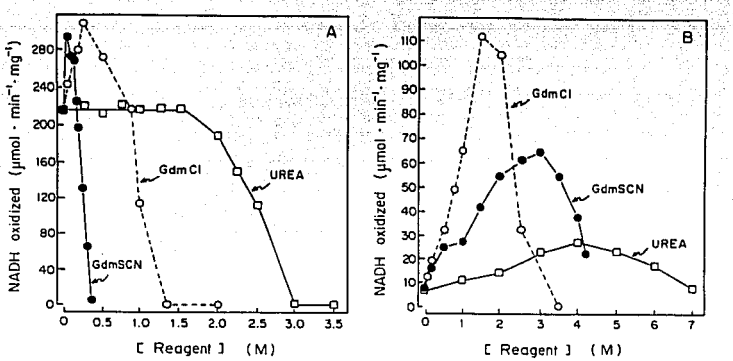

Fig. 2. Effect of urea, GdmCl and GdmSCN on the activity of lactate dehydrogenase in standard water media (A) and in the TPT System (B). (A) The activity was measured as described under Methods with the indicated concentrations of urea (□——□), GdmCl (□- - - -□) and GdmSCN (●——●). (B) The transfer of the enzyme to the TPT system and the rates of activity were measured as in Fig. 1 with 3.8% (by vol.) water that contained the indicated concentrations of urea (□——□), GdmCl (□- - - -□), and GdmSCN (●——●).

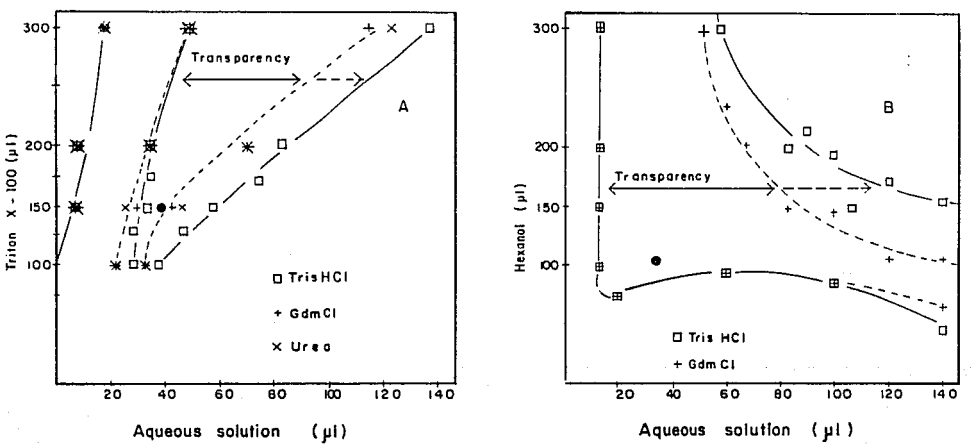

Fig. 3. Solubility diagrams of water and aqueous solutions of either GdmCl or urea in (A) TPT and (B) cetyltrimethylammonium bromide/hexanol/*n*-octane systems. (A) To several tubes that contained 0.85 ml toluene with 10 mg phospholipids, the amount of Triton X-100 indicated was added. To these tubes (□) aqueous buffer solution (40 mM phosphate or 20 mM Tris/HCl pH 7.4) and (+) 1.5 M GdmCl or (×) 4 M urea solutions were added stepwise. After the different solutions were added, the tubes were stirred in a vortex and allowed to stand for 20 min. The region where transparency was observed is indicated; transparency was also observed in the left side of the diagram. (B) The same procedure was followed except that the tubes contained 1.0 ml *n*-octane with 0.2 M cetyltrimethylammonium bromide and the amount of hexanol indicated. The region of transparency for (□) aqueous buffer and (+) 1.25 M GdmCl solution are indicated. Titrations were also made with hexanol to tubes that contained the indicated amounts of aqueous solutions. The results in both cases were very similar. The closed circles indicate the region where measurements of enzyme activity were carried out.

amount of water, but it is noted that even with the highest amount of water tested (6.0% by vol.), the activity was about 25 times lower than in all-water mixtures. With increasing amounts of a 1.5 M GdmCl solution, the activity curve was bell-shaped with a maximum at about 4–4.5% (by vol.) 1.5 M GdmCl solution. It is clear that in the two conditions there

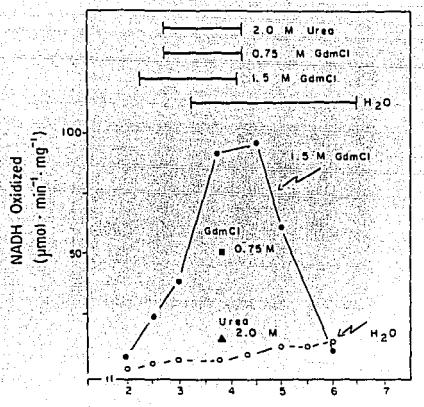

Fig. 4. Activity of lactate dehydrogenase in the TPT system with increasing amounts of water or 1.5 M GdmCl solution, and 2.0 M urea and 0.75 M GdmCl: regions of transparency with aqueous buffer, 0.75 M GdmCl, 1.5 M GdmCl and 2.0 M urea. Enzyme activity was measured as in Fig. 1, except that the tubes contained the indicated amounts of either (- - - - -) water or (●——●) 1.5 M GdmCl. Activity was also measured in the presence of (■) 0.75 M GdmCl and (▲) 2.0 M urea as indicated. The regions where transparency was observed with the various aqueous solutions is depicted by bars; these were determined as in Fig. 3.

are differences, both in the activity of the enzyme and in the region of reverse micelles. Thus, it could be that the higher activity with GdmCl was due, or related, to changes in the organic solvent system, particularly if it is considered that with increasing amounts of 1.5 M GdmCl solution there was a drop of activity that coincided with a transition in the phase boundary (see region of reverse micelles with 1.5 M GdmCl and activity curve). Moreover there are reports [43, 44] that indicate that phase boundary transitions are accompanied by marked changes in enzyme activity.

To explore further whether, in the presence of denaturants, changes of the micellar system correlated with changes of enzyme activity, we searched for conditions in which similar solubility diagrams were obtained with aqueous solutions of different composition. With 0.75 M GdmCl and 2.0 M urea, nearly identical solubility diagrams were obtained (top bars in Fig. 4). In the regions where reverse micelles existed, the activity of lactate dehydrogenase was measured; it was more than three times higher with GdmCl than with urea (see triangle and square in Fig. 4). Thus, although in the presence of denaturants there are distinct phase changes, the data also suggest that enzyme activity does not depend solely on such a factor; particularly if it is considered that with almost identical solubility diagrams, markedly different activities were obtained by varying the components of the water phase.

With respect to the activity and the effect of denaturants on the activity of lactate dehydrogenase in the cetyltrimethylammonium bromide/hexanol/*n*-octane system, it was found that with 3.0% water, the enzyme exhibited an activity of about 18 μmol min^{-1} mg^{-1}. The addition of either 0.5 M or 1.25 M GdmCl produced an immediate increase in activity to values of around 80 and 115 μmol min^{-1} mg^{-1}, respectively, which were constant with time for 20 min (for experimental details see Materials and Methods). These activity levels were comparable to those observed in the TPT system with equivalent GdmCl concentrations at 3.8% water. Also as in the TPT system, the plot of activity versus GdmCl was bell-shaped with a maximum at 1.25 M GdmCl (not shown). Urea also activated the enzyme; the activity increased progressively with the concentration of urea up to a value of 47 μmol min^{-1} mg^{-1} with 4.5 M urea (not shown). The latter was the highest urea concentration that could be introduced into this system. Hence, in both the TPT and the cetyltrimethylammonium bromide/hexanol *n*-octane systems, the effect of GdmCl and urea were markedly similar.

As shown in Fig. 3B, the solubility diagrams of water or a GdmCl solution in the cetyltrimethylammonium bromide system were almost indistinguishable in the lower phase boundary, but there were differences in the upper boundaries. The point of the reverse micelle system at which the effect of denaturants on enzyme activity was measured was close to the lower phase boundary (closed circle in Fig. 3B).

It was also found that GdmCl did not affect the distribution of hexanol at the interphase (and the water space) of the reverse micelle as determined by the phase boundary method (for a detailed discussion of this method, see Hilhorst et al. [29]). In the absence or presence of GdmCl, the titration curves of *n*-octane and hexanol were almost identical; the intercepts at the ordinate (the amount of hexanol at the interphase and the water space) were 114 μl and 112 μl hexanol, without and with GdmCl respectively. In both cases, the slope (amount of hexanol in the solvent) was 29.3 μl ml octane. Also as expected, denaturants did not affect the partition of pyruvate, NADH, or ATP between water and organic solvents (either toluene or a mixture of hexanol and *n*-octane). More than 99% of the compounds were localized in the water phase.

Therefore, the overall findings indicate that denaturants produce alterations of the phase diagrams of the reverse micelle systems employed. However, the data also indicate that the enhancement of enzyme activity by denaturants is not strictly related to such changes.

With respect to the favorable effect of denaturants on enzyme activity, there is a point that deserves comment. This deals with the distribution of either the enzyme and/or the substrate within the water pool of a reverse micelle, i.e. it is possible that substrate molecules, or the enzyme [45], may bind to the internal surface of the reverse micelles and that denaturants cause their displacement into the micellar water pool. This could lead to an increase in the availability of substrate to the enzyme, or to a detachment of the enzyme from the polar micellar surface, and thus the increase in activity.

In the evaluation of the latter possibility, two experimental conditions are pointed out. One concerns the nature of the low-water systems employed. The TPT system was formed with the non-ionic Triton X-100 and phospholipids which, according to their composition (40% phosphatidylcholine, 29% phosphatidylethanolamine, 14% monophosphoglycero-inositol, 4% phosphatidylserine, and small amounts of lysophospholipids [46]), have a negative charge at pH 7.0. In contrast, the internal surface of the micelles of the cetyltrimethylammonium bromide system is positively charged. As a strikingly similar activating effect of denaturants was observed

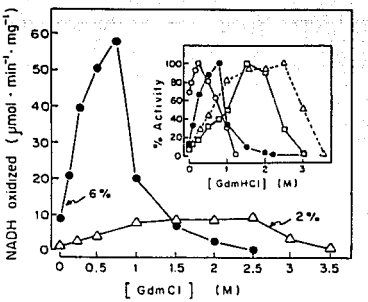

Fig. 5. Effect of different GdmCl concentrations on the activity of lactate dehydrogenase in the TPT system that contained different amounts of water. The experiment was made as in Fig. 1, except that the system contained (○——○) 2.0% or (●——●) 6.0% (by vol.) water and the indicated concentrations of GdmCl. The inset shows the effect of different concentrations of GdmCl on the activity of the enzyme in (△——△) 100% aqueous media and in the TPT system with (●——●) 6.0%, (□——□) 3.8% and (△- - -△) 2.0% water. The data with 100% and 3.8% water were taken from the experiments of Fig. 2; the data were normalized by considering the maximal activity at each water concentration as 100%.

in both systems, it seems improbable that a denaturant-induced substrate, or enzyme redistribution, accounted for their effect on activity. Also, with respect to substrate redistribution, it is noted that enzyme activities were measured at substrate concentrations in which maximal or near maximal velocities were attained. Thus substrate availability would not seem to be the factor that accounted for the marked increase of enzyme activity as induced by denaturants in low-water system.

Effect of GdmCl on lactate dehydrogenase activity in the TPT system with various amounts of water

The aforementioned data indicate that denaturants produce changes of the micellar system and that these may be related to enzyme activation by denaturants. However, it has been reported that several enzymes are activated by denaturants in all water media [18–21]; thus enzyme activation in the low-water systems could also be due to this effect of denaturants. Of particular interest are the observations with NADP–malate dehydrogenase from chloroplasts. The dark-oxidized form of the enzyme is inactive, but shows considerable activity in the presence of GdmCl, i.e. 25% of the activity of the active reduced form of the enzyme [22]. Also Ma and Tsou [47] recently showed that GdmCl activates cross-linked lactate dehydrogenase (the activity of the preparation was about 60% of the activity of the native enzyme) under conditions in which the native enzyme is not (see following paper [37]).

These findings suggest that significant activation by denaturants takes place when enzyme catalysis is restricted. In the light of the work of Rupley et al. [1], Finney and Pool [2], Bone et al. [48], Klibanov et al. [5], and Garza-Ramos et al. [4, 41] on the role of water in the flexibility of enzymes, it was thought that in the low-water system denaturants induced activation through release of constraints of enzyme activity,

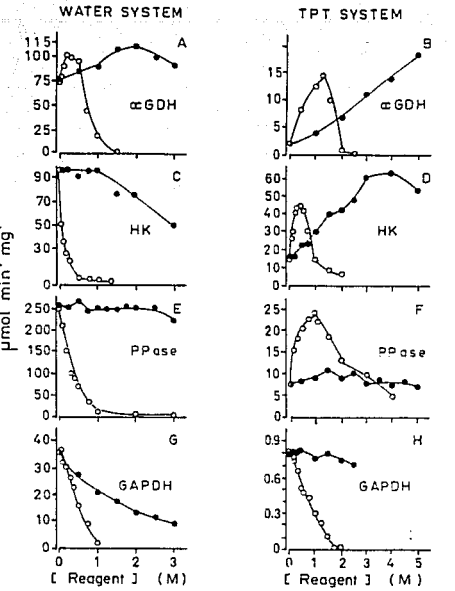

Fig. 6. Effect of urea and GdmCl on the activity of various enzymes in standard water media and in the TPT system with 3.8% water. In all water media and in the TPT system, the activities were measured as described under Methods; the mixtures contained the indicated concentrations of (○——○) GdmCl or (●——●) urea. The graphs on the left and right sides refer to standard water media and TPT mixtures, respectively. The enzymes tested were glycerol-3-phosphate dehydrogenase (α-GDH), hexokinase (HK), inorganic pyrophosphatase (PPase), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

and this could be related to the amount of water in contact with the enzyme.

The experiments of Fig. 5 illustrate the effect of denaturants on enzyme activity at various water concentrations. In the TPT system with 2.0% and 6.0% water, increasing concentrations of GdmCl enhanced enzyme activity in a bell-shaped type of curve. With 2.0% water, the curve was rather flat with a broad range of GdmCl concentrations (around 1.5–2.5 M) at which activity was near maximal. With 6.0% water, the bell-shaped curve was sharp with a peak at 0.75 M GdmCl. The inset of Fig. 5 summarizes the data on the effect of different concentrations of GdmCl on lactate dehydrogenase activity at various water concentrations, including standard aqueous media. The activities of the various water concentrations were normalized in order to illustrate that the concentration of GdmCl required to produce maximal activation and half-maximal inactivation was inversely related to the amount of water. In other words, as the content of water was increased, the concentration of GdmCl at which maximal activation took place approached that which in all water media produced activation. Therefore by varying the amount of water, it is

possible to have an enzyme with a distinct response to a given concentration of denaturing agent.

We would also like to call attention to the role of water and a GdmCl solution as solvents for enzyme function. The data of Fig. 4 show that, for the case of water, the activity progressively increased as the water content was raised. This probably reflects the dependence of enzyme activity on the amount of water. The dependence of activity on the amount of solvent is also observed with a GdmCl solution up to about 4.0% (by vol.). However within this solvent range, activity was much higher with GdmCl. The difference most probably reflects the different characteristics of the two solvents.

Effect of GdmCl and urea on the activity of various enzymes placed in the TPT system with 3.8% water

We also explored if, in addition to lactate dehydrogenase, other enzymes were activated by denaturants when placed in a low-water environment. The activities of glycerol-3-phosphate dehydrogenase, hexokinase, inorganic pyrophosphatase and glyceraldehyde-3-phosphate dehydrogenase were studied in the TPT system with 3.8% water. Except for inorganic pyrophosphatase, all the activities were assayed at saturating substrate concentrations; at half or twice the concentrations indicated in the legend to Fig. 6 with or without GdmCl or urea, the activities did not vary significantly from those shown.

In the TPT system, inorganic pyrophosphatase was assayed in a concentration range of 0.2–9 mM Mg[^{32}P]PP$_i$. As described by Kunitz [49] in all-water media, the activity increased almost linearly with pyrophosphate concentration. At all substrate concentrations, GdmCl increased the activity by nearly the same percentage (not shown); in the experiment of Fig. 6, the activity was assayed with 3 mM Mg[^{32}P]PP$_i$.

For the enzymes tested, the activities were around 30–40 times lower than in all-water mixtures, except with hexokinase which was about 8 times lower (compare data of left and right sides of Fig. 6). In the TPT system, GdmCl activated glycerol-3-phosphate dehydrogenase, hexokinase, and inorganic pyrophosphatase; of these three, the first two were also activated by urea. Some other features of the results are noteworthy. (a) Not all enzymes were activated to the same extent by denaturants. (b) Maximal activation of the various enzymes was attained with different concentrations of denaturants. (c) GdmCl increased the activity of hexokinase and inorganic pyrophosphatase, even though in all-water systems low GdmCl concentrations caused inactivation. (d) For glycerol-3-phosphate dehydrogenase and hexokinase, the activating effect of urea was higher than with GdmCl. (e) Glyceraldehyde-3-phosphate dehydrogenase was not activated by the denaturants; instead, GdmCl and urea brought about inactivation at relatively low concentrations.

The differences in activation of the various enzymes by GdmCl and urea most likely reflect the action of denaturants on proteins with distinct structural and kinetic features. The following paper [37] describes the relevance of the latter factors on the effect of water and denaturant solutions on the activity of lactate dehydrogenase isoenzymes. The data of Fig. 6 are also of interest in the sense that with different enzymes, maximal activities were reached with different concentrations of denaturants; this indicates that drastic changes of catalytic activity do not necessarily coincide with transitions of the phase boundaries of the low-water system.

In addition, the data of Fig. 6 also suggests that, in low-water systems, one of the earliest events in the action of denaturants on enzymes is the release of constraints to catalysis. In the latter respect, it is interesting that of the enzymes tested, only glyceraldehyde-3-phosphate dehydrogenase was not activated by denaturants. According to the data of Tsou and coworkers in the standard water system [50, 51], GdmCl and relatively high temperatures drastically affected the activity of the lobster enzyme before changes in its circular dichroic spectrum were detected, and before dissociation of subunits had taken place. Similar findings were reported for ethanol dehydrogenase [52], creatine kinase [53], phosphofructokinase [54], and lactate dehydrogenase [37, 47]. Apparently with these enzymes, one of the first events in denaturant action is also the perturbation of enzyme catalysis by denaturants.

In conclusion, from the experimental results it is apparent that denaturants increase the activity of different enzymes when placed in a low-water systems. Denaturants affect the characteristics of the low-water systems, and this may reflect in the expression of enzyme catalysis. However according to the known actions of denaturants on proteins [7, 12, 15], it would seem that the principal factor in enzyme activation in low-water systems is a denaturant-induced release of constraints of enzyme activity imposed by the low-water environment. In fact, it is known that protein conformational mobility is drastically affected by water [1, 2], and here it was found that the effect of denaturants on activity depended on the amount of water. Therefore it is possible that, in a limited water space, denaturants promote solvent–protein interactions and thereby support the expression of relatively high catalytic rates.

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