Analytica Chimica Acta, 76 (1975) 193–198

SHORT COMMUNICATION

Application of resonance Raman spectrometry to the determination of vitamin B_{12}

CHENG-WEN TSAI* and MICHAEL D. MORRIS
Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48104 (U.S.A.)
(Received 4th October 1974)

The resonance Raman effect has been known for many years[1]. Its potential for trace analysis was pointed out by Brandmüller[2] in 1959. However, full exploitation of resonance Raman spectrometry did not begin until the development of high power, multiline gas lasers as Raman excitation sources. The recent application of tunable dye lasers as excitation sources[3] promises even broader applications of resonance Raman spectrometry.

The resonance Raman effect is extremely sensitive. Rimai *et al.*[4] have observed resonance Raman spectra from β-carotene solutions as dilute as 10^{-7} *M*. Moreover, Raman bands are typically quite narrow, 10–20 cm^{-1} half-width. This width corresponds to 0.3–0.6 nm at 500 nm. Thus, while resonance Raman spectrometry has a sensitivity comparable to that of spectrophotometry, it offers a selectivity which spectrophotometry cannot match.

In the present work, resonance Raman spectrometry was investigated as an alternative to spectrophotometry for the determination of vitamin B_{12}. The standard spectrophotometric determination at 360 nm offers good sensitivity, but suffers from many potential interferences[5]. Microbiological methods have detection limits approaching 10^{-9} *M*, but require very careful control of experimental conditions in order to achieve reproducibility[6].

Recently, Hester *et al.*[7–9] have examined the resonance Raman spectra of vitamin B_{12}, cyanocobalamin. Similar, less extensive work has been reported by George and Mendelsohn[10]. Wozniak and Spiro[11] have shown that various cobalt corrins have resonance Raman spectra similar to that of cyanocobalamin.

Although several bands of the cyanocobalamin Raman spectrum are resonance-enhanced, the strongest is a ring stretching vibration at 1504 cm^{-1}. This band can be strongly enhanced using either the 488.0 nm or 514.5 nm line of an Ar^+ laser. Both of these lines lie in the α–β band system of the absorption spectrum of cyanocobalamin.

Previous researchers have worked with solutions in the 10^{-4}–10^{-3} *M* range. Such concentrations yield excellent spectra in which all the minor bands

* Present Address: Department of Chemistry, Texas Technological University, Lubbock, Texas 79409, U.S.A.

are clearly visible. In the present paper, it is shown that the resonance Raman spectrum of cyanocobalamin is detectable at submicromolar concentrations, and the effect of pH and of other water-soluble vitamins on the major band is described.

Experimental

All Raman spectra were obtained on a Spex "Ramalog" spectrometer, with a slit width of 9 cm^{-1} (400 μm) and a scan speed of 25 cm^{-1} min^{-1}. A cooled (−30°C) RCA C31034 photomultiplier was used as the detector. Both photon counting and d.c. current measurements were employed with chart recorder display. The excitation source was the 488.0-nm line from a Coherent Radiation CR 5 argon ion laser. Laser power was limited to 2 W at the laser head to avoid sample destruction. Standard 1.8-mm o.d. melting point capillaries were used as sample cells.

Cyanocobalamin, thiamine hydrochloride, pyridoxine hydrochloride, riboflavin and ascorbic acid were all U.S.P. grade and were used as received. Niacin (Diamond Shamrock, assay 99.5%) and folic acid (Calbiochem) were used without further purification. All other reagents were A.C.S. reagent grade. Distilled water was used to prepare all solutions.

Most experiments were run in acetic acid–sodium acetate buffers of pH 5. Buffers based on hydrochloric acid, phthalic acid, tris(hydroxymethyl)aminomethane, carbonate, phosphate and boric acid were used as required for control of pH. Riboflavin-containing solutions were made 0.001 *M* in mercury(II) ion and exposed to laser light (488 nm) for 10 min before measurements were made. This procedure reduced riboflavin fluorescence to tolerable levels.

The vitamin B_{12} 1504-cm^{-1} line was examined by scanning the spectrum from *ca.* 1450 to 1540 cm^{-1} and measuring the peak intensity relative to a baseline at 1530 cm^{-1}. The 1050-cm^{-1} line of 0.1 *M* nitrate was employed as an internal standard. Data are reported as the ratio of scattering intensity at 1504 cm^{-1} to intensity at 1050 cm^{-1}. For solution concentrations above about 10^{-6} *M*, the data are reproducible to about ±2%.

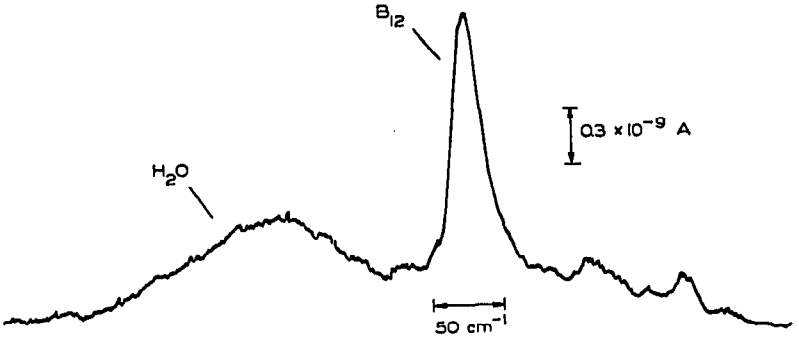

Fig. 1. Resonance Raman Band of vitamin B_{12} at 1504 cm^{-1}. Excitation at 488 nm. Resolution 9 cm^{-1}. $5 \cdot 10^{-6}$ *M* vitamin B_{12}.

Results and discussion

The 1504-cm^{-1} band of vitamin B_{12} is shown in Fig. 1. Also visible is the broad, weak water band at 1645 cm^{-1}. The full width at half-height of the 1504-cm^{-1} band is 20 cm^{-1}, about 0.6 nm. This width is independent of pH, concentration and laser frequency or power.

The pH dependence of the relative intensity of the 1504 cm^{-1} line ($6 \cdot 10^{-5}$ *M* Vitamin B_{12}) is shown in Fig. 2. In acidic solution the benzimidazole group of vitamin B_{12} is protonated and the benzimidazole–cobalt bond is broken. The change in relative intensity occurs around pH 3, where deprotonation of benzimidazole and formation of the cobalt–ligand bond is known to occur[12]. The inflection at *ca.* pH 8 apparently corresponds to deprotonation of the co-ordinated water molecule[12].

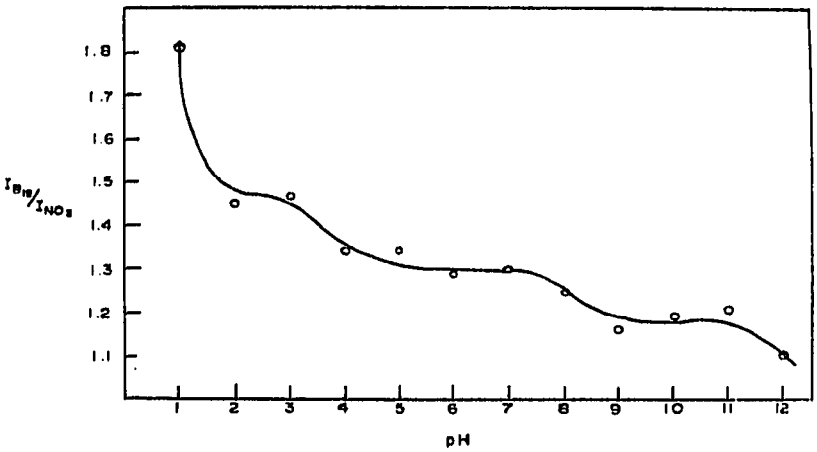

Fig. 2. pH dependence of 1504-cm^{-1} intensity. $6 \cdot 10^{-5}$ *M* vitamin B_{12}. Excitation at 488 nm. Resolution 9 cm^{-1}.

The causes of the large rise at pH 1 and the further dip in intensity at pH 12 are not known. These intensity changes are reproducible and reversible. Adjustment of the solution pH to any given value produces the same relative intensity, regardless of the prior history of the solution. This suggests that hydrolyses of the phosphate ester linkage and other irreversible reactions involving break-up of the molecule did not occur.

Because pH 5 is in a flat region of the intensity–pH curve, this pH was chosen for further experiments.

Table I shows the concentration dependence of the intensity of B_{12} scattering at pH 5. Scattering intensity is linear with concentration over the range $2 \cdot 10^{-6}$–$2 \cdot 10^{-5}$ *M*. Above $2 \cdot 10^{-5}$ *M*, reabsorption of scattered light causes negative deviations from linearity. Below $2 \cdot 10^{-6}$ *M* scattering intensity (per unit concentration) appears to increase. However, the signals in this region are sufficiently small that systematic errors in the photon-counting system and in baseline measurement techniques may be significant and partly responsible for the increase. Below

TABLE I

CONCENTRATION DEPENDENCE OF VITAMIN B_{12} SCATTERING AT 1504 cm^{-1}

$c_{B_{12}}$ ($\cdot 10^{-6}$ M)	$I_{B_{12}}/I_{NO_3}$ [a]
80	2.00
60	1.50
40.0	1.07
20.0	0.54
9.0	0.260
8.0	0.240
7.0	0.210
5.00	0.144
4.00	0.120
2.00	0.057
1.00	0.038
0.40	0.017
0.20	0.10

[a] 0.1 *M* KNO_3, pH = 5, photon counting.

$2 \cdot 10^{-7}$ *M* signals are too small (less than twice background fluctuations) to allow measurement. The background appears to be due to the presence of residual fluorescent impurities and to scattering by water. Lower detection limits are not obtained by increasing laser power.

The effect of other water-soluble vitamins on the vitamin B_{12} resonance Raman signal is shown in Table II. With the exception of riboflavin, the presence of at least a ten-fold excess of any given vitamin has only a small effect, usually a slight depression of less than 10%.

In order to reduce the intense fluorescence of riboflavin to manageable levels, mercury(II) ion was added to the solution to complex the riboflavin. After exposure to laser light for about 10 min, the fluorescence background decays to a signal whose intensity is about equal to that from $5 \cdot 10^{-5}$ *M* vitamin B_{12}. In the absence of mercury(II) nitrate, riboflavin fluorescence is *ca.* 1000 times stronger than the Raman scattering from $5 \cdot 10^{-5}$ *M* vitamin B_{12} and makes the measurement of that signal impractical if not impossible. The orange color persists, since only a very small volume of the solution is in the laser beam and subject to photodecomposition. The remaining fluorescence is due to a mixture of photodecomposition products and free and complexed riboflavin supplied by diffusion from the remainder of the solution. Since the undecomposed riboflavin and its complex have absorption spectra which overlap the B_{12} emission, these species can reabsorb the scattered light and attenuate the B_{12} signal. This reabsorption accounts for the strong attenuation of the B_{12} signal in the presence of even small amounts of riboflavin and is a serious impediment to the use of resonance Raman spectrometry in the analysis of vitamin B_{12} in multivitamin preparations.

An attempt was made to use resonance Raman spectrometry to assay vitamin B_{12} in multivitamin preparations, including standard decavitamin tablets. However, the concentration of riboflavin is sufficiently high and the concentration of vitamin B_{12} is so low (*ca.* $1 \cdot 10^{-6}$ *M* after extraction and dilution to volume) that

TABLE II

EFFECT OF OTHER WATER-SOLUBLE VITAMINS ON VITAMIN B_{12} SCATTERING

Added vitamin[a]	*Concentration (M)*	*Relative signal*
None	—	1.00
Thiamine hydrochloride	$0.51 \cdot 10^{-4}$	0.93
	$1.01 \cdot 10^{-4}$	0.93
	$2.02 \cdot 10^{-4}$	0.93
	$4.04 \cdot 10^{-4}$	0.93
Pyridoxine hydrochloride	$0.30 \cdot 10^{-4}$	0.94
	$1.60 \cdot 10^{-4}$	0.95
	$3.2 \cdot 10^{-4}$	0.96
	$6.4 \cdot 10^{-4}$	0.91
Folic acid	$1.13 \cdot 10^{-6}$	0.93
	$2.25 \cdot 10^{-6}$	0.87
	$9.0 \cdot 10^{-6}$	0.88
Niacin	$0.200 \cdot 10^{-4}$	0.93
	$0.50 \cdot 10^{-4}$	0.93
	$0.99 \cdot 10^{-4}$	0.87
	$1.98 \cdot 10^{-4}$	0.87
Riboflavin	$1.00 \cdot 10^{-6}$	0.78
	$2.50 \cdot 10^{-6}$	0.70
	$5.0 \cdot 10^{-6}$	0.71
	$10.0 \cdot 10^{-6}$	0.62

[a] Vitamin $B_{12} = 5.5 \cdot 10^{-6}$ *M*, pH = 5.0, 0.1 *M* KNO_3 internal standard.

residual fluorescence and reabsorption obscure any vitamin B_{12} signal.

Several groups have attempted time resolution of fluorescence emission from Raman scattering[13–15]. The technique gives only modest improvements in signal quality and is not capable of rejecting fluorescence signals which are orders of magnitude larger than the Raman signal. Practical application of resonance Raman scattering to analysis of vitamin B_{12} will have to await either improved fluorescence rejection techniques or a simple, highly efficient separation of vitamin B_{12} and riboflavin.

We wish to thank Ms. Betty Silver, Diamond Shamrock Chemical Company for samples of several vitamins.

REFERENCES

1 J. Behringer, in H. A. Szymanski (Ed.), *Raman Spectroscopy*, Plenum, New York, 1967, pp. 168–223.
2 J. Brandmüller, *Z. Anal. Chem.*, 170 (1959) 29.
3 T. C. Strekas and T. G. Spiro, *J. Raman Spectrosc.*, 1 (1973) 387.
4 L. Rimai, R. G. Kilponen and D. Gill, *J. Amer. Chem. Soc.*, 92 (1970) 382.
5 M.-U.-L. Hashmi, *Assay of Vitamins in Pharmaceutical Preparations*, Wiley, London, 1973, pp. 243–245.

6 M.-U.-L. Hashmi, *Assay of Vitamins in Pharmaceutical Preparations*, Wiley, London, 1973, pp. 267–276.
7 E. Mayer, D. J. Gardiner and R. E. Hester, *Biochim. Biophys. Acta*, 297 (1973) 568.
8 E. Mayer, D. J. Gardiner and R. E. Hester, *Mol. Phys.*, 26 (1973) 783.
9 E. Mayer, D. J. Gardiner and R. E. Hester, *J. Chem. Soc., Trans. Farad. Soc. Part II*, (1973) 1350.
10 W. O. George and R. Mendelsohn, *Appl. Spectrosc.*, 27 (1973) 390.
11 W. T. Wozniak and T. G. Spiro, *J. Amer. Chem. Soc.*, 95 (1973) 3402.
12 J. M. Pratt, *The Inorganic Chemistry of Vitamin* B_{12}, Academic Press, New York, 1972, pp. 130–153.
13 P. P. Yaney, *J. Opt. Soc. Amer.*, 62 (1972) 1297.
14 R. P. Van Duyne, D. L. Jeanmaire and D. F. Shriver, *Anal. Chem.*, 46 (1974) 213.
15 F. E. Lytle and M. S. Kelsey, *Anal. Chem.*, 46 (1974) 855.