

BBM 00648

# A continuous wave laser T-jump apparatus and its application to chemical reactions in hemoglobin single crystals

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(Received 17 September 1987)

(Accepted 22 December 1987)

## Summary

A laser temperature jump apparatus is constructed where the T-jump is achieved by means of the direct absorption of continuous laser radiation of low intensity by a solid sample. The final temperature in the irradiated volume element is reached when the absorbed radiation power equals the dissipation of heat by heat conduction. The time range from the beginning of irradiation to the stationary state depends on the geometry of the irradiated volume element and is less than 10 ms. The heating laser beam is simultaneously used to detect the relaxation to the new chemical equilibrium in the sample. Relaxation processes with relaxation rates between  $10^2 \text{ s}^{-1}$  and less than  $10^{-3} \text{ s}^{-1}$  on samples with volumes less than  $10^{-3} \text{ mm}^3$  may be investigated using this T-jump method. One application of this method is the determination of reaction rates of ligand reactions in hemoglobin single crystals. Rate constants obtained for the reaction of thiocyanate with crystallized horse methemoglobin are presented.

Key words: Laser temperature jump; Hemoglobin crystal; Methemoglobin thiocyanate

## Introduction

A laser heated temperature jump apparatus offers several advantages over the conventional joule heating apparatus. The most obvious is the possibility for studying very fast reactions due to the rapid heating times achievable using Q-switched laser pulses [1,2]. The second is the removal of any constraint upon the conductance of the sample, with the possibility of using nonaqueous solutions or even non-conducting solids as samples. We present the construction of a continuous

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wave laser T-jump apparatus and its application to studies of biochemical reactions in hemoglobin single crystals. The joule heating method could hardly be used in this case because the crystals could not be fixed and an optical detection of the absorbance change after the T-jump was impossible. However, Q-switched lasers are very expensive and we wanted to restrict our investigations to comparatively slow reactions with relaxation times in the ms and s range. So we constructed a laser heated temperature jump apparatus, where the T-jump is achieved by means of the direct absorption of continuous laser radiation of low intensity (1–10 mW) by a small volume element of the sample. So the heating is not achieved by a short pulse. By means of continuous irradiation of the sample the stationary state is reached, which is characterized by the equilibrium between absorbed radiation power and the dissipation of heat by heat conduction. After this equilibrium is reached the average temperature of the irradiated sample volume is constant with time and its stability depends only on the stability of the laser output power. The geometric conditions of irradiation are chosen in such a manner that the time interval from the beginning of the irradiation to the stationary state is in the ms time range.

A second speciality of the method presented here is the fact, that the heating laser beam is simultaneously used to detect the relaxation to the new chemical equilibrium. The unavoidable temperature gradient in the course of the detecting beam is therefore known and can be calculated in the case of a quantitative analysis of the relaxation amplitude. However, if the determination of the relaxation rates is the only aim, and if we restrict temperature changes to small rises, the gradient is of no importance. The method developed here may be applied to the investigation of chemical reactions in solid samples with volumes less than  $10^{-3}$  mm<sup>3</sup>. Using this equipment, we have studied ligand reactions in methemoglobin single crystals.

The measurements are of great interest because structural factors play an important role in the reactions of hemoglobin with ligands. The structure of crystallized hemoglobin is precisely known from X-ray measurements [3] and so a comparison of the reactivity of methemoglobin towards ligands in solution and in the crystallized form may point out the difference in the structure and dynamics of these systems [4–6]. A light beam of a He-Ne laser was chosen as heating source and for the optical detection of the chemical relaxation. The extinction coefficient of methemoglobin at the He-Ne wavelength, 633 nm, depends strongly on the species of the sixth ligand of Fe<sup>3+</sup>. On the other hand, this extinction coefficients are large enough to allow a sufficient absorption of the laser radiation in a sample volume element of the right geometry. We have used this apparatus to study the binding of SCN<sup>-</sup> to horse methemoglobin in the crystal state. The structure of this complex is known from X-ray measurements [7] and there are some data available from literature concerning this binding reaction in solution [8,9].

## Experimental

### *Materials*

The preparation of the oxyhemoglobin from fresh horse blood followed the methods of Benesch et al. [10]. The oxidation of the hemoglobin to methemoglobin

was achieved by the addition of a threefold amount of  $K_3(Fe(CN)_6)$ . The sample was desalted by running through a column of Sephadex G-25. Single crystals were prepared by mixing the solution of methemoglobin with buffered ammonium sulfate solutions according to the method of Perutz [11]. Optically clean crystals were selected from the preparations after two or three weeks and washed several times with 1.2 M  $Na_2SO_4$  solution. The pH of this solution was adjusted to 7 with a phosphate buffer.

Temperature jump measurements have been carried out on the monoclinic (space group C2) form of methemoglobin, which crystallized with a platy habit flattened on (001). Since the (001) face contains the  $b$ -axes, which are the twofold rotation axis of the crystal, light remains plane polarized when it propagates through this crystal with its electric vector parallel or perpendicular to this axes, and extinction is observed when the crystal is examined between crossed polarizers [12]. At a wavelength of 633 nm, the maximum crystal extinction coefficients have values of  $4000 M^{-1} \cdot cm^{-1}$ . The heme concentration in the crystal is about 0.05 M. A peak optical density of about 1 requires crystals having a thickness on the order of 50  $\mu m$ . Crystals of this size have lateral dimensions of about  $200 \times 200 \mu m^2$ . The T-jump experiments were done in a 1 mm cuvette with the crystal lying in a 1.2 M  $Na_2SO_4$  solution, to which amounts of NaSCN were added to give the desired concentrations of free  $SCN^-$ .

#### *Apparatus*

Fig. 1 is a block diagram of the complete system. A He-Ne laser (Spectra Physics, Model 124B) with a maximum output power of 20 mW serves as the light and heating source. After the light beam has passed the electromechanical shutter S it is split into two beams of equal intensity at mirror M1. One part is reoriented at mirror M2 and focused by lens L1 so that its cross-section in the sample plane leads to a sufficiently high energy density and a sufficiently small irradiated volume element of the sample. The reason for this requirement will be discussed in the next section. Lens L2 collects the light passed through the sample and focuses it on the receiver diode D1 (BPW 34). The second part of the beam coming from M1 passes a wedge filter G and reaches the reference diode D2. The signals from D1 and D2 are subtracted by a differential amplifier (Tektronix AM 502) so that a noise reduction of the laser mode interference noise is achieved of about 30 dB. A Nicolet 1072 transient recorder serves both as an analog-to-digital converter and as a data buffer. The transient recorder is triggered by the current driving the shutter S. The data processing was carried out with the help of a CBM 8296 computer system. The maximum intensity  $p_0$  of the laser light in the sample plane was measured with an Ulbricht sphere type photometer. To determine the radius  $w$  of the beam in this plane it was enlarged by a lens of 100 dpt and scanned with a photodiode covered with a 10  $\mu m$  iris. With the system described here it is possible to detect absorbance changes of the sample as low as  $\Delta E/E = 10^{-4}$ .

#### *Theory*

Fig. 2 shows a schematic diagram of the irradiation and the absorption geometry in a hemoglobin single crystal. The intensity distribution of a single mode laser

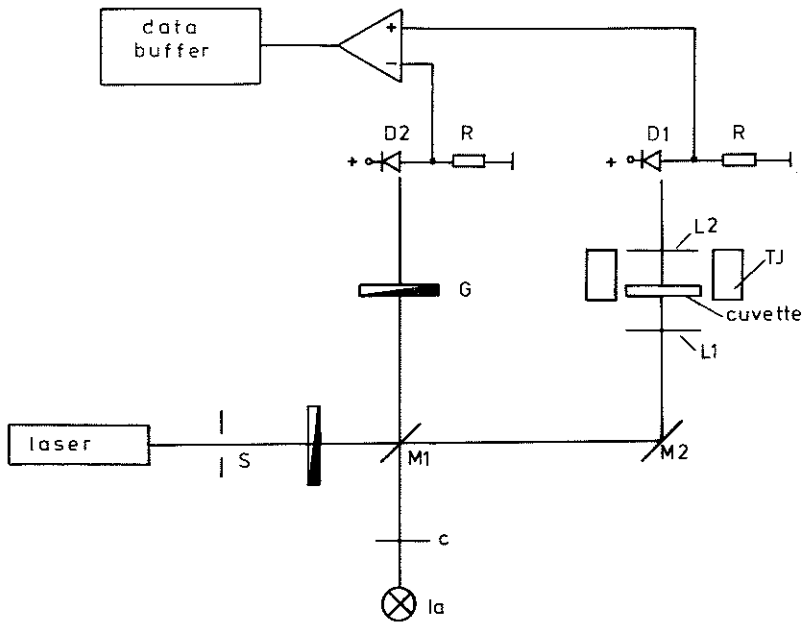


Fig. 1. Simplified block diagram of the laser T-jump apparatus: S, shutter; L, lens; G, wedge filter; D, photodiode; la, lamp; M, mirror; c, condenser; cuvette, cuvette with sample; TJ, tempering jacket; R, resistor.

follows a Gaussian distribution. Provided the validity of the Lambert-Beer's law, the irradiation  $p(\rho, t)$  within the absorbing crystal is given by

$$p(\rho, z, t) = p_0 \exp(-2(\rho/w)^2) \exp(-\alpha z) \quad (1)$$

with  $p_0$ , maximum intensity;  $w$ , radius of the beam at the  $e^{-2}$  points;  $\alpha$ , absorption coefficient;  $\rho$ , radius in the  $x, y$ -plane.

Outside the crystal the irradiation is constant with respect to  $z$ . The absorption of light leads to a temperature rise in the sample. For temperature calculations we assume that heat conduction is the leading process of heat flow. This assumption will be proved later. To determine the temperature rise  $T(x, t)$  the inhomogeneous heat conduction equation

$$\delta T / \delta t - \kappa \Delta T = \epsilon / \bar{\rho} \cdot c \quad (2)$$

with  $\kappa = \beta / \bar{\rho} \cdot c$ , has to be solved [13].  $T$  being the temperature,  $t$  the time,  $\bar{\rho}$  the density of the medium,  $c$  the specific heat and  $\beta$  the heat conductivity. The term  $\epsilon$  represents the externally added heat energy density per time and thus the so called source term in the heat conduction equation. The energy flux density deposited in the crystal with the geometry shown in Fig. 2 results in

$$\epsilon(\vec{r}, t) = -\frac{\delta p}{\delta z} = \begin{cases} \alpha p_0 \cdot \exp\{-2(\rho/w)^2 + \alpha z\} & \text{for } 0 \leq z \leq d \\ 0 & \text{otherwise} \end{cases} \quad (3)$$

In solving eq. (2) we have to consider the boundary conditions. Since the temperature is held constant on the surface of the sample cell by a thermostat and the size

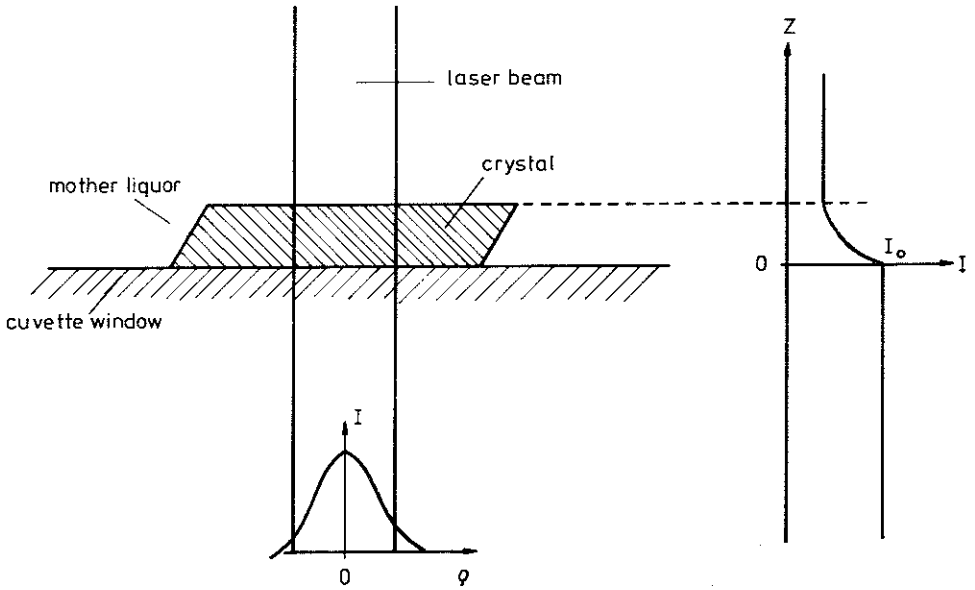


Fig. 2. Schematic diagram of the irradiation and absorption geometry in a layer with constant absorption coefficient irradiated by a single mode laser beam.

of the irradiated and absorbing volume element is small in comparison to the sample cell dimensions, the boundary conditions of an 'infinite solid' are almost perfectly realized. For this situation the solution of Eq. 2 is given by Birngruber [14,15]:

$$T(\vec{r}, t) = T(\rho, z, t) = \frac{\alpha p_0}{2\rho c} \cdot \exp\{-\alpha z\} \cdot \int_0^t dt' \cdot \frac{\exp\{\alpha^2 \kappa t' - 2\rho^2/(w^2 + 8\kappa t')\}}{1 + 8\kappa t'/w^2} \cdot \operatorname{erf}\left\{\frac{z}{2\sqrt{\kappa t'}} - \alpha\sqrt{\kappa t'}\right\} - \operatorname{erf}\left\{\frac{z-d}{2\sqrt{\kappa t'}} - \alpha\sqrt{\kappa t'}\right\} \quad (4)$$

with the notation

$$\operatorname{erf} x = 2/\sqrt{\pi} \int_0^x d\xi \cdot \exp(-\xi^2) \quad (5)$$

The integration with respect to time was carried out numerically. To calculate the temperature distribution  $T(\rho, t)$  in the crystal a number of parameters must be known. These are the thermal conductivity  $\kappa$ , the absorption coefficient  $\alpha$ , the maximum intensity  $p_0$  and the radius  $w$  of the  $e^{-2}$  points in the sample plane.

The thermal conductivity was determined by examining the behaviour of the thermal relaxing of homogeneously heated hemoglobin crystals as a function of their thickness [16]. A value of  $\kappa = 0.17 \text{ mm}^2/\text{s}$  was found. Because the crystals show double refraction birefringence, the absorption coefficient  $\alpha$  depends on the orientations between the vector  $\vec{E}$  of the polarized light beam and the crystal axes.

From the isotropic extinction coefficient of methemoglobin determined from solution measurements, and the known angles between the heme planes and the crystal axes [12], the crystal extinction coefficients may be calculated.

We get  $\epsilon_{b633} = 4400 \text{ l}/(\text{mol} \cdot \text{cm})$  and  $\epsilon_{a633} = 2000 \text{ l}/(\text{mol} \cdot \text{cm})$  for light polarized parallel to the  $b$  and  $a$  crystal axes, respectively. With the concentration of  $5 \times 10^{-2} \text{ mol/l}$  of methemoglobin in the crystal [12], the absorption coefficients are  $\alpha_{b633} = 5.06 \times 10^4 \text{ m}^{-1}$  and  $\alpha_{a633} = 2.3 \times 10^4 \text{ m}^{-1}$ .

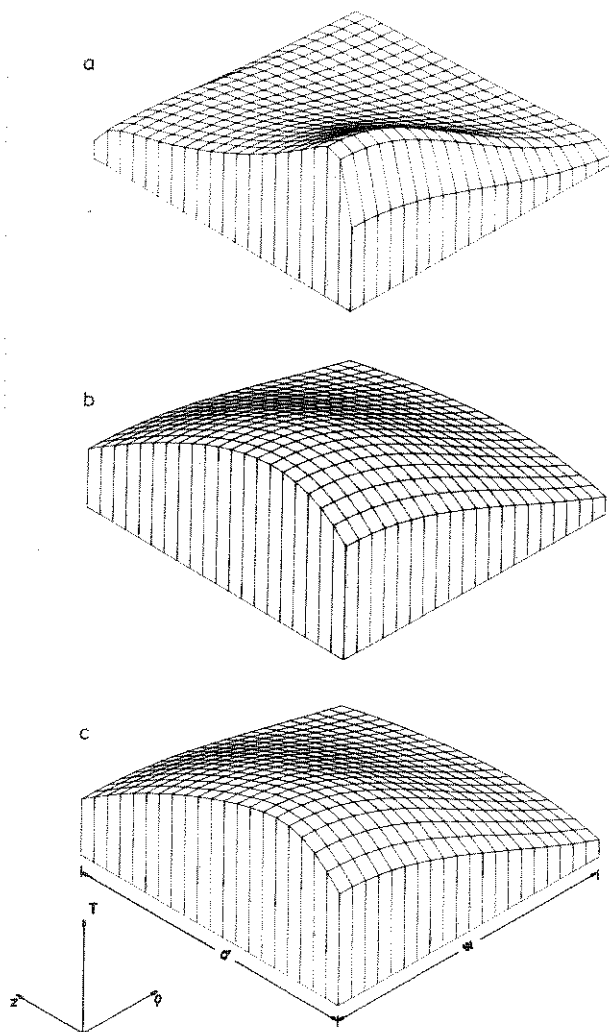


Fig. 3. Temperature profiles of a layer with constant absorption coefficient ( $\alpha = 5 \times 10^4 \text{ m}^{-1}$ ,  $d = 30 \mu\text{m}$ ) irradiated by a laser beam with  $2w = 60 \mu\text{m}$ . The temperature profiles are shown for three periods after beginning the irradiation: (a)  $t = 10 \mu\text{s}$ ,  $T_{\text{max}} = 0.09 \text{ K}$ ; (b)  $t = 1 \text{ ms}$ ,  $T_{\text{max}} = 3.0 \text{ K}$ ; (c)  $t = 500 \text{ ms}$ ,  $T_{\text{max}} = 6.9 \text{ K}$ .

For three values of  $t$ , the time from the beginning of the irradiation, the temperature profiles  $T(\rho, z)$  in a methemoglobin single crystal were calculated, and are shown in Fig. 3.

At  $t = 10 \mu\text{s}$  (Fig. 3a) the temperature diffusion has hardly influenced the temperature distribution, the profile is given by the intensity distribution of the light beam in the crystal. At  $t = 1 \text{ ms}$  (Fig. 3b) the temperature profile is essentially determined by heat diffusion, and the final state is almost reached as can be seen by comparison with Fig. 3c. The chemical relaxation according to the temperature rise  $T$  in the crystal is detected by the change of the intensity of the light beam after it has passed the sample. The temperature rise in the irradiated volume as well as the laser beam intensity are functions of  $\rho$  and  $z$ . So we have to define an apparent temperature rise  $T_{\text{app}}$  in the following way: the averaged values  $\overline{T(\rho)}$  with respect to  $z$  are weighted with the laser beam profile  $I(\rho)$  to yield

$$T_{\text{app}} = \frac{\int_0^{\infty} \overline{T(\rho)} \cdot I(\rho) \cdot \rho \cdot d\rho}{\int_0^{\infty} I(\rho) \cdot \rho \cdot d\rho} \quad (6)$$

$T_{\text{app}}$  and the maximum value of  $T_{\text{max}}$  are given in Fig. 4 for a number of irradiation geometry parameters as a function of time. The only variable we can measure is  $T_{\text{app}}$ . The knowledge of the ratio of  $T_{\text{max}}/T_{\text{app}}$  is important to avoid partial denaturation in protein samples. This ratio can be calculated. We get  $T_{\text{max}}/T_{\text{app}} < 1.4$  for a crystal of  $50 \mu\text{m}$  thickness, irradiated with light polarized parallel to the crystal  $b$ -axes.

## Results

As a test of the method presented here, the value of  $T_{\text{app}}$  was measured as a function of time. It is known that methemoglobin in solution shows a fast relaxation process [17], which could also be observed in methemoglobin single crystals [16]. For  $\lambda = 633 \text{ nm}$  the relaxation amplitude was determined by the authors to be  $\Delta E/(E\Delta T) = 2.2 \times 10^{-3} \text{ K}^{-1}$  and  $1.9(\pm 0.2) \times 10^{-3} \text{ K}^{-1}$ , respectively. The relaxation rates in both cases were greater than  $10^5 \text{ s}^{-1}$ . This relaxation process is used to determine the temperature rise  $T_{\text{app}}$  in the crystal. Because of the great relaxation rate, the absorbance change will follow the temperature rise directly. Assuming that this fast relaxation process is the only process detectable in the time range up to 1 s, the time course of the absorbance change  $\Delta E(t)$  should directly follow the temperature rise  $T_{\text{app}}$  in the sample. Fig. 4 shows the resulting  $T_{\text{app}}$  in a crystal of  $23 \mu\text{m}$  thickness in a time range up to 1 s. Excellent agreement is obtained between this experimental time course and the calculated plots of  $T_{\text{app}}$ , so we conclude that the developed theory is adequate to describe the observed temperature rise. Examinations in time ranges up to 1000 s gave no evidence that convection processes in the mother liquor covering the crystal contribute to the heat transport from the irradiated crystal volume. So a great stability of the temperature in the irradiated

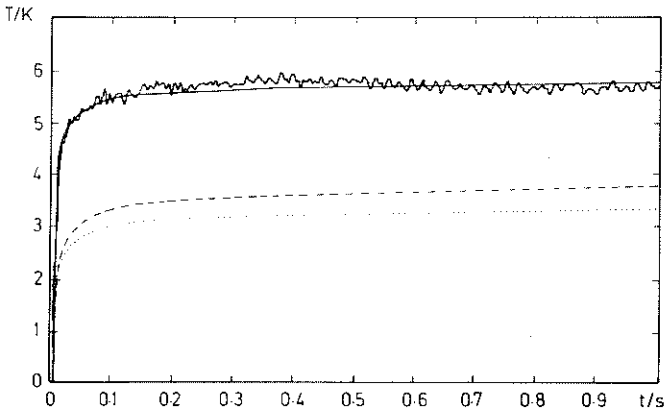


Fig. 4. The temperature rise  $T_{app}$  as a function of time calculated for a laser beam power of 1 mW. The parameters are: (—)  $2w = 44 \mu\text{m}$ ,  $d = 23 \mu\text{m}$ ,  $\alpha = 5.1 \times 10^4 \text{ m}^{-1}$ ,  $T_{max}/T_{app} (t = 1 \text{ s}) = 1.28$ . The noisy figure is the experimentally determined  $T_{app}$  for a hemoglobin crystal with the geometrical parameters given above. (-----)  $2w = 90 \mu\text{m}$ ,  $d = 50 \mu\text{m}$ ,  $\alpha = 5.1 \times 10^4 \text{ m}^{-1}$ ,  $T_{max}/T_{app} (t = 1 \text{ s}) = 1.38$ , and (.....)  $2w = 90 \mu\text{m}$ ,  $d = 23 \mu\text{m}$ ,  $\alpha = 5.1 \times 10^4 \text{ m}^{-1}$ ,  $T_{max}/T_{app} (t = 1 \text{ s}) = 1.32$ .

volume element is achieved if the laser output power is constant in the time range under investigation. With a spot diameter of  $2w = 90 \mu\text{m}$ , the experimentally determined value  $T_{app}$  increases linearly with increasing beam power up to about 2 mW resulting in a temperature rise  $T_{app}$  of 6 K.

#### *Application to the interaction of methemoglobin single crystals with ligands*

The water content of methemoglobin single crystals is 52% [3], and the heme group is accessible to a number of different ligands [4–6,12]. In the following we shall discuss diffusion as the movement of the ligand to the active site of the hemoglobin through a water phase. After a temperature jump in a volume element of the hemoglobin crystal, this area serves as a source or sink of ligands according to the change of the chemical equilibrium. Since the volume of the irradiated crystal element is much less than the volume of the whole sample (crystal plus covering mother liquor), the surroundings of the heated area act as a ligand buffer, and the initial concentration will again be achieved by a diffusion process. For an estimation of the relaxation time of this diffusion process, the irradiated volume element is approximated by a sphere of radius  $w$ . We have calculated the time course of the average concentration  $\bar{c}$  in this sphere, whose initial uniform concentration had been  $c_i$ , while its final concentration is  $c_f$ :

$$\begin{aligned} c &= c_i \neq 0 & \text{for } 0 < w & \text{ and } t = 0 \\ c &= c_f \neq 0 & \text{for } r = w & \text{ and } t = 0 \end{aligned} \quad (7)$$

where  $c_i$  may be larger or smaller than  $c_f$ . With this initial and boundary condition, the solution of the diffusion equation is [13]:

$$\frac{c_i - \bar{c}}{c_i - c_f} = 1 - \frac{6}{\pi^2} \sum_{h=1}^{\infty} \frac{1}{h^2} \exp(-h^2 \pi^2 D t / w_0^2) \quad (8)$$



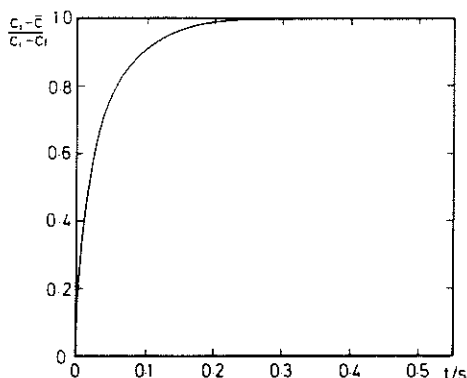


Fig. 5. Average concentration for a sphere of radius  $w = 22 \mu\text{m}$  according to Eq. (8).

$D$  is the diffusion coefficient of the ligands in water and is set to  $D = 10^{-3} \text{ mm}^2/\text{s}$ . The time course of the term  $(c_i - \bar{c})/(c_i - c_f)$  is shown in Fig. 5 for a sphere of radius  $w = 22 \mu\text{m}$ . After a time of 0.1 s, the average concentration  $\bar{c}$  in the sphere is already 90% of the final concentration  $c_f$ . So, if we deal with chemical relaxations with relaxation times  $\tau$  greater than 0.1 s, the concentration of the ligands in the irradiated volume element is practically constant.

Assuming a simple biomolecular reaction between the methemoglobin (methb) and the ligand L



the concentration dependence of the inverse of the relaxation time for the sample geometry discussed above is of the form

$$1/\tau = k_{-1} + k_1[\text{L}]. \quad (10)$$

For relaxation times  $\tau$  in the range of 0.1 s and less, the diffusion process has to be taken into account leading to a modified relaxation equation [18].

#### *The reaction with thiocyanate*

Transient records of temperature jumps for horse methemoglobin single crystals plus  $\text{SCN}^-$  were obtained by following the change in absorbance of the laser light. No transients were observable for buffer plus  $\text{SCN}^-$  alone. Methemoglobin single crystals alone show relaxation processes with negative values of  $\Delta E/\Delta T$  and with typical relaxation times of  $\tau > 10 \text{ s}$  and  $\tau < 100 \mu\text{s}$ , time ranges which are far apart from the observed methb – SCN relaxation times. A typical transient record of the reaction in 1.2 M  $\text{Na}_2\text{SO}_4$  with 30 mM NaSCN is shown in Fig. 6. For  $t > 0.05 \text{ s}$  the data points are excellently fitted by a single exponential over an amplitude range of about two decades. For  $t < 0.05 \text{ s}$  the data points deviate from this exponential. The amplitude of this second relaxation depends on the concentration of SCN. Since the heating relaxation (cf. Fig. 4) as well as the diffusion relaxation (Fig. 5) fall into this

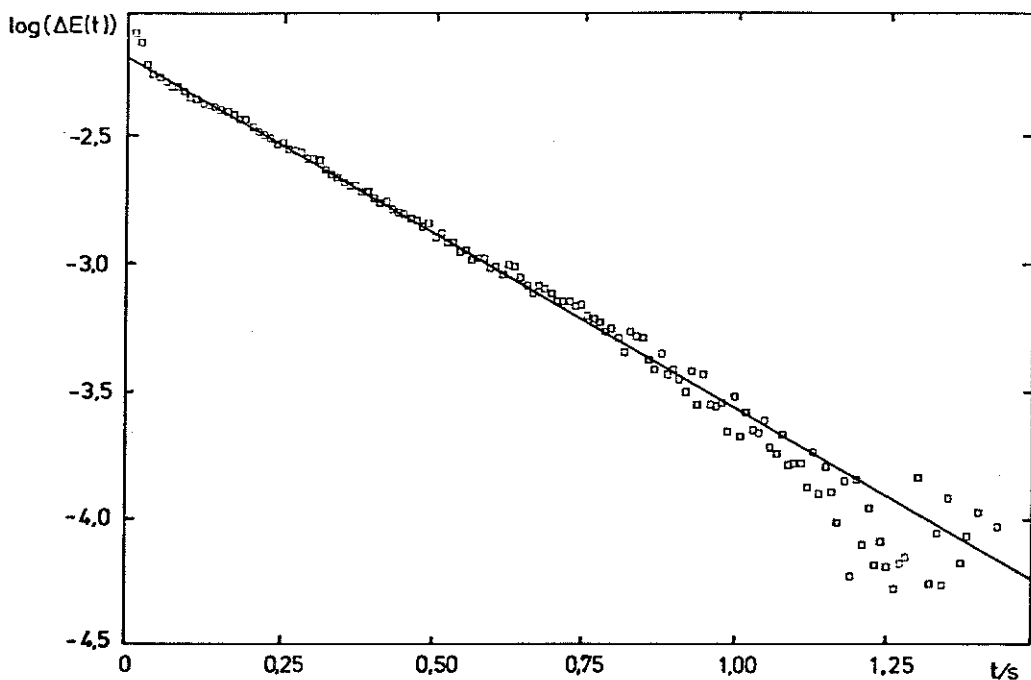


Fig. 6. The logarithm of a typical transient record of the reaction of crystallized methemoglobin in 1.2 M  $\text{Na}_2\text{SO}_4$  with 30 mM NaSCN. The change in extinction  $\Delta E(t)$  is defined as  $\Delta E(t) = E(t = \infty) - E(t)$ . The other parameters are: extinction of the crystal  $E = 0.54$ , temperature  $T = 292$  K, temperature rise  $T_{\text{app}} = 3.2$  K.

time range, we have to consider these processes as the reason for this deviation. A calculation shows that both processes lead to a deviation from the single exponential reducing the absolute value of the slope in this time range. Instead of this, we

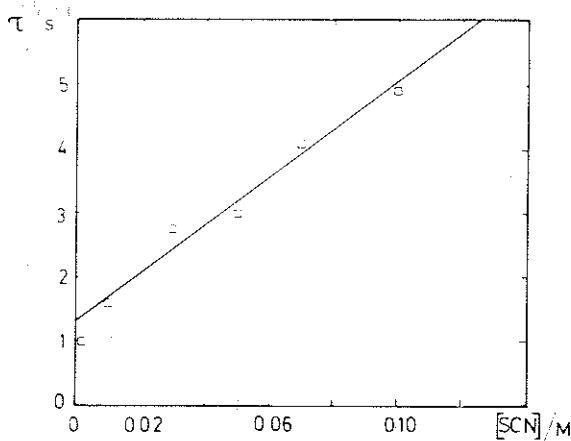


Fig. 7. Dependence to  $1/\tau$  on  $[\text{SCN}^-]$ .

observe an absolute value of the slope greater than that in the range  $t > 0.05$  s. These facts lead to the conclusion that we are observing at least a biphasic transition. This conclusion may be supported by the observations that the time course of the combination of SCN with methemoglobin in solution is heterogeneous [8,9]. Correspondingly, the velocity constant for this reaction with the isolated ferric chains is different, being higher for the  $\beta$  chains. The rate constant of the fast process in our experiment cannot be resolved because it coincides with or is even greater than the rate of heating.

The inverse of the relaxation time obtained for the slow process is shown in Fig. 7 as a function of  $[\text{SCN}^-]$  in the mother liquor.  $\tau^{-1}$  appears to be linearly dependent on the ligand concentration. The value for the rate constants obtained from the graph are  $k_{-1} = 1.3 \text{ s}^{-1}$  and  $k_1 = 38 \text{ M}^{-1} \cdot \text{s}^{-1}$  and the resulting  $K_D = k_1/k_{-1} = 29 \text{ M}^{-1}$ .

## Discussion

The agreement between the calculated average temperature rise and the observed temperature rise is very good (cf. Fig. 4). This agreement indicates that the computer simulation gives a valid representation of the laser heating method. For the absorption geometry given above, this method may be applied if one restricts the investigations to relaxation processes with rates less than  $10^2 \text{ s}^{-1}$ . Other values of the absorption coefficient  $\alpha$  and beam radius  $w$  would further shorten the typical heating times. The temperature rise obtained is large enough, the construction is simple, and the operation is stable, a condition which is important for the investigation of very long relaxation times. A relaxation process with rates of  $1/\tau < 0.01 \text{ s}^{-1}$  in acid methemoglobin single crystals is presently under investigation [16]. The fact that the heating beam is simultaneously used to detect the relaxation to the new chemical equilibrium makes it possible to limit the T-jump to a very small volume element of the sample. The detection limit is of the order  $\Delta E = 10^{-4}$  (with an electronical bandwidth of 1 kHz) and may further be reduced by using a single mode laser. This method might be used to investigate chemical equilibria in protein single crystals or living cells. For the examination of chemical reactions between indiffusible and diffusible substances the diffusion process has to be taken into account leading to a reduction of the upper limit of the detectable relaxation rates.

As an application the association of crystallized horse methemoglobin with  $\text{SCN}^-$  is examined. The results indicate that these substances combine in a heterogeneous reaction as is reported for this reaction in solution [8,9]. The slower process is excellently fitted by an exponential over an amplitude range of about two decades. Its relaxation rate is linearly dependent on the concentration of SCN in the mother liquor as is observed with the slower process in solution [9]. A further comparison of the reactivity in solution and in the crystal requires additional experiments concerning the influences of ionic strength and pH.

## Simplified description of the method and its applications

A laser temperature jump apparatus is described where the T-jump is achieved by means of the direct absorption of continuous laser radiation by a solid sample. The final temperature in the irradiated volume element is reached when the absorbed radiation power equals the dissipation of heat by heat conduction. The heating laser beam is simultaneously used to detect the relaxation to the new chemical equilibrium in the samples. Relaxation processes with relaxation rates between  $10^2 \text{ s}^{-1}$  and less than  $10^{-3} \text{ s}^{-1}$  in samples with volumes less than  $10^{-3} \text{ mm}^3$  may be investigated using this T-jump method. One application of this method is the determination of reaction rates of ligand reactions in hemoglobin or myoglobin single crystals.

## Acknowledgements

I would like to thank Professor Dr. A. Redhardt who supported this work with his experience of many years in questions of biophysical methods. The assistance of Mrs. H. Schlegel in preparation of the manuscript is gratefully acknowledged.

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