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# Simple and Sensitive Technique for Investigation of Desorption Properties

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The technique of thermally stimulated pressure (TSP) is described and has been applied to study the state of bound water on crystallised lysozyme at hydration levels of 0–24 mg of water per gram of protein. If it is assumed that for the low densities used the molecules of water are bound to independent sites in the macromolecule of lysozyme, first-order kinetics can be used to fit the experimental pressure *versus* temperature graphs. The activation energy is 37.63 kJ mol$^{-1}$.

*Keywords*: *Bound water*; *lyszoyme*; *thermally stimulated pressure*; *activation energy*; *desorption*



Since the middle of the nineteenth century,[1] biologists have studied the state of water molecules in biological systems. In 1855 Nägeli[2] pointed out that some of the water in extruded cytoplasm from plant and animal tissues is bound to the constituent macromolecules. More recently several techniques and properties have been used to study the state of water in macromolecules and membranes: cryoscopy,[3] specific conductivity,[4] differential scanning calorimetry,[4] nuclear magnetic resonance spectroscopy,[5] dielectric relaxation,[6,7] X-ray spectrometry,[8] thermally stimulated depolarisation current,[9,10] sorption of water vapour[11,12] and others.[13–16] In spite of the existence of so much literature on the subject, few data on the energies involved in the process have been reported.

We describe here the technique "thermally stimulated pressure" (TSP), which consists in the measurement of the water vapour pressure, in a fixed volume, as a function of temperature while the sample is subjected to a linear heating rate. Graphs of pressure of water vapour *versus* sample temperature at different hydration levels are presented that give information about the activation energy and the corresponding relaxation time of the desorption process.

## Theoretical

We assume that the water molecules are bound to different classes of sites, $j$, in the macromolecule, which are independent at these hydration levels (from 0–25 mg of water per gram of protein). Any physical quantity relative to a class will be denoted by the subscript $j$, and the main characteristics of a class of sites are its free energy of interaction with the water molecule, denoted by $\Delta F_j$, and the transition probability, $W_j$, from the bound to the free state. The rate of desorption of water molecules from class $j$ at a given temperature $T$, assuming first-order kinetics and a linear heating rate, *i.e.*, $\mathrm{d}t = (1/b)\mathrm{d}T$, where $b$ is the heating rate will be

$$\frac{\mathrm{d}N_j(T)}{\mathrm{d}T} = -\frac{1}{b} W_j(T)\, N_j(T) \qquad (1)$$

We assume that the Arrhenius activation law[15] is valid:

$$W_j(T) = W_{j0}\exp(-\Delta F_j/kT) \qquad (2)$$

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where $W_{j0}$ is the characteristic frequency of the desorption process, $k$ the Boltzmann constant and

$$\Delta F_j = \Delta E_j - T\Delta S_j \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

$\Delta E_j$ is the activation energy and $\Delta S_j$ is the activation entropy. At the initial state of the process ($T = T_0$), all molecules are bound. At a given temperature, $T > T_0$, the conservation law gives

$$N_j(T) + N_{fj}(T) = N_{0j} \quad .. \quad .. \quad .. \quad .. \quad (4)$$

where $N_{fj}(T)$ is the number of free water molecules and $N_{0j}$ the total number of water molecules. The solution of equation (1), with the law (2), is

$$N_j(T) = N_{0j} \exp\left[-\frac{1}{b} \cdot W_{j0} \int_{T_0}^{T} \exp(-\Delta F_j/kT')\, dT'\right] \quad .. \quad .. \quad (5)$$

The pressure $P(T)$ measured inside the flask is given by

$$P_j(T) = \frac{N_{fj}(T)}{V} .kT_w \quad .. \quad .. \quad .. \quad .. \quad (6)$$

where $V$ is the volume of the flask and $T_w$ is the temperature of the wall of the flask (the temperature of the water vapour). Solving equation (4) for $N_{fj}$, using (5), we obtain

$$P_j(T) = P_{fj}\left\{1 - \exp\left[-\frac{1}{b} .W_{j0} \int_{T_0}^{T} \exp(-\Delta F_j/kT')\, dT'\right]\right\} \quad .. \quad (7)$$

where $P_{fj} = \frac{N_{0j}kT_w}{V}$ is the final pressure when all molecules of class $j$ are free. The measured pressure, $P(T)$, will be a sum over $j$ of expressions such as equation (7):

$$P(T) = \sum_j P_j(T) \quad .. \quad .. \quad .. \quad .. \quad .. \quad (8)$$

Differentiating equation (7) with respect to $T$, and considering equation (3), we obtain, for one class of sites

$$L = \left[\frac{b}{P_{fj} - P_j(T)}\right] \frac{dP_j(T)}{dT} = W_{j0} \exp(\Delta S_j/k) \exp(-\Delta E_j/kT) \quad .. \quad (9)$$

By plotting the logarithm of the left-hand side of equation (9) against $1/T$, the activation energy can be determined. On extrapolating to infinite temperature we obtain $W_{j0}\exp(\Delta S_j/k)$.

## Experimental

Commercial lysozyme (Grade 1) was obtained from Sigma (crystallised three times, dialysed and lyophilised) and was used as received. Tablets, approximately 1 × 13 mm diameter, were made by compressing the powder in a Carver, Model C, laboratory press. The best tablets were obtained with a density of 0.9 g cm$^{-3}$. To obtain a better result it was necessary to dehydrate the powder before and during the pressing. The main reason for choosing lysozyme powder was its high resistance to thermal denaturation and for continuous cycles of sorption and desorption of water molecules.[14] The dry state of the tablets was obtained by extrapolating to zero time the graphs of mass *versus* the hydration time. Before being weighed, the samples were left to dehydrate under vacuum for 24 h at 70 °C. The "humid" state was obtained by leaving the tablets to hydrate on the balance plate.

The tablet support and the heater system are shown in Fig. 1. The heater system consisted of a resistance (*ca.* 700 Ω) inserted in a porcelain cylinder, connected to a variable a.c. source with a suitable voltage (25–50 V) to obtain a constant heating rate of 0.10 K s$^{-1}$. The

tablet support consisted of a hollow cylinder, in which the heater system was inserted. The upper part (185 mm high) and the walls (1 mm thick) were made of stainless steel; the lower part (35 mm high) had a parallelepiped shape and was made of copper to facilitate heat conduction. The tablet support was inserted in a cylindrical glass plate (100 mm diameter, 20 mm high) for access to the wiring (see Fig. 2). The system, as shown in Fig. 2, will rest on the mouth of a Duran 50, Schott Mainz Jena ER Glas, flask (5 l). The mouth of the flask was ground and a flat brass ring with the same diameter, possessing a circular guide for insertion of a rubber ring to prevent leaks, was attached to it with Araldite epoxy resin. The whole flask was immersed in a solid thermostat maintained at 200 °C to prevent sorption of water molecules on to the walls of the flask.

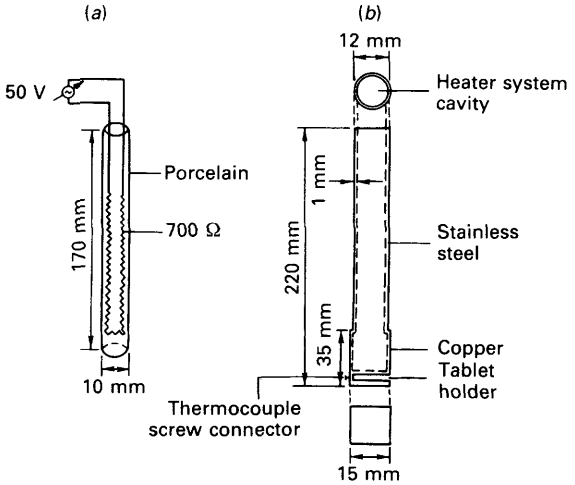


Fig. 1. (*a*) Heater system, single resistance inside heater system cavity to vary temperature. (*b*) Tablet support, holder chosen to minimise any temperature gradient in sample.

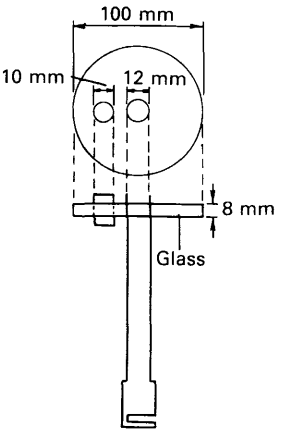


Fig. 2. Top of 5-l flask that contains sample. Walls of the flask kept at 200 °C to avoid adsorption of water molecules.

The pressures at sample temperatures were recorded with a Hewlett-Packard 700 4B *X* - *Y* recorder. The flask had a lateral exit on its neck which was connected through a rubber tube to a stainless-steel T-connector, one end of the connector being coupled to a mechanical pump and the other to a Varian NRC 531 thermocouple vacuum gauge which was energised by a Varian 856-A source. This system was previously calibrated with a Stockes McLeod gauge. There were two more thermocouples (copper - constantan thermocouples, Leeds & Northrup Co.): one was connected through a screw to the tablet support (see Fig. 1) and allowed measurement of the temperature of the sample; the other was left free inside the flask to measure the temperature of the water vapour, which was identical with that of the wall of the flask. Both were connected to Philips DC PM2436 micrometers.

## Results and Discussion

The first molecules adsorbed on a dehydrated macromolecule of lysozyme (bound-water molecules) are connected by hydrogen bonds to hydrophilic amino acids on the surface. These bonds are:

$$\text{O—H} \cdots \text{O}$$

$$\text{N—H} \cdots \text{O}$$

Lysozyme, like the globulins, conforms to the principle of "hydrophobic in, hydrophilic out" or the oil-drop model of a protein. After completing the bonds with the macromolecules, the water molecules start to form monolayers[13]; 300 mg of water per gram of protein are sufficient to cover the surface of the lysozyme molecule with a single monolayer of water.[8]

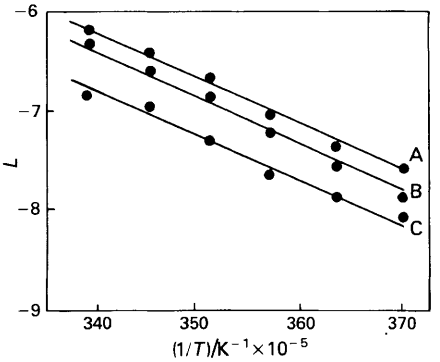


Fig. 3. Representation of equation (9). A, $h = 9.0$; B, $h = 14.0$; and C, $h = 24.0$ mg of water per gram of protein.

Fig. 3 shows the graph of $L$ *versus* $1/T$ [equation (9)], from which the parameters to be used in equation (7) can be calculated. Solving equation (7) then gives the solid points shown in Fig. 4 together with the experimentally measured graphs. It is important to note, however, that equation (9) can be applied only to monomolecular kinetic processes with one activation energy and one corresponding relaxation time, which means one class of sites. In the general case, with several classes of sites, the adjustment of the parameters is more complicated and a numerical programme is needed in order to minimise the mean square errors in an iterative method using the general equation (8).

With linear heating of the hydrated samples (250-370 K), the hydrogen bonds break. In Fig. 4, the continuous lines show the experimental pressure of water vapour formed by desorption of bound water molecules *versus* the sample temperature.

The energy obtained, 9.03 kcal mol$^{-1}$, agrees with the Monte Carlo simulation of Hagler and Moult[15] for waters in close contact with the protein and within 10% of the average energies for six water molecules inside the lysozyme enzyme calculated by Clementi *et al.*[16] Finally, we should point out that the ratio of the hydrations must be equal to the ratio of final pressures for any pair of curves, $h_i/h_j = P_{f_i}/P_{f_j}$. This is true within an error of 4%.

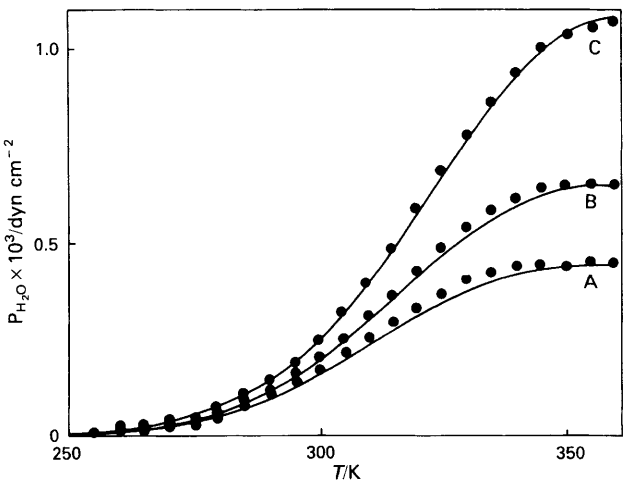


Fig. 4. Vapour pressure of water *versus* sample temperature. Full lines show experimental curves. The circles show the results obtained by the present model, which uses one class of sites. The agreement is very reasonable. A, $h = 9.0$; B, $h = 14.0$; and C, $h = 24.0$ mg of water per gram of protein.

These results were compared with thermogravimetric analysis (TGA) by application to the same system. This technique consists in the continuous measurement of the mass of a sample as a function of the linear increase in temperature. Owing to the high sensitivity of the thermogravimetric scale (Rigaky type, Cat. No. 8076D1) it is possible to detect the variations observed in TSP measurements.

Using the same theoretical basis we have from the sample mass

$$N(T) = N_0[m(T) - m_f] \quad .. \quad .. \quad .. \quad .. \quad (10)$$

where $N_0$ is Avogadro's number, $m(T)$ is the sample mass at temperature $T$ and $m_f$ the final mass when the sample is dehydrated. For only one class of sites, substituting $N(T)$ given by equation (10) in equation (1), we obtain

$$W(T) = b\frac{d}{dT}\left\{\ln[m_f - m(T)]^{-1}\right\} \quad .. \quad .. \quad .. \quad (11)$$

Therefore, by applying this expression to TGA curves, the energy and corresponding relaxation time can be determined. The results obtained with TGA agree with those of TSP, as shown in Table I.

TABLE I

PARAMETERS OBTAINED BY TSP AND TGA TECHNIQUES

| Hydration/mg of water per gram of protein | Heating rate/ K $s^{-1}$ | $\Delta E$/eV TSP | $\Delta E$/eV TGA | $(1/W_{10})\exp(-\Delta S_1/k)$/s $\times 10^{-4}$ |
|---|---|---|---|---|
| 9.0 | 0.101 | 0.388 | | 1.15 |
| 14.00 | 0.103 | 0.388 | | 1.43 |
| 24.00 | 0.100 | 0.388 | | 2.05 |
| 40.00 | 0.035 | | 0.380 | 3.00 |
| 60.00 | 0.034 | | 0.340 | 3.20 |

In conclusion, the technique of thermally stimulated pressure can clearly provide valuable information on the dehydration properties of biological materials. The sensitivity of the technique is high and it can be readily carried out using simple apparatus at a relatively low cost.

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