[*Photoacoustic spectroscopy (PAS) involves the acoustic monitoring of the nonradiative deexcitation mode of energy that follows the initial light absorption by a sample. PAS thus offers a nondestructive method of studying absorption spectral features of optically 'opaque' samples such as solids, surfaces and intact biological specimen such as cells, tissues and microorganisms in vivo. Prof. D. Balasubramanian, who has been actively pursuing PAS studies on chemical and biological systems, discusses some of the biological applications in this article. He discusses the use of PAS in screening cells for chromophores, studying the interaction of drugs on microorganisms, measuring the thermal properties of the specimen, analysing photochemical processes for their energy storing steps, reaction times and the calorimetry of the photoreactions as well as measuring the action spectrum of photoprocesses such as solid-state photopolymerization reactions and photosynthesis.*]

BIOLOGICAL APPLICATIONS OF PHOTOACOUSTIC SPECTROSCOPY

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WHEN light of appropriate energy falls on a sample, the latter may absorb the light and be excited to higher energy states. The wavelength dependence of this process forms the basis of optical absorption spectroscopy. The intensities of the light incident on (I_0), and transmitted (I) from the sample are measured, and the ratio yields the absorptivity of the sample. The absorbing system has, in principle, several options open for it in the excited state: (a) it might emit light and return to the ground state, *i.e.* luminescent decay, (b) it might utilise the energy stored to undergo photoprocesses, *e.g.*, photochemical reactions or transfer the energy to another molecule (energy transfer), or (c) the system might dissipate the energy through nonradiative modes of relaxation simply as heat and return to the ground state. Since many molecules neither luminesce nor undergo any photoinduced transformations, the de-excitation mode they adopt for returning to the ground state is that of nonradiative (heat) decay. It can thus be seen that information can be obtained about the absorption spectral features of molecules, either by the absorption spectroscopic method or by a measurement of the heat emanating from the sample as a function of wavelength ('photothermal' method).

While conventional optical absorption spectroscopy is easy, accurate and has a high resolution, it is beset with difficulties when the transmitted photon intensity (I) has to be measured from samples that are not transparent. Samples in the solid state, gels, opaque materials, surface coatings, colloids or biological specimen tend to scatter the light incident on them and hence the collection and measurement of photons transmitted from the sample after partial absorption are prone to error. Special sample pretreatment and the measurement of the amount of light reflected from the sample are resorted to in the diffuse optical reflectance spectroscopic method, in the case of certain types of samples, in order to

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study their optical absorption characteristics.

The alternate method of measuring the wavelength dependence of the amount of heat emission upon photon incidence was originally devised by A G Bell in 1880, who termed it the photophone method. It has since been revived, improved upon, renamed as photoacoustic (PA) spectroscopy and has been used with considerable success during the last eight years on optically difficult samples.

THE PRINCIPLE

In photoacoustic spectroscopy (PAS), the incident light that falls on the sample is 'chopped', or intensity-modulated, at an acoustic frequency (usually 10–2000 Hz) using mechanical or electronic methods. The molecules of the sample, upon absorbing light of appropriate energy, will be excited to higher states. When the system relaxes to the ground state *via* the nonradiative mode, the consequent heat liberated will diffuse from the points of absorption; when it reaches the sample surface, it will cause temperature changes in the immediate gas medium (usually air) that surrounds the sample. Since the light incident on the sample is modulated at an audiofrequency, absorption and the subsequent temperature changes will also be modulated at the same audiofrequency. Such a periodic thermal change will result in pressure oscillations in the gas layer surrounding the sample, which would act as an acoustic piston leading to an acoustic signal as the final consequence of light absorption by the sample; this phenomenon is termed the PA effect. The PA signals are detected by a sensitive microphone attached to the sample cell and amplified by a lock-in amplifier which, apart from improving the signal/noise ratio, enables one to measure both the magnitude of the PA signal and its phase with respect to that of the light source modulation signal. The wavelength dependence of the PA signal of the sample yields the PA spectrum which, under appropriate conditions, corresponds to its optical absorption spectrum. Since it is the heat effect that is measured and not photons, samples that scatter light are amenable to PAS. *In situ* and *in vivo* spectroscopy is thus possible using the PA effect; several recent reviews discuss the details, methodology and applications of PAS[1–4].

The types of information obtained by PAS are (i) absorption spectrophotometric, (ii) thermal, such as thermal diffusivity of the sample and calorimetry of photo-initiated processes, (iii) temporal, such as decay times of photointermediates, excited state lifetimes or delay times of signals from deeper layers of the sample, (iv) photochemical, such as energy storage in photochemical intermediates and the action spectra of photoreactions, (v) luminescence efficiencies, *i.e.*, absolute fluorescence quantum yields in solids, (vi) depth profile analysis of chromophores in solids and (vii) phase transitions occurring in the sample.

Ganguly and Rao[4] have recently reviewed some of the theories and applications of PAS to solids and surfaces. In our laboratory we have employed PAS for monitoring the acidity of the catalyst surfaces for the first time and have detected three acidic sites in a silica-alumina catalyst by this method[5]. In this article, we shall illustrate the versatility and the applications of PAS specially in biological areas, quoting from published work of recent years, as well as from work currently in progress in our own laboratory. The PAS method is far more convenient and quicker than conventional methods. Some other studies on catalyst surfaces have been reported by Jagannathan *et al.*[6]

SPECTRA *IN VIVO*

In figures 1 and 2 we illustrate the PAS of two intact biological specimens, recorded in our laboratory. Figure 1 is the *in vivo*

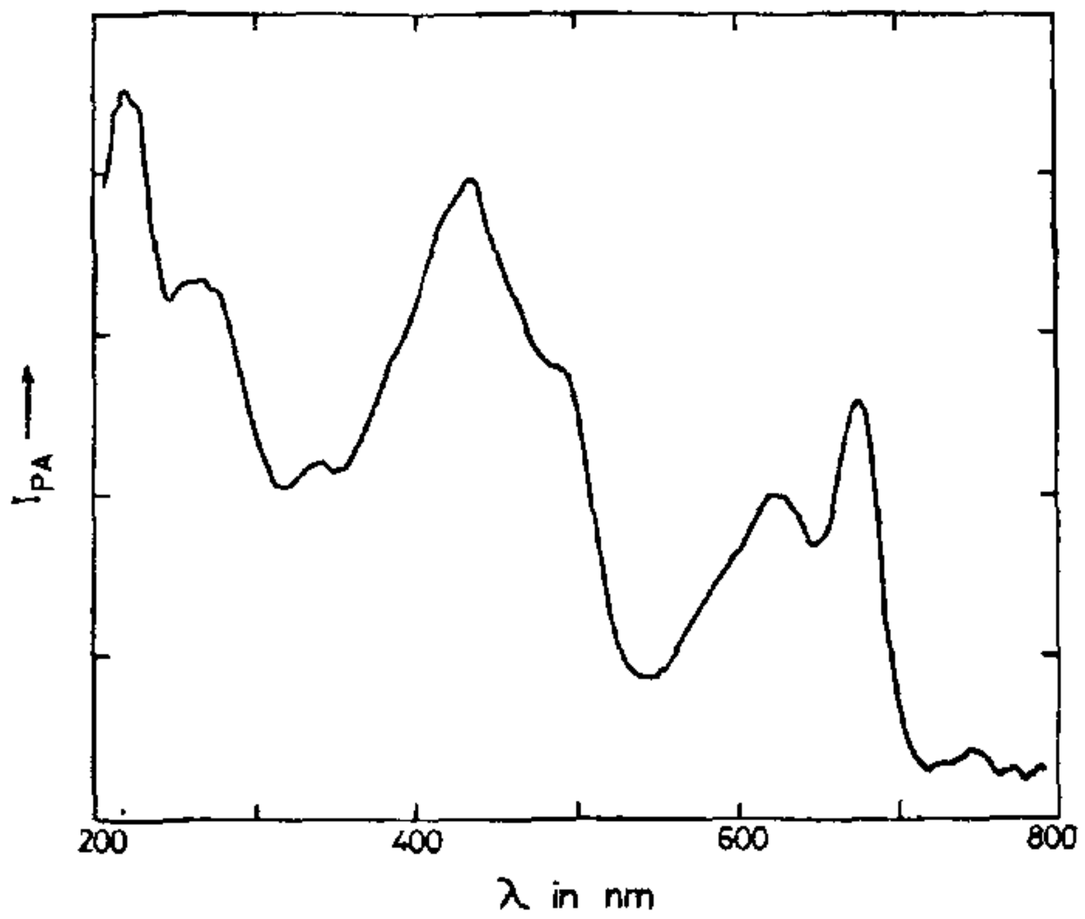

Figure 1. Photoacoustic spectrum of aqueous suspensions of the blue-green bacteria *synechococcus sp.* run in the E. G. & G Princeton Applied Research Corp. model 6001 photoacoustic spectrometer. Room temperature; modulation frequency 40 Hz.

spectrum of the blue-green *synechococcus sp.*, a photosynthesising bacterial cell, that clearly displays absorption spectral bands due to chlorophyll a (660 nm), phycocyanin (615 nm), Soret bands of the porphyrin ring (around 400 nm) and the cellular proteins (280 nm). Conventional absorption spectrosocopy of the intact cell is normally not possible in this range since the size of the cell is comparable (larger) to the wavelength of the radiation, leading to light scattering. The PA spectrum is remarkable for its details and for the fact that no special preparatory procedures are required on the sample for the measurement.

The use of PAS to screen several batches or specimen for such 'fingerprint' spectral features is evident. Indeed, a recent report[7] involves the use of PAS to identify cyanobacteria, diatoms, *thiocapsa sp.*, and *chromatium sp.* on the surface and in an interior layer of algal mats on stromatolite deposits in the North Sea.

Current work in our laboratory on the PAS of *synechococcus* involves analysis of the pigments present, of the nature and thickness of the photoprotective surface layer of the cell, thermal properties of the system, and a bioenergetic analysis of the photosynthetic activity of the intact cell, using some of the methods pioneered by Cahen and coworkers[1].

In figure 2 we show the PAS of the lyophilised cells of the malarial parasite, isolated from mice wherein they were cultured. The effect of antimalarial drugs on the hemozoin pigment contained in the parasite is revealed, implicating a possible mode of action of the drugs on the organism *in vivo*. The PAS results indicate a complex formation between the drugs and the hemozoin pigment in the parasites. They also show the pigment to be hemin-bound to the proteins *in situ* and not free hemin coexisting with free proteins, as suspected by some workers. So far there has been only evidence for the coexistence of (and not interaction between) the drugs and the pigment inside the food vacuoles of the parasite. These new results of this simple comparative PAS study corroborate those

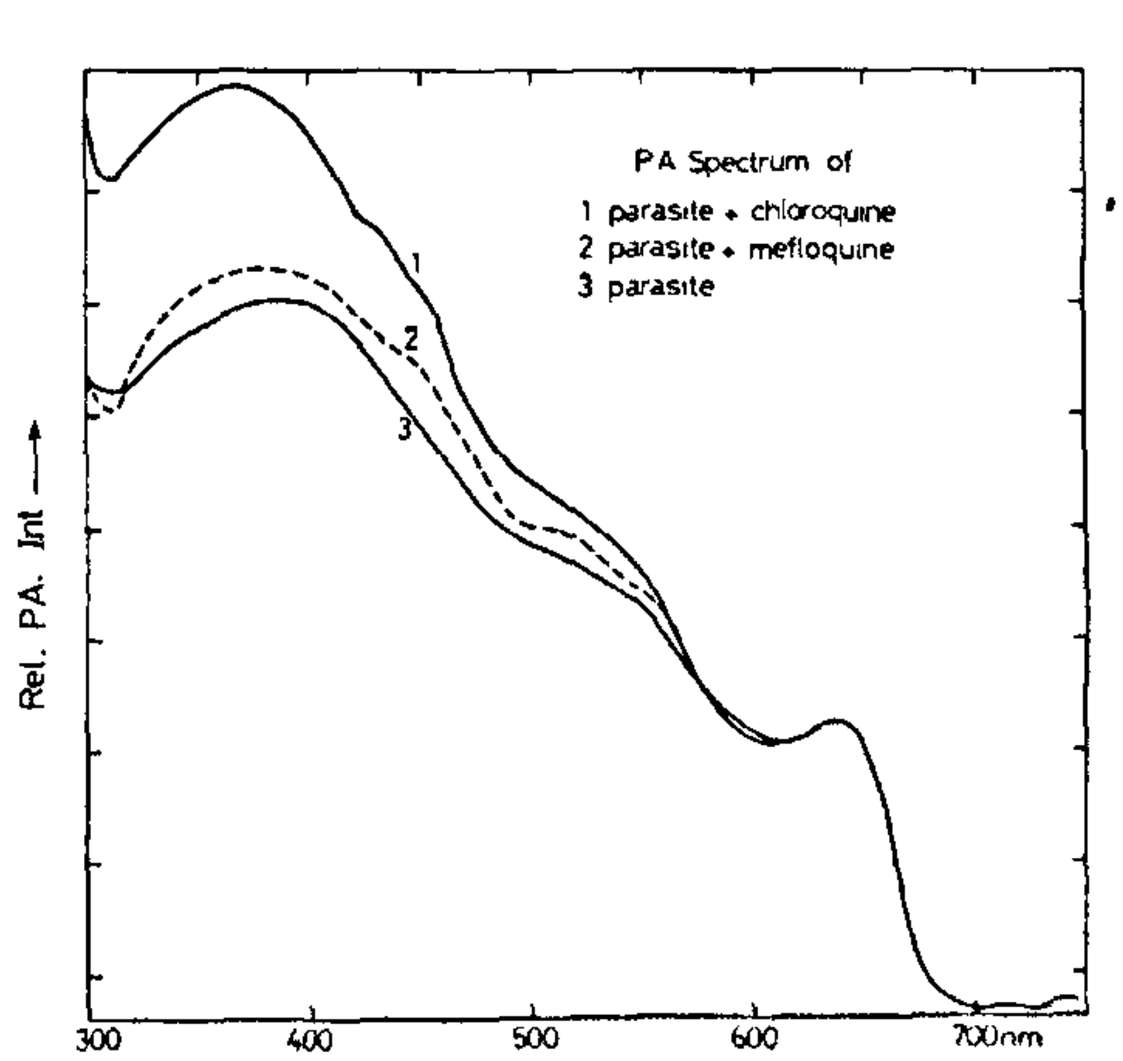

Figure 2. P. A. spectra of isolated and lyophilised cells of the malarial parasite grown in mice. Curves 1 and 2 are for the parasites isolated from mice treated with the antimalarial drugs chloroquine and mefloquine respectively, while curve 3 is for the parasite isolated from untreated mice. All curves have been arbitrarily normalised for the same intensity in the 630 nm region. Same instrument and same operation conditions.

from other detailed spectroscopic studies, currently in progress, namely that the action of the drug need not be on the DNA of the parasite alone, but that direct intermolecular interaction occurs with the pigment.

THERMAL CHARACTERISTICS OF SAMPLES

Quantitative information about the spectral and thermal characteristics of a sample can be obtained upon an analysis of the variation of the PA signal on some relevant parameters; this variation is shown in table I. Notice that PA spectra can be obtained on optically and thermally "difficult" (high β and low μ_s) samples, which under suitable conditions, correspond to their optical absorption spectra. With some samples, it might be possible to reduce the thickness L and make them optically transparent and amenable for well-resolved PAS. With powdered or finely dispersed samples that are highly absorbing, dilution by dispersing in an inert medium (MgO or quartz powders) solves the PA saturation problem. While μ_β is not an alterable experimental parameter, the thermal diffusion length μ_s is. Since it varies as $\omega^{1/2}$, changing the modulation frequency can make a sample thermally thin or thick as needed. Also, the frequency dependence (ω^{-1} or $\omega^{-3/2}$) of I_{PA}, shown in table 1, implies a change of slope in the plot of I_{PA} versus ω at a frequency ω_0 corresponding to the situation when $\mu_s = L$. Thus, from a knowledge of ω_0 and L, it should be possible to obtain the thermal diffusivity α of the sample, using PAS. In an application of this method[8], the thermal diffusivity of the mineral brown tourmaline has been determined to be 0·15 $cm^2 s^{-1}$ (with $L = 0{\cdot}044$ cm, $\mu_\beta = 0{\cdot}08$ cm and $\omega = 25$ Hz), and that of ruby as 0·12 $cm^2 s^{-1}$. In a similar fashion, the μ_s of a leaf of *Artemisia lactiflora* was estimated as 80–120 μm, based on an assumed value[9] of α.

On samples of copper phthalocyanine

TABLE 1

Optical absorption length $\mu_\beta = \beta^{-1}$	Thermal diffusion length $\mu_s = [2\alpha/\omega]^{1/2}$	P. A. Intensity I_{PA}
Optically transparent	(i) *Thermally thin*	
$\mu_\beta > L$	$\mu_s \gg L$, $\mu_s > \mu_\beta$	$I_{PA} \propto \beta L$
	(ii) $\mu_s \geqslant L$, $\mu_s < \mu_\beta$	$I_{PA} \propto \omega^{-1}$
	(iii) *Thermally thick*	
	$\mu_s < L$, $\mu_s \ll L\ \mu_s$	$I_{PA} \propto \beta\, \mu_s$; $\propto \omega^{-3/2}$
Optically Opaque	(i) *Thermally thin*	
$\mu_\beta < L$	$\mu_s \gg L$, $\mu_s \gg \mu_\beta$	I_{PA} Independent of β (PA Saturation)
	(ii) *Thermally thick*	
	$\mu_s < L$, $\mu_s > \mu_\beta$	$I_{PA} \propto \omega^{-1}$
	(iii) *Thermally thick*	
	$\mu_s \ll L$, $\mu_s < \mu_\beta$	$I_{PA} \propto \beta\mu_s$; $\propto \omega^{-3/2}$

β = *Optical absorption coefficient in* cm^{-1}[3,4]; L = *Sample thickness;*
ω = *Modulation frequency;* α = *Thermal diffusivity (= Thermal conductivity / density × specific heat)*

films coated on glass surface, a monomolecular layer of the substance was detectable by PAS. The thickness of a 50-monolayer film of the material was derived to be 40 nm, based on the estimated thermal diffusion length[10].

PHASE ANGLE MEASUREMENTS

Another important handle available in PAS is the phase angle difference, θ, between the measured PA signal and the source modulation frequency. While the conversion of the absorbed photon energy into heat is rapid, the flow of the heat to the sample-gas interface is slower, causing a phase shift $\theta = x\,[\omega/2\,\alpha]^{1/2}$ where x is the distance between the point of heat generation and the surface. Since the heat escape to the surface from different depths of a sample would take different times, correspondingly different values of the phase angle shifts are to be expected. Thus, King and Kirkbright[11] have separately measured the PA spectra of the waxy cuticle surface layer and of the interior chloroplast pigments in an intact spinach leaf, by observing the signals at different θ values. Likewise, Ganguly and Rao[4] have been able to do a depth profile analysis of the red and green pigment layers in an intact croton leaf.

Again since θ varies with ω as shown above, it has been possible to measure the thickness x of polymer layers adsorbed onto glass surfaces, by examining the variation in the phase shift of the PA signal with the modulation frequency[12]. Also, using the simplified relationship $\tan\theta = \omega\tau$, it is possible to estimate the lifetime τ of the excited state. In the case of the 24000 cm^{-1} band seen in brown tourmaline, τ was 0·3 ms, helping in the assignment of the band to be the d-d transition of the constituent Fe^{3+} ions[8].

FLUORESCENCE EFFICIENCIES

In PAS, one measures the efficiency of the thermal decay of the excited state. If the system were to lose its excited state energy by other modes, such as luminescent emission or photochemical processes, the intensity of the PA signal under these conditions would be correspondingly quenched. This feature offers a method of measuring the emission quantum yields of luminescent samples. It follows that the ratio of the PA intensity of a sample under conditions of fluorescence and of the total quenching of its emission (with quencher added) should yield its emission quantum yield. This technique has been used to measure the quantum yields of several compounds in solution, and a slightly modified method to measure the property in the solid state[13]. This point underscores the complementarity of PAS and fluorescence spectroscopy; naturally this would be valid only when other excited state processes, *e.g.* photochemistry or energy transfer do not occur.

PHOTOCHEMISTRY USING PAS

The other possibility of photochemistry in the excited state (and no fluorescence) can be monitored using a similar argument. We illustrate this by describing the solid-state photopolymerisation that occurs in diacetylene monomers upon excitation to a higher electronic state, which we have studied in detail[14] on the compound (3 butyloxy carboxyl methyl urethane)—diacetylene (3-BCMU). This compound, as single crystals or adsorbed on filter paper strips, was *in situ* irradiated by the source lamp of the PA spectrometer and the spectral features of the photoproduct, poly (3-BCMU), analysed to yield a wealth of information on the reaction. The time course of the polymerisation was followed and the rate was found to depend on the amount of monomer taken. Pulse excitation experiments revealed the reaction to be not autocatalysed. The spectra revealed the planar conformation of the polymer and the orientation of the long chains of the molecules

along the major crystal axis. Photocalorimertry of the reaction, using PA methods, showed that the heat liberated in the reaction was offset by the PA heat generated due to the higher absorption by the polymer. Out of this work also has emerged a general method to determine the *action spectrum*, *i.e.* the wavelength dependence of 'photoaction', obtained by comparing the conventional optical absorption (OA) spectrum of the photoreaction with its PA spectral intensity. While the OA intensity of absorption varies directly as the power of the absorbed light (P_{abs}), that of PA varies as (P_{abs}. K) where K is the efficiency of nonradiative return to ground state. A plot of I_{OA}/I_{PA} versus wavelength would show peaks at those wavelengths corresponding to the action spectrum in such cases where excited state photochemistry occurs; in case no photoaction occurs but fluorescence does, the plot locates the excited states from where emission occurs. The action spectrum of the photopolymerisation of 3-BCMU obtained in this manner is more convenient and rigorous, and agrees with that obtained by the conventional sample analysis method.

We have also devised the necessary polariser optics and measured linearly the dichroic PA spectra of oriented systems. That the chains of poly (3-BCMU) are oriented along the major axis of the monomer crystal was concluded by such linear dichroic PA spectral studies.

PAS OF PHOTOSYNTHESIS

Pioneering PAS work on photosynthesis has been done by Cahen and coworkers [15,16], who compared the PA signals of spinach chloroplasts at 680 nm with the corresponding I_{OA}, and concluded that the quantum yield of photosynthesis is low at 720 nm, and that the largest photochemical loss occurs at 680 nm ('the red drop'). Using chloroplast membranes under constant (unchopped) background light as the reference, they could estimate the relative photochemical losses at several wavelengths. This is an excellent system to use as reference since it has the same optical and thermal properties but is not photoactive (saturated), and also since the background light is unchopped, it would not yield any PA signals. The ratio of the energy stored per photon to that absorbed was obtained by dividing the difference between the steady state PA signals of the system when the light was switched on and off by the signal with the background light constantly on. The energy storage so obtained showed the red drop above 690 nm in the presence of an electron acceptor such as methyl viologen. But in the absence of the acceptor, they could see a 'red rise', suggesting that there is a hitherto undetected photoactivity of the Photosystem I, perhaps a cyclic electron flow. Monitoring the fluorescence of the system appears to reflect the state of the Photosystem II as it is balanced by a linear electron flow to Photosystem I.

HALOPHILIC BACTERIA-PHOTOCALORIMETRY

The purple membrane protein bacteriorhodopson (bR) of the bacterium *Halobacterium Halobium* displays, upon illumination, the photocycle $bR_{570} \rightleftharpoons K_{590} \rightarrow L_{550} \rightarrow M_{412} \rightarrow N_{530} \rightarrow O_{660} \rightarrow bR_{570}$, which causes a pumping of H^+ ions to the extracellular medium. Cahen and coworkers have studied this system as well by PA methods[17,18] and measured the modulation frequency dependence of I_{PA} at 550 nm and 415 nm and concluded that the M_{412} intermediate decays slowly (milliseconds). Use of constant side illumination for reference purposes (see above) revealed that the bacterium *in vivo* displayed energy storage when illuminated in the 540–620 nm region of absorption. They have analyzed the PA signals of the

bacterium (corrected with respect to a photoinactive reference) in terms of the modulation frequencies and differentiated the various steps involved in the photocycle and estimated the kinetic constants. This photocalorimetric analysis has shown that the 5 ms step seen was associated with the O_{660} intermediate, the 3 ms step with M_{412}, 1 ms with N_{530} and the fastest one (0·5 ms), which has no optical absorption parallel, has been attributed to a conformational change in the protein. In contrast, the dried membrane fragments did not exhibit any calorimetric changes, and hence, no energy conversions. (A similar use of the PA method for photocalorimetric purposes, in the photopolymerization reaction of a diacetylene has been alluded to the above[14]).

The quantum yield of the photocycle in bacteriorhodopsin has been estimated[19] by PAS to be pH independent and of value 0·3. Comparing this value with that of the proton pumping step (which is pH dependent), they could conclude that 2 protons are pumped per photocycle in acid pH range while 1 proton is pumped out to the medium at alkaline pH.

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References

1. Cahen, D., Bults, G., Garty, H., and Malkin, S., *J. Biochem. Biophys. Methods*, 1980, **3**, 293.
2. Rosencwaig, A., *Annu. Rev. Biophys. Bioeng.*, 1980, **9**, 31.
3. Balasubramanian, D. and Mohan Rao, Ch., *Photochem. Photobiol.*, 1981, **34**, 749.
4. Ganguly, P., and Rao, C. N. R., *Proc. Indian Acad. Sci (Chem. Sci.)*, 1981, **90**, 153.
5. Mohan Rao, Ch. and Balasubramanian, D., *Spectrosc. Lett.*, 1980, **13**, 329.
6. Jagannathan, K., Ganguly, P., Rao, and C. N. R., *J. Catal.*, 1981, (under publication, cited in ref. 4 above).
7. Schubert, W., Giani D., Rongen, P., Krumbein, W. E. and Schmidt, W., *Naturwiss.*, 1980, **67**, 129.
8. Sugitani, Y. and Fujinami, M. *Bull. Chem. Soc. Jpn*, 1981, **54**, 722.
9. Inoue, Y., Watanabe, A., and Shibata, K., *FEBS Lett.*, 1979, **101**, 321.
10. Hursh, D. and Kuwana, T., *Anal. Chem.*, 1980, **52**, 646.
11. King, A. A., and Kirkbright, G. F., *Lab. Practice*, 1976, **25**, 377.
12. Adams, M. J. and Kirkbright, G. F., *Analyst*, 1977, **102**, 678.
13. Adams, M. J., Highfield, J. G. and Kirkbright, G. F., *Anal. Chem.*, 1980, **52**, 1260.
14. Mohan Rao, Ch. and Balasubramanian, D., *J. Phys. Chem.*, 1981 (in press).
15. Cahen, D., Malkin, S. and Lerner, E. I., *FEBS Lett.*, 1978, **91**, 339.
16. Lasser-Ross, N., Malkin, S. and Cahen, D., *Biochim. Biophys. Acta*, 1980, **593**, 330.
17. Cahen, D., Garty, H. and Caplan, S. R., *FEBS Lett.*, 1978, **91**, 131.
18. Garty, H., Cahen, D. and Caplan, S. R., *Biochem. Biophys. Res. Commun.*, 1980, **97**, 200.
19. Renard, M. and Delmelle, M., *FEBS Lett.*, 1981, **128**, 245.