SPECTRA

All Done by Resonance

from our Molecular Biology Correspondent

YET another spectroscopic technique has, it seems, been cast up on the wilder shores of biochemistry. This is a variant, long familiar to spectroscopists, of the Raman effect, the possibilities of which have been greatly widened since the introduction of laser sources. Lest all readers forthwith lower their lorgnettes and look for less rarefied topics on which to exercise their minds, it should be added that the technique, where it is applicable, is devoid of the two great drawbacks of Raman, and for that matter, infrared spectroscopy as applied to biological systems; namely, the complexity of the spectra, and the distressingly large concentrations of the solutions that are needed.

The method makes use of a resonance phenomenon, whereby anomalously large scattering is produced when the wavelength of the incident light falls within an electronic absorption band of the molecule. The intensity is such that spectra can be observed under these circumstances from species present at concentrations several orders of magnitude lower than otherwise necessary. With a choice of rare gas lasers available, there is a good chance of finding a wavelength that fulfills the resonance condition for coloured molecules.

One of the most attractive prospects for this technique is the possibility that it may be used to isolate the Raman spectrum of a ligand binding to a macromolecule, such as a protein, from which something of the nature of the binding and the state of the bound species might then be divined. The first report of an exploration of such a system comes from the stronghold of molecular spectroscopy in Ottawa, and concerns the interaction of methyl orange with bovine serum albumin. Carey, Schneider and Bernstein (*Biochem. Biophys. Res. Commun.*, **47**, 588; 1972) have used an argon laser to excite within the visible absorption band of the dye in aqueous solution at a concentration of only 10^{-5} molar. A series of lines in the Raman spectrum has been assigned in terms of the structure, including azo-group modes, and a phenyl-nitrogen and a C-N stretch. In the solid state, the bands are shifted to shorter frequencies, and the spectrum comes closely to resemble that of the dye in solution in the presence of a large molar excess of albumin.

From this emerges the first conclusion, that in its bound state the dye is in a non-aqueous environment, and therefore presumably seated in a crevasse in the protein. The angular relation of the phenyl groups is evidently unchanged on binding, for any twist about the azo bond (such as has been suggested to occur) would be strongly manifested in shifts of a sensitive frequency. Indeed no anomaly is observed in any of the frequencies involving azo or amino nitrogens, apart from the small environmental effect. The dye also contains a sulphonic acid group, one frequency having been identified as relating to its state, and from a comparison with arsonical analogues, it is deduced that water molecules or ions in its vicinity are asymmetrically disposed. Carey *et al.* note that an analysis of intensities of aromatic ring frequencies might allow one to infer interactions with aromatic side chains in the protein.

Now whereas these findings are not in themselves such as to send protein chemists into any paroxysms of excitement, they plainly point the way the resonance Raman technique might very usefully develop. There are many protein-ligand systems of greater biological cogency to which it might be applied, some indeed where it is known, or thought, that the ligand binds in a configurational state different from that in free solution, or where it is involved in multiple interactions with subsites on the protein. Another possible application has been perceived by Strekas and Spiro (*Biochim. Biophys. Acta*, **263**, 830; 1972), who have used an argon-krypton laser, emitting at 5682 Å, to excite resonance Raman spectra of the prosthetic group in haemoglobin in various states. The spectra of deoxy-, oxy- and carboxyhaemoglobins and methaemoglobin (ferric) derivatives are largely similar, though there are considerable differences in intensity.

Two bands in particular, however, seem to reflect the state of the iron, for they are present in both oxy- and carboxyhaemoglobin, but are missing in deoxyhaemoglobin. In methaemoglobin azide they are again prominent, but in the acid form of methaemoglobin, where the sixth iron ligand is water, they are weak. The distinguishing feature between these forms is that the iron atom lies in the plane of the haem ring in low-spin derivatives, but appreciably out of plane in those of high spin. The last include deoxyhaemoglobin, where the crystallographic analyses of Perutz show the iron to protrude 0.8 Å above the plane, and acid methaemoglobin, where it is 0.3 Å out of plane: Strekas and Spiro have found that the sensitive bands serve as a marker for the acid-base titration of the methaemoglobin.

The frequencies in question are thought to arise from carbon-carbon double bonds in the haem, which respond to the movement of the iron, by way of a pucker induced in the ring system. The bands might well be put to the same kind of use as structural indicators as the hyperfine-shifted resonances in the NMR spectra of haem proteins. It is somewhat disappointing that no frequencies arising from the iron-bound ligands are to be seen: evidently the resonance conditions in these cases are not fulfilled.

Phytochrome Intermediates *in vivo*

THE plant photomorphogenic pigment phytochrome is responsible for developmental processes initiated by brief treatments with low irradiances of red light, or by continuous irradiation with high energies of broad spectrum light (the so-called high energy reaction). Attempts to account for this reaction have centred principally on the fact that the two photoconvertible forms of phytochrome (P_R and P_{FR}) have overlapping absorption spectra and thus under continuous irradiation a photostationary state is established with characteristic proportions of the two forms depending on the wavelengths.

Most workers have assumed that P_{FR} is the active form of phytochrome and that treatments which maintain P_{FR} for long periods of time will have maximum morphogenic effect. It is known from flash spectroscopic studies that several intermediates are formed during the photoconversions of P_R and P_{FR}, some of which have a relatively long half-life (Linschitz *et al.*, *J. Biol. Chem.*, **241**, 3395; 1966). Although the existence of these intermediates *in vivo* was shown by Briggs and Fork in 1969 (*Plant Physiol.*, **44**, 1081), inadequate instrumentation has up till now prevented the estimation of how much of the total phytochrome exists as intermediates during continuous irradiation. In its turn this has prevented the determination of $P_{FR}:P_{total}$ proportions.

Fortunately, C. J. P. Spruit, of Wageningen, who designed the instrument which first proved the presence of P_{FR} in dark-imbibed seeds (Boisard *et al.*, *Meded. LandbHoogesch. Wageningen*, 68–17; 1968) has successfully developed an *in vivo* spectrophotometer equal to this new task, and in next Wednesday's *Nature New Biology* (June 28) Spruit and R. Kendrick report some results achieved with this instrument.

Spruit and Kendrick clearly demonstrate for several tissues that under continuous incandescent white light, up to 30 per cent and more of the total phytochrome is present as conversion intermediates. Furthermore, the proportion in the intermediate forms varies with light intensity. The high levels of intermediates will come as a shock to many workers in this field and are clearly of importance to an understanding of phytochrome action in the high energy reaction.