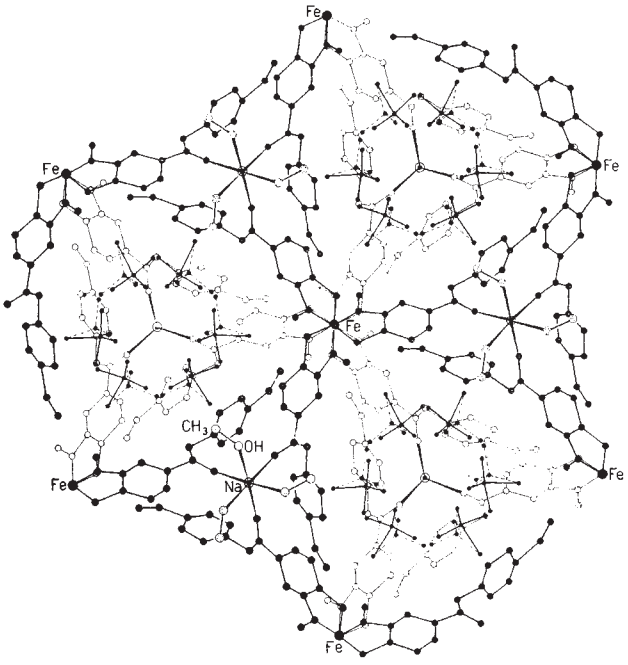

Fig. 2. Projection of about half of the crystal structure of ferroverdin I along axis *c*.

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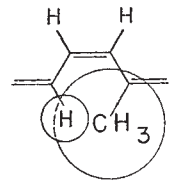
Relationship between Absorption Spectrum and Molecular Conformations of 11-*cis*-Retinal

THE absorption spectrum of 11-*cis*-retinal is strongly influenced by the presence of a *cis* bond between carbon atoms 11 and 12. The *cis* bond in this position results in two important spatial properties of the molecule: first, steric hindrance between the C_{13}-methyl group and the C_{10}-hydrogen atom; second, a bend in the system of conjugated double bonds. Both the steric hindrance and the bend in the molecule give rise to particular absorption bands. We now report the results of experiments which allow the identification of these absorption bands in the 11-*cis*-retinal spectrum.

The absorption spectra of carotenoids containing the sterically hindered *cis* configuration



have features which those of their nonhindered *cis* and *trans* isomers do not possess[1–11]. The solution spectrum of 11-*cis*-retinal at room temperature (Fig. 2, heavy line) shows, for example, three distinct bands at 27,030 cm^{-1}, 35,460 cm^{-1}, and 39,600 cm^{-1}, respectively. The maximal decadic molar absorption coefficient (ε_{max}) of the low wavenumber band of 11-*cis*-retinal, like that of sterically hindered carotenoids, is much smaller than the ε_{max} values of the nonhindered *cis* and *trans* retinals. This property was first detected in the spectra of isomers of *retro*-vitamin A methylether by Oroshnik, Karmas and Mebane[5], who attributed the low absorption of one isomer to a break in coplanarity of the conjugated system resulting from steric hindrance. If Oroshnik *et al.* are correct, the absorption bands for the segments of the conjugated system of carotenoids should appear at higher wavenumbers than those of the main band. Wald and coworkers[10] measured the spectra of three sterically hindered compounds, including 11-*cis*-retinal, and observed that the size (oscillator strength) of the low wavenumber bands increased markedly on cooling (see also ref. 12). If cooling of a sterically hindered carotenoid restores conjugation by restoring coplanarity, then it follows that the high wavenumber bands which arise from a break in coplanarity should decrease on cooling. The lack of reliable data for the interesting high wavenumber region of sterically hindered carotenoids at low temperatures prompted us to measure the spectrum of 11-*cis*-retinal at low temperature.

The absorption spectra of 11-*cis*-retinal in diethyl ether–2-methylbutane–ethanol (5 : 5 : 2, by volume; EPA) at room temperature and liquid nitrogen temperature (Fig. 2) are characterized by the opposite changes in size of the two prominent absorption bands (A and C, D) with temperature. When the temperature is decreased, the low wavenumber band (A) goes up, and the high wavenumber band (C, D) simultaneously goes down. When the cooled sample is heated back to room temperature, the original spectrum reappears. For the low wavenumber band A the ratio of ε_{max} at 77 K to ε_{max} at 295 K is 1·75, and the ratio of the oscillator strengths (f_{77}/f_{295}) is approximately 1·35. At low temperatures the maxima of the two major absorption bands are shifted to lower

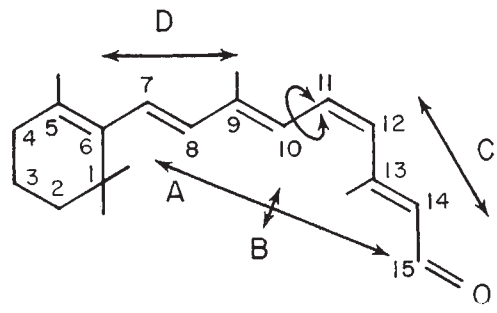


Fig. 1. Structural formula of 11-*cis*-retinal. The arrows indicate the directions of the transition moments for the different absorption bands (see Fig. 2). The transition moments for the bands A and B are supposed to be in an identical plane. B designates the *cis*-peak. Thermal excitation of vibrations around the C_{10}–C_{11} bond breaks the coplanarity of the conjugated chain giving rise to the two new segments, C and D, the transition moments of which are in different planes. The $C_5 = C_6$ bond in β-ionylidene compounds is generally not completely in plane with the conjugated side-chain[3].

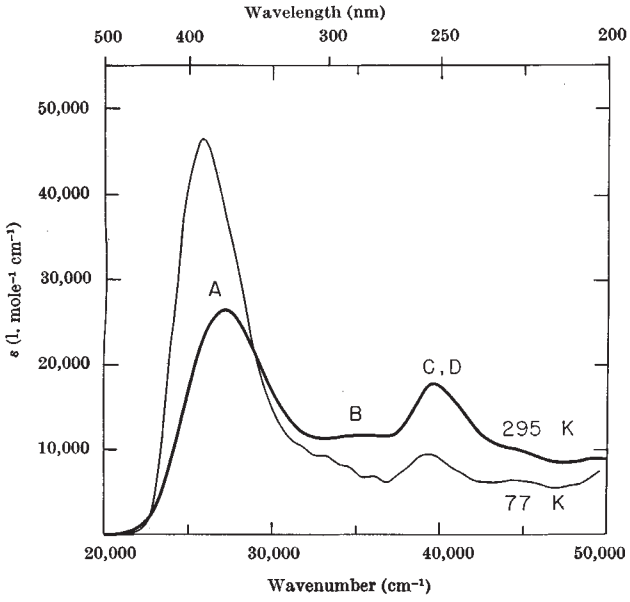


Fig. 2. Absorption spectrum of 11-*cis*-retinal in EPA at 22° C (heavy line) and liquid nitrogen (light line) temperature. Optical path length, 1 mm; concentration, $2{\cdot}69 \times 10^{-4}$ M at 22° C. Contraction factor of EPA for cooling from 295 K to 77 K, 0·771.

wavenumbers, and the region between these two bands has distinct fine structure, with an equal distance of 1,450 cm^{-1} between the bands. In contrast, the main absorption band of all-*trans*-retinal in EPA (Fig. 3) shows only a small change in the maximal extinction ($\varepsilon_{77}/\varepsilon_{295} = 1{\cdot}15$) and no detectable change in the oscillator strength ($f_{77}/f_{295} = 1{\cdot}0$). The minor absorption bands at higher wavenumbers are nearly unchanged in their size. Other features of the low temperature spectrum of all-*trans*-retinal are, however, the same as for 11-*cis*-retinal. The maximum of the main band is also shifted to a lower wavenumber with decreasing temperature, and fine structure with an interband distance of approximately 1,500 cm^{-1} appears.

The two spectra in Fig. 2 suggest that dramatic changes in the π-electron system of the 11-*cis*-retinal molecule occur with change of temperature. Models reveal that steric hindrance between the C_{10}-hydrogen and the C_{13}-methyl could be relieved by rotation around the C_{10}—C_{11}, C_{11}=C_{12} or C_{12}—C_{13} bond (or a combination of these). We interpret the changes in the absorption band sizes of 11-*cis*-retinal as arising from a thermal excitation of torsional vibrations* predominantly around the C_{10}—C_{11} bond of the molecule (Fig. 1, circular arrow). The partial double-bond character of single bonds in conjugated systems of this length normally restricts rotation around these bonds at room temperature. This holds for all-*trans*-retinal, but in 11-*cis*-retinal the steric hindrance caused by the overlapping of the C_{13}-methyl group and the C_{10}-hydrogen atom lowers the resonance energy—which is normally gained by the conjugation of six double bonds—and the thermal energy at room temperature is sufficient to force the C_{11}/C_{15} segment out of conjugation with the C_1/C_{10} segment. Steric hindrance is maximal at low temperatures when the resonance system is in a planar form; at higher temperatures steric hindrance is relieved and two new partial chromophores are created, each with three conjugated double bonds (C and D, in Fig. 1; combined spectral band, C, D, Fig. 2). The absorption spectra of analogous polyenes with three double bonds are known. *trans*-β-Ionylidene-ethanol, which represents the C_1/C_{10} segment, has a broad absorption band[13] with two maxima at 38,600 cm^{-1} ($\varepsilon_{max} = 13{,}500$ l. $mole^{-1}$ cm^{-1}) and 42,300 cm^{-1} (13,400), respectively. *trans*-3-Methylpenta-2,4-dienal, representing the C_{11}/C_{15} segment, has maximum absorption at about 38,700 cm^{-1} (23,000)[14]. The band C, D of 11-*cis*-retinal (Fig. 2, room temperature spectrum) shows maximal absorption at 39,600 cm^{-1} in EPA, which agrees quite well with the absorption maxima of these model compounds.

Patel[15] recently concluded from NMR data that the steric strain between the C_{10}-hydrogen and C_{13}-methyl is relieved by a skew geometry around the C_{12}—C_{13} bond with the C_{10}—C_{11} bond remaining *s-trans* planar. Our spectroscopic data do not agree with Patel's assignment. A break in conjugation at the C_{12}—C_{13} bond would result in a partial chromophore with two double bonds which should absorb at higher wavenumbers than our spectra show. (The $\tilde{\nu}_{max}$ of crotonaldehyde is 46,950 cm^{-1} in cyclohexane.) Furthermore, we found that 11-*cis*-retinol has a steric hindrance band at 42,900 cm^{-1} in EPA, the size of which is temperature dependent. Because the steric properties of 11-*cis*-retinol should be similar to hose of the retinal, the appearance of a partial chromophore band at 42,900 cm^{-1} (two double bonds) would again support a vibration around the C_{10}—C_{11} bond. We feel that the discrepancy between our and Patel's interpretation needs further investigation.

(Patel apparently uses a static model in which all 11-*cis*-retinal molecules would be equally twisted around one of the single bonds. We picture a dynamic equilibrium between the planar ground state and vibrationally excited states with many of the molecules in the ground

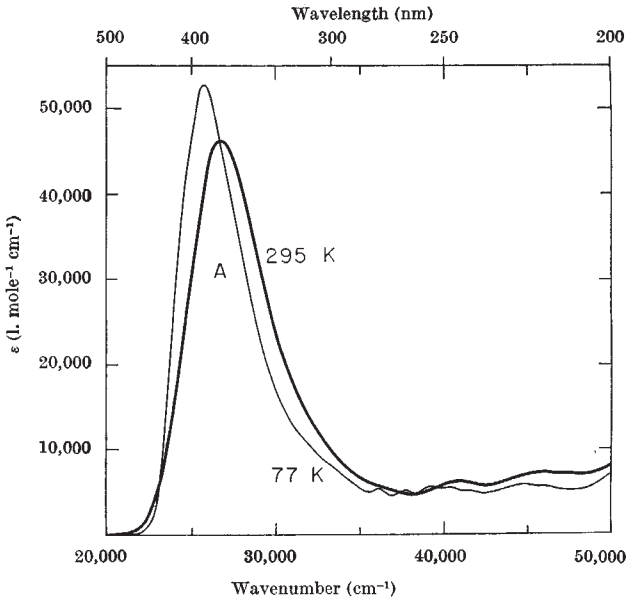


Fig. 3. Absorption spectrum of all-*trans*-retinal in EPA at 22° C (heavy line) and liquid nitrogen (light line) temperature. Optical path length, 1 mm; concentration, $2{\cdot}32 \times 10^{-4}$ M at 22° C.

* In an isolated 11-*cis*-retinal molecule the energy used to excite torsiona vibrations around the C_{10}—C_{11} bond should be strictly quantized, and only the occupation of distinct states should be allowed. The distribution of molecules between two vibrational states is determined by the Boltzmann factor $e^{-\Delta E/kT}$, where ΔE is the energy difference between these states. Each of these states has its intrinsic electronic absorption spectrum. At very low temperatures (near 0 K) most of the vibrations are in their lowest energetic state, which in the case of 11-*cis*-retinal corresponds to a planar or nearly planar conformation of the whole conjugated system. In solution (as opposed to the case of the isolated molecule) rapid energy exchanges between 11-*cis*-retinal and the solvent molecules do not allow the full development of vibrationally excited states with energies of the order of kT. A shorter vibrational lifetime will broaden ΔE. If the molecule passes through more than one minimum of potential energy during one vibration, atropisomers might form depending on the temperature and the size of the energy barrier between the minima.

Loeb *et al.* (ref. 10, page 618, Fig. 5) measured the temperature-dependence of ε_{max} for 11-*cis*-retinal from room temperature to liquid nitrogen temperature, and found that on lowering the temperature "ε_{max} rises in two linear stages", with a "break" the significance of which they could not explain. Under the assumption that most of the molecules are already in their lowest energy state at liquid nitrogen temperature—ε_{max} is near its highest value—it is possible by using the data of ref. 10 to calculate ΔE as $2{\cdot}5 \times 10^{-14}$ erg/molecule and simulate the curve obtained by Loeb, Brown and Wald.

state at any time. Fast exchanges between different states at room temperature would average the spin coupling constants of all these states. The probability distribution function for the deviation of the C_{10} and the C_{11}-hydrogen atoms from the planar conformation in the first vibrationally excited state should not have its maximum too far from the planar conformation. According to Karplus[18] the coupling constant $J_{10,11}$ would decrease only slightly for the first 20 to 30 degrees rotation around the C_{10}—C_{11} bond. Because of the averaging effect and the high proportion of the molecules in the planar ground state, the decrease of $J_{10,11}$ might be relatively small.)

The disruption of the conjugated system at the C_{10}—C_{11} bond by thermal vibrations does not seem to be restricted to the 11-*cis*-retinal molecule, but probably has a bearing on the properties of rhodopsin as well. Yoshizawa and Wald[16] found that when rhodopsin in a glycerol–water mixture was cooled from room temperature to liquid nitrogen temperature the extinction of the 498 nm band rose about 1·6 times while λ_{max} moved to 505 nm (the oscillator strength also changes with temperature). This suggests that the Schiff base of 11-*cis*-retinal in rhodopsin has conformational properties similar to 11-*cis*-retinal in solution: vibrations around the C_{10}—C_{11} bond at room temperature result in a lower light absorption by rhodopsin in the visible region than theoretically possible if the chromophore were in a plane.

The 11-*cis* bond in the molecule causes a bend in the conjugated system and gives rise to an absorption polarized in a direction perpendicular to the main axis of the molecule. This "*cis* peak" has alternatively[8,9] been assigned to the 39,600 cm^{-1} and the 35,460 cm^{-1} band. Our assignment of the 39,600 cm^{-1} band to partial chromophore absorption suggests that the remaining band at 35,460 cm^{-1} represents the *cis* peak. No experimental evidence has yet been given. Eckert and Kuhn[17] identified the *cis* peak of another *cis*-carotenoid, the non-hindered 15,15′-*cis*-β-carotene, by measuring the dichroism of the carotene dissolved in a stretched polyethylene film.

In the case of 11-*cis*-retinal the identification of the *cis* peak is experimentally more difficult: the *cis* peak appears at a higher wavenumber in the ultraviolet

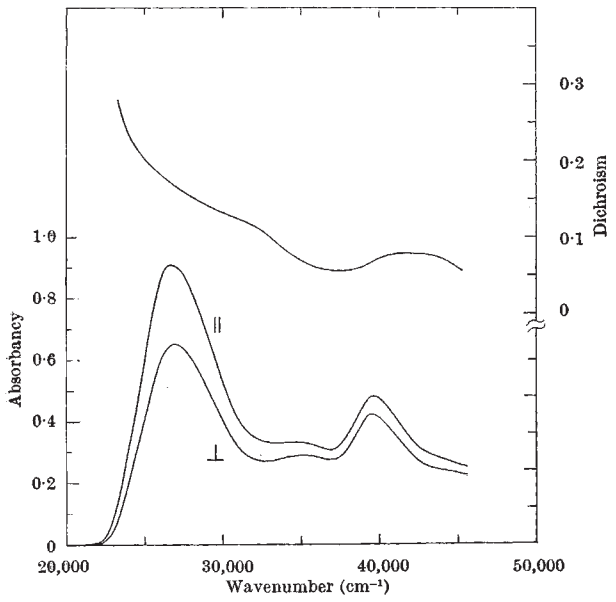


Fig. 4. Absorption spectrum of 11-*cis*-retinal in stretched polyethylene film measured with the electric vector of the light beam parallel (||) and perpendicular (⊥) to the stretching direction. The upper curve shows the dichroism $Di = \frac{E\| - E\perp}{E\| + E\perp}$. Optical path length, 0·07 mm. 22° C.

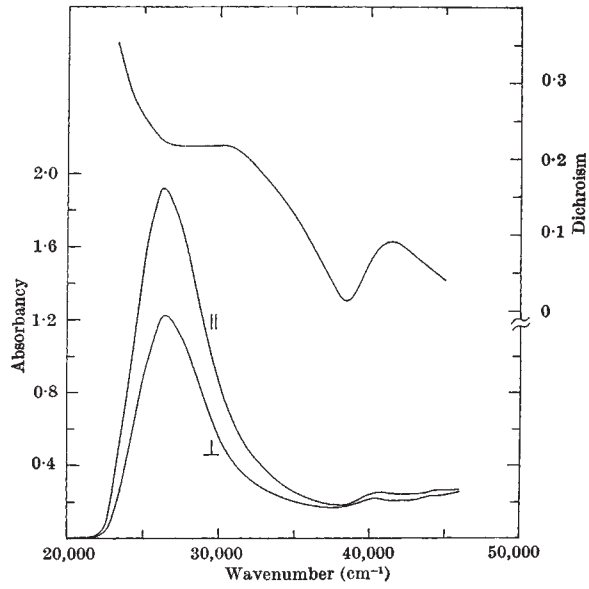


Fig. 5. Absorption spectrum of all-*trans*-retinal in stretched polyethylene film measured with the electric vector of the light beam parallel (||) and perpendicular (⊥) to the stretching direction. The upper curve shows the dichroism $Di = \frac{E\| - E\perp}{E\| + E\perp}$. Optical path length, 0·07 mm. 22°C.

region; it is relatively small, and strongly overlapped by both the main absorption band and the steric hindrance bands. Absorption spectra of 11-*cis*- and all-*trans*-retinal in stretched polyethylene film are presented in Figs. 4 and 5. Fig. 4 includes the spectrum of 11-*cis*-retinal measured with light with its electric vector polarized parallel (||) and normal (⊥) to the stretching direction. The upper curve shows the dichroism $Di = \frac{E_{\|} - E_{\perp}}{E_{\|} + E_{\perp}}$ plotted against the wavenumber. $E_{\|}$ is the absorbance ($E = \log I_0/I$) of the film measured with light parallel, $E_{\perp}$ with light perpendicularly polarized to the stretching direction. Fig. 5 shows the corresponding data for all-*trans*-retinal. It is reasonable to assume that both all-*trans*- and 11-*cis*-retinal orient themselves with their long geometric axis parallel to the polyethylene molecules in the stretching direction of the film. Figs. 4 and 5 demonstrate that, in agreement with theoretical expectations[17], the transition moment of the main absorption band lies in the direction of the main geometric axis for both retinals. For both all-*trans*- and 11-*cis*-retinal the dichroism Di is very large at low wavenumbers, diminishes with increasing wavenumber, and levels off in the region of the main absorption band. The dichroism curve for 11-*cis*-retinal begins to form a shallow trough at 32,000 cm^{-1}, goes through a broad minimum between 36,000 cm^{-1} and 38,500 cm^{-1}, and levels off at about 40,000 cm^{-1}. The all-*trans*-retinal Di curve sharply decreases above 31,000 cm^{-1}, goes through a minimum at about 38,500 cm^{-1}, and forms a positive peak at 41,400 cm^{-1}.

The trough of the 11-*cis*-retinal Di curve represents an absorption band oppositely polarized from the main band A. Because it also falls into the region of band B, this band is apparently the *cis* peak. The spectrum above 39,000 cm^{-1} is predominantly the sum of the absorptions by the partial chromophores C and D, the third electronic transition (second overtone), and residual *cis* absorption. The steric hindrance bands C and D should be polarized differently from both the main absorption band and the *cis* band (see Fig. 1). Overlapping of these differently polarized bands results in unspecific and low dichroism above 39,000 cm^{-1}.

The origin of the all-*trans*-retinal dichroism band at 38,500 cm^{-1}, which is differently polarized from the main

absorption band, is not immediately evident from the structure of the molecule. One possible explanation is that a predominantly *s-cis* configuration of the C_6—C_7 bond in all-*trans*-retinal causes a deviation from linearity of the conjugated system, so allowing higher electronic transitions which are perpendicularly polarized to the main absorption band and which are forbidden in a completely linear conjugated system. In contrast, 11-*cis*-retinal may prefer the *s-trans* configuration at the C_6—C_7 bond, a configuration which could be indirectly forced on the ring by the steric hindrance of the C_{13}-methyl and the C_{10}-hydrogen (the energetic difference between *s-cis* and *s-trans* configuration in the β-ionylidene group is small and favours one or the other in different compounds[3]). An *s-cis* configuration in all-*trans* and an *s-trans* configuration in 11-*cis-retinal* would aid in explaining why the value of $\tilde{\nu}_{max}$ of 11-*cis*-retinal (EPA, 25,790 cm^{-1}, 387·5 nm) is low compared with that of all-*trans*-retinal (25,820 $cm^{-1} \triangleq 387{\cdot}2$ nm) at liquid nitrogen temperature (normally one would expect a higher wavenumber maximum for the *cis* than for the *trans* form). The reason for the high *Di* value for both retinals in the low wavenumber region is probably a small difference in the absorption of retinal molecules lying parallel or perpendicular to the polyethylene molecules. This would have its most noticeable effect on the dichroism at the low-absorbing sides of absorption bands.

Fig. 1 summarizes our assignments of the three principal absorption bands of 11-*cis*-retinal at room temperature to the corresponding electronic transitions. The arrows indicate the directions of the transition moments for the different bands. There is no doubt[17] that the main band (A) at 27,030 cm^{-1} corresponds to absorption by the whole conjugated system. The small band (B) at 35,460 cm^{-1} arises from the bend within the plane of the conjugated system (*cis* peak), whereas the band at 39,600 cm^{-1} results from the absorption of partial chromophores arising as a consequence of steric hindrance. The transition moments of A and B are supposed to be in the same plane; those of C and D are in different planes.

Polyethylene film, 0·125 mm thick, was stretched in a stream of warm air to about 400 per cent of its original length, presoaked in *n*-heptane for one day, soaked in the retinal solution for 1 h and dried. To measure the absorption spectra, the films were placed in quartz cuvettes containing water as an immersion medium. The spectra were corrected with a baseline run with a blank polyethylene film prepared under identical conditions. All spectra, including those in EPA, were measured with a Cary 14 spectrophotometer.

All-*trans*-retinal was purchased from Distillation Products, Eastman, Rochester. 11-*cis*-Retinol was prepared by a modification of the methods of Dieterle and Robeson[8] and Brown and Wald[9], 11-*cis*-retinal by reducing the aldehyde with KBH_4[9]. Seed crystals of 11-*cis*-retinal were kindly provided by Y. Maeda and T. Isemura (Division of Physical Chemistry, Institute of Protein Research, Osaka University).

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Physicochemical Studies of L-Asparaginase from *Erwinia carotovora*

SOME L-asparaginases (L-asparagine amidohydrolase, EC 3.5.1.1) are able to destroy asparagine dependent tumours in animals and bring about regression in some cases of acute lymphatic leukaemia in man[1]. The anti-tumour property of this enzyme was first recognized by Broome[2] with the asparaginase from guinea-pig serum and has since been observed with the enzyme from chicken liver[3]. Most of the clinical work, however, has been carried out with the more readily available asparaginases from bacteria. An asparaginase from *Escherichia coli*[4], and more recently the enzyme from *Erwinia carotovora*[5], are both being used in clinical trials on leukaemia. One interesting aspect of the clinical use of asparaginases is that not all preparations are effective against tumours. For example, the enzymes from guinea-pig liver[6], *Bacillus coagulans*[4] and yeast[7] are ineffective. In fact, only one of the two asparaginases present in *E. coli* exhibits this property. Furthermore, those which are effective differ in their efficacy towards the 6H3HED tumour in mice which is normally used for the assessment of anti-tumour activity. Information on molecular structure may shed light on those features of the asparaginase which confer this property and suggest ways of enhancing it.

The molecular weight of the active asparaginase from *E. coli* has been reported as 139,000 by Whelan and Wriston[8] using sedimentation equilibrium. We have now determined the molecular weight of the *E. carotovora* asparaginase by the same method.

When dialysed against a solution containing sodium phosphate buffer ($I = 0{\cdot}1$; *p*H 7·2) and NaCl ($I = 0{\cdot}1$), the *Erwinia* asparaginase produced a symmetrical velocity boundary at s_{20w}, $7{\cdot}3 - 7{\cdot}5$ in the ultracentrifuge which did not show a marked dependence on concentration in the range 1–10 mg/ml. Estimates of 94–98 per cent homogeneity were obtained from measurements of the refractive increments across the sedimenting boundary and the refractive index of the enzyme solution against its diffusate. Some instability was indicated by the gradual decrease to 85–90 per cent homogeneity when solutions were stored at 4° C for more than 2–3 days.

The molecular weight was determined on freshly dialysed solutions by the "short column" sedimentation method using Rayleigh interference optics with high speed centrifugation to establish equilibrium[9]. Using concentrations of 0·05–1·0 mg/ml. and centrifuging at 17,250 r.p.m. for 17 h in 2·0–2·5 mm columns, Mr D. Miller (Microbiological Research Establishment) obtained molecular weights in the range 132,900–145,000. This was confirmed by Dr P. A. Charlwood (National Institute for Medical Research, London), who obtained values of 128,400–142,100 using low speed methods[10,11] and concentrations of 1·5–3·5 mg/ml.

The active asparaginases from both *E. coli*[12] and *E. carotovora* have been crystallized and large crystals of the latter enzyme have been grown in alcoholic solutions.