Spectral characteristics of the blood of streptozotocin diabetic rats using photoacoustic technique

T. T. Shreedevi, P. S. Padayatti*, M. S. Kala, J. Philip and C. S. Paulose*

University Science Instrumentation Centre and Department of Physics, *Centre for Biotechnology, Cochin University of Science & Technology, Cochin 682 022, India

Optical absorption characteristics of rat blood affected by diabetes has been studied using photoacoustic (PA) technique. PA spectrum of blood depends on the molecular structure of haemoglobin. The peak value ratio γ/β increases with increase in the diabetic state. Externally added glucose to normal blood does not show any increase in γ/β ratio as seen in the diabetic condition. The increase in γ/β ratio may be due to the decrease in DPG level and the resultant shift from $R \rightarrow T$ conformation of majority of diabetic haemoglobin.

PHOTOACOUSTIC spectroscopy (PAS) is a valuable technique increasingly used for analysing biological samples[1-4]. It enables one to obtain spectra, similar to optical absorption spectra, of any type of solid, semi-solid or liquid material. Its capability lies in the fact that absorbed light is converted into sound through nonradiative deexcitation of the sample. Several samples which are highly light-absorbing or light-scattering cause many difficulties in conventional spectroscopic techniques; but they do not cause any problem in PAS. Many biological samples occur naturally in a soluble state, but many others are bound membranes or part of bone or tissue structures. PAS is a useful technique for such samples. Because of its capability to provide optical data on intact biological material and even on optically opaque materials, PAS holds great promise both as a research and diagnostic tool in biology and medicine.

Photoacoustic technique has earlier been used to investigate the optical absorption characteristics of human blood[5,6]. The oxygen-carrying protein of red blood cells, haemoglobin, displays a strong and characteristic absorption spectrum[7]. PA spectrum of a smear of whole blood exhibits the characteristic spectrum of oxyhaemoglobin as clearly as in PA spectrum of red blood cells and even of the extracted haemoglobin itself. So it is possible to study haemoglobin directly in whole blood *in situ*, without extraction procedures. PA detection has been used to study oxygen-haemoglobin interaction on a red cell suspension[8]. Poulet *et al.*[9] measured the sedimentation of red cells in the plasma in

less than two minutes by recording the time evolution of the PA signal produced by a whole blood sample. PA detection offers a means to study the size of red cells and the blood viscosity rather than the aggregation of erythrocytes as observed by the usual sedimentation methods[10,11]. From the PA spectra, taking peak ratio as a method for interpreting molecular changes especially for heme in disease condition where the haemoglobin is affected by malaria parasites and with the addition of drugs has been reported[12]. There are some distinct differences in the PA spectra from the blood of patients suffering from anemia, leukemia and methemoglobin compared to those of healthy people[2,5]. PA technique can be used conveniently and quickly to diagnose the haemotonosis with the lower density of haemoglobin and haemoglobinopathy with the abnormal structure of haeme. The clinical and pathological potentials of PAS is expected to be clear in the near future as considerable research is currently underway in this area.

In this work we have carried out a systematic investigation of the optical absorption characteristics of rat blood affected during various stages of diabetes using PA technique. The results point to the use of PA technique as a diagnostic tool to be used with patients affected by diabetes. In the following we present the experimental details and the results obtained.

Experimental

The optical absorption spectra of rat blood samples affected by varying stages of diabetes have been recorded with a photoacoustic spectrometer.

Sample collection

For all the experiments female Sprague-Dawley rats of the same age and weight (≈ 200 g) were used. The rats were induced diabetic by a single infrafemoral vein injection of Streptozotocin (35 mg/kg of body weight) which was dissolved in citrate-buffered vehicle of pH 4.5. Rats injected with vehicle alone served as controls.

The rats were provided with food and water *ad libitum*. The blood samples were drawn from tail between 9 and 10 hours and the blood glucose was estimated using glucose oxidase enzyme kits (Merck). Rats with blood glucose above 100 mg/dl were used for all experiments. The experiments were later repeated with male rats.

Photoacoustic spectrometer

The PA spectrometer consists of a 1 kW high pressure Xenon arc lamp (Müller electronik-optik model SVX 1000) as the polychromatic light source. A grating monochromator (Oriel model 7240) selects the wavelength of the incident radiation which is intensity-modulated with a mechanical chopper (PTI model OC4000) before falling on the sample kept inside the photoacoustic cell. The photoacoustic cell has a sample compartment and a separate microphone compartment which are acoustically coupled. The volume of the sample compartment can be adjusted to have maximum amplitude for the generated photoacoustic signal. PA signals detected by the microphone (Knowles model BT 1759 electret microphone) were analysed with a single phase lock-in amplifier (Stanford model SR510).

Experimental procedure

The photoacoustic spectrometer was mounted on a vibration-free table. The sample dish made of glass which can be inserted in the sample compartment, was thoroughly cleaned. The spectrometer was calibrated with carbon black as the sample and the power spectrum of the light source was recorded. The experiment was then done with the samples. The photoacoustic amplitude for different incident wavelengths was measured and normalized with the power spectrum of the source. The normalized PA amplitude was plotted against wavelength. Experiments were repeated for all the samples discussed below.

A modulation frequency of 20 Hz was used. The optical absorption coefficient of blood is not sufficiently large for the thermal diffusion length to be greater than the optical path length at this frequency[1].

Experimental results

The photoacoustic spectra of the blood of a number of normal as well as diabetic rats have been recorded with an accuracy of 4%. As has been observed by Rosencwaig[1] in human blood, we have observed three peaks, viz. a γ-peak between 390 and 410 nm, a β-peak between 520 and 540 nm and an α-peak between 555 and 575 nm in all the blood samples. A range of values is given rather than a single wavelength for the three peaks because of experimental limitations. Typical PA
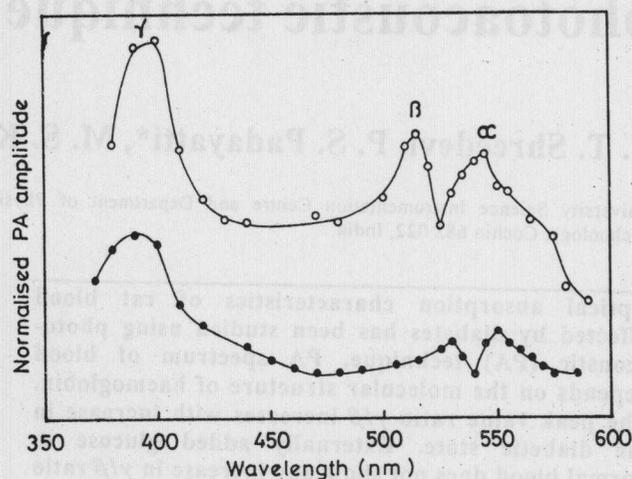

Figure 1. Photoacoustic spectra of the blood samples of a slightly diabetic rat (glucose level –181.51 mg/dl) (-o-) and a highly diabetic rat (glucose level –536.69 mg/dl) (-•-)
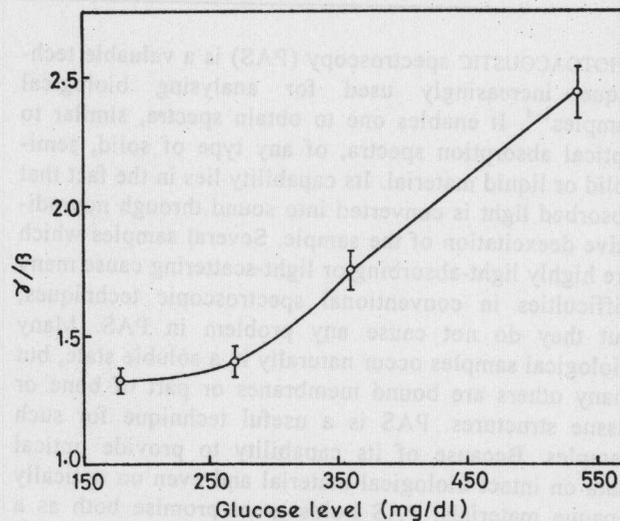

Figure 2. Variation of γ/β ratio with blood glucose level.

spectra of the blood samples of a slightly diabetic rat and a highly diabetic rat are shown in Figure 1 with the γ, β and α peaks indicated. The experiments have been repeated on diabetic rat blood at various blood glucose levels. Experiments have been repeated with male rat blood as well and it is found that the results are similar in all respects. Although the spectra shown in Figure 1 indicate that normal rat blood gives rise to stronger PA signal, it is not of much significance because the PA signal amplitude very much depends on the experimental arrangement. What is of interest to us is the ratio γ/β as explained below.

From the plotted PA spectra, the peak ratio γ/β was determined from each of the plots obtained for the same

rat with different levels of diabetes. In Figure 2 we plot the variation of the photoacoustic amplitude ratio γ/β as a function of the blood glucose level in one rat. It can be seen from Figure 2 that γ/β increases with the blood glucose level. Similar results were obtained from a number of rats, including male ones, confirming that the result shown in Figure 2 is reproducible.

The optical absorption spectrum of diluted rat blood was also recorded with a UV-Vis-NIR spectrophotometer. The position of the three peaks could also be identified in these spectra but a quantitative analysis is difficult, indicating the superiority of the PA technique over conventional absorption spectroscopy in the study of biological systems.

To verify whether glucose added externally to blood gives rise to similar variation for the γ/β ratio, we investigated the variation of PA spectrum of four normal blood samples of the same rat with different concentrations of externally added glucose. γ/β ratios in this case have random values lying in the same range without any systematic increase with glucose level. This leads to the conclusion that the variation of γ/β with glucose level (Figure 2) is clearly due to the diabetic condition of rats.

Discussion

The present results show that as the diabetic state advances, the PA spectrum of blood shows changes resulting in a net increase in the γ/β ratio. But external addition of glucose to normal blood does not show any systematic increase in γ/β ratio as seen in diabetic rats.

Since Ditzel[13] first demonstrated functional changes in the microcirculation of diabetes, many alterations in circulating constituents have been reported. But how much of these changes can be attributed to insulin deficiency and how much to hyperglycemia is yet to be clarified. In long-standing diabetes, glycosylation of haemoglobin has been reported[14] due to interaction of glucose with the β-chain of haemoglobin at the N-terminal valine residue[15]. Such nonenzymatic interaction between the aldehyde or ketonic groups of sugars with amino groups, called Maillard reaction, has been demonstrated. A similar explanation was offered in the case of other body proteins, such as those which accumulate in the renal mesangium or those in diabetic nerve, which lead to delayed conduction velocity or to delayed axo-plasmic flow[16]. But all these diabetes-related complications can be observed in long-standing diabetes. Our observations show that changes in γ/β ratio can be seen during the first week of diabetes itself.

Diabetic haemoglobin shows decreased oxygen-carrying capacity, which can be indirectly attributed to decreased levels of 2,3-diphosphoglycerate (DPG)[13]. The action of DPG is brought about by stabilization of deoxyhaemoglobin quarternary structure R (relaxed or oxy form)[17] by crosslinking the β-chains[18]. The PA spectral characteristics of haemoglobin can be directly attributed to structural characteristics of the molecule. In the diabetic state concentration of DPG molecule is less and most of the haemoglobin molecules remain in quarternary structure from T (tense or deoxy form). This R → T shift of the majority of haemoglobin molecules increases as a result of decreased level of DPG, giving rise to a change in the PA spectrum and an increase in the γ/β ratio. Thus our studies show that the altered spectral characteristics of haemoglobin is not due to glycosylation of haemoglobin. We attribute the observed increase in γ/β ratio in the PA spectrum to binding of molecules like DPG and the altered conformation of haemoglobin molecule.

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