in ref 12. The Arrhenius plot has a slope given by

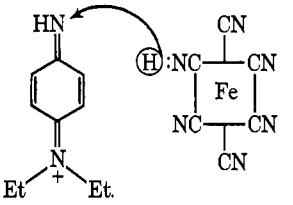
$$\frac{d \ln k_{obsd}}{d 1/T} = \Delta E_1 + \Delta H \quad (26)$$

which gives ΔE_1, the activation energy for reaction involving k_1, the value 6.7 kcal/mol.

Mechanism. The reaction leading to SQ formation in stage I is not subject to promotion by acids as observed for the Michaelis reaction (*i.e.*, plots analogous to that of Figure 4 for this reaction have zero slope). In addition, a kinetic isotope effect ($k_{H_2O}/k_{D_2O} = 1.10 \pm 0.03$) was observed at pH 5.62 by comparison of rates in normal and heavy water. Despite these observations a proton must be involved in the transition state as shown by the dependence of rate on (H^+) in Figure 8.

These observations are consistent with a mechanism involving SQ formation by atomic hydrogen transfer from protonated ferrocyanide[13] through the transition state II. The observed isotope effect is consistent with this mechanism since the experimental pH region is above the pK_a for protonation of ferrocyanide.

The atom transfer would be slower, but the degree of ferrocyanide protonation greater in heavy water than in normal water. Thus, a cancellation could occur giving the observed isotope effect near unity.



transition state II

The rate data in Tables IV and VI show that $Fe(CN)_6^{4-}$ competes favorably with PPD to reduce QDI at pH below pK_R. Above this pH the order of reactivity is reversed; therefore, ferrocyanide should not exhibit any catalytic effects on the reduction of QDI in the alkaline region. The rate constants for oxidation of PPD by ferricyanide (k_2 and k_4 in Table VI) limit the rate of nucleophilic reactions of QDI which may be studied when QDI is prepared by oxidation of PPD by ferricyanide in the presence of nucleophile.

Acknowledgments. The authors are grateful to Mr. W. Gass of these laboratories for preparation and purification of the azides used in this work, and to Dr. Bryant Rossiter for many helpful discussions.

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Raman Spectra of Dilute Solutions of Some Stereoisomers of Vitamin A Type Molecules

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Abstract: Laser-excited Raman spectra of retinals (*trans*, 9-*cis*, 13-*cis*), retinols (*trans*, 13-*cis*), and *trans*-retinoic acid in octanol solution are reported. The terminal group is easily identified by the frequency of the line at 1580–1590 cm^{-1}, while the isomer is uniquely characterized by the lines in the 1100–1400-cm^{-1} range. The observed spectra are mainly contributed by the conjugated segment and not by the saturated part of the molecule. A discussion of possible mode assignments is also presented.

The observation of intense, resonance-enhanced, Raman spectra[1-5] of carotenoids[6] in very dilute solution[7] and in live tissues[8] prompted us to investigate the Raman spectra of a number of vitamin A type molecules.[9-13] The importance of the stereoisomerism of these molecules[14,15] to the mechanism of vision[16-18] and the possibility of identifying stereoisomers by their vibrational spectra[10,19-26] further motivated the present work.

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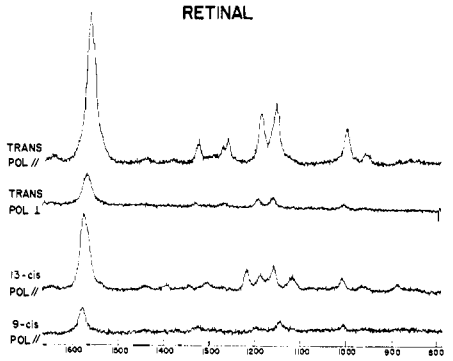

Figure 1. Raman spectra of retinal isomers dissolved in octanol. Excitation at 6328 Å, incident power 30 mW, spectral slit ~3 cm^{-1}, scan speed 20 cm^{-1}/min.

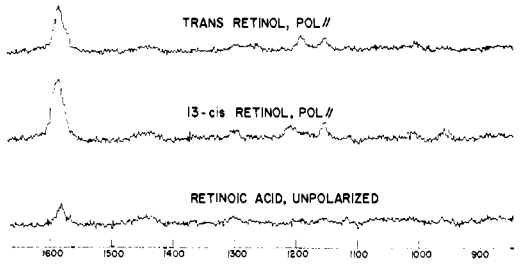

Figure 2. Raman spectra of retinol isomers and of *trans*-retinoic acid dissolved in octanol. Conditions identical with those of Figure 1.

We report the spectra of dilute solutions of some commercially available isomers of retinal and retinol. The spectra were excited in the red at a wavelength far removed from the first absorption band, yet preresonance intensity enhancement[1-5] was already quite apparent. The spectra indicate that the red light did not induce stereoisomerization.[10,15] The spectral features distinguishing the stereoisomers were obtained and are discussed in terms of possible vibrational assignments.

The enhanced Raman intensities of the solutions excited in the blue-green made it clear that the technique was applicable to the detection of vitamin A complexes in native tissue. This expectation was fulfilled in our experiments on whole cattle retinas at low temperatures.[27] In section I the experimental technique is briefly reviewed. In section II we present the data. Section III reviews possible assignments of the vibrational frequencies and in section IV possible analytical applications are mentioned.

I. Experimental Section

Isomers of retinol$_1$, retinal$_1$, and retinoic acid, purchased from Sigma Chemical Co., were dissolved in octanol at approximate concentrations of 1% by weight. β-Carotene was dissolved in dichloroethane.

The solutions at room temperature were excited by ~30 mW of 6328-Å radiation from a He–Ne laser. The beam, 2 mm in diameter, was focused into the sample by an f = 40-cm lens; a section of the sample, approximately 1 cm in length, near the focus

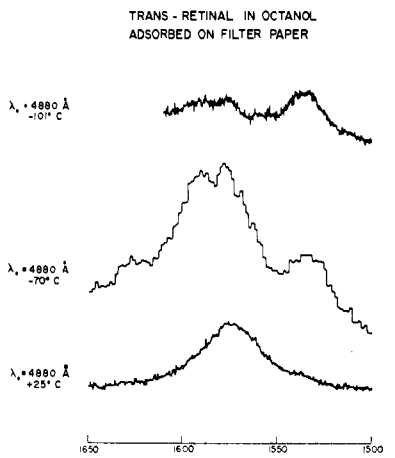

Figure 3. Raman spectra of *trans*-retinal in 1% octanol solution adsorbed on filter paper. Temperatures −101, −70, and 25°. Excitation at grazing incidence, 4880 Å, 15 mW.

of the illuminating lens was imaged onto the monochromator entrance slit with ~ ×2 magnification.

The data illustrating the temperature dependence of the *trans*-retinal spectrum were obtained with 4880-Å excitation from an Ar ion laser attenuated to ~15 mW. The exciting beam was still focused by the same lens, but it grazed rather than penetrated the surface of the sample.[28] The latter was either the 1% octanol solution contained in a thin cuvet made of microscope slides, or a drop of the same solution adsorbed on a piece of Whatman filter paper and pressed by a microscope slide against a copper block cooled by a flow of cold N_2 gas. It was found that while the samples frozen in bulk octanol exhibited strong fluorescence[29,30] (a badly slanted base line), the latter was quenched by adsorption to the paper with no ill effect on the Raman spectrum.

The monochromator was a Jarrell-Ash double Czerny-Turner, f = 75 cm, and the detecter was an FW 130, ITT photomultiplier operating at room temperature. Its output was processed in a typical photon counting combination of a pulse preamplifier and height discriminator. The pulses were counted in a resettable scaler whose output was temporarily stored in a binary shift register. A reset pulse, provided by a binary countdown from an audio signal generator, was used to periodically reset the counter and at the same time channel its contents into the shift register. The shift register fed a recorder through a digital-to-analog converter. The reset timing determined the integrating time for data collection. The dynamic range of the system was practically infinite, because each time the storage limit of the register was reached, loading started anew from zero. This feature permitted the full amplification of weak Raman lines even in the presence of intense background (*e.g.*, fluorescence). The dynamic range was limited by the maximum overall counting rate of the sytem, which was roughly 10^6 pps.

The spectra presented here were all taken with a scanning speed of 20 cm^{-1}/min. The counter reset time was varied between ~0.1 sec and ~5 sec, as can be seen on the discrete steps in the noise pattern of some spectra reproduced in Figure 1. The polarization of the beam was always perpendicular to the scattering plane. The polarization of the scattered light could be selected with a polaroid analyzer. Under the aforementioned conditions we have observed no time-dependent changes in the spectra that would indicate photoisomerization.[10] The latter should have been easily detected, since the spectra are characteristic of the particular isomers.

II. Results

Recorder tracings of the Raman spectra are shown in Figures 1–3. All the Raman lines observed below 2000 cm^{-1} are exclusively contributed by the vitamin or carotenoid solute molecules. The solvent lines in this region are at least four times weaker than the weakest of the solute lines. The opposite occurs in the

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Table I. Vibrational Frequencies (cm^{-1}) and Depolarization Ratios (DR, $I_\perp/I_\parallel$) of Raman Lines of Retinals[1] (*trans*, 9-*cis*, and 13-*cis*), Retinol[1] (*trans*, 13-*cis*), and *trans*-Retinoic Acid Dissolved in 1-Octanol, and of β-Carotene in Dichloroethane[a]

trans-Retinal	13-*cis*-Retinal	9-*cis*-Retinal	*trans*-Retinol	13-*cis*-Retinol	*trans*-Retinoic acid	β-Carotene
			Lattice Modes			
			Top of Acoustic Branches			
865	799	845		870		
	841					
888	886	885		890		
			Middle Branch			
1115 (sh)	1119, 0.17	1116	1117	1117	1119	
1161, 0.15	1161, 0.2	1145, 0.30	1156	1157	1153	1155, 0.10
1195, 0.17	1189, 0.25	1235	1195		1191	1186
1267, 0.16	1219, 0.15	1255	1269	1213	1276	1213
1279, <0.2	1271	1290	1305	1298	1301	
1331, 0.2	1308	1326		1309	1342	
1387	1348	1368		1369	1365	
	1396					
			Upper Branch			
1538	1540	1553	1575 (?)			1522, 0.14
1570, 0.13	1579, 0.22	1579, 0.20	1592, 0.17	1588	1582	
			Group Modes			
			Methyl-to-Chain C–C Stretch			
1005, 0.15	1010, 0.2	1005	1005	1015	1013	
			C–H Bending of Chain Hydrogens			
964, 0.33	965, 0.4	965	957	963	963	
1443, 0.4	1443	1442	1445	1440	1439	
			C=O Stretch			
1650, <0.1	1650, <0.1	1645				

[a] All the lines for which the DR is not given are polarized. The frequencies are grouped according to our *tentative* assignments, which are by no means conclusive. The terminal group (alcohol, aldehyde, or acid) can be identified by the frequency at 1570–1590 cm^{-1} (lattice mode, upper branch), while the isomer is characterized by the (middle branch) frequencies at 1100–1400 cm^{-1}. The (group) mode at 1005–1015 cm^{-1} is characteristic of the side methyls and is tentatively assigned to methyl-to-chain C–C stretching. The mode at 965 coincides with the intense ir-active out-of-plane *trans*-ethylene C–H bending. The 1440 line is assigned to asymmetrical C–H bending within the side methyl groups rather than to CH_2 scissoring in the methylenes, which are remote from the conjugated chain.

spectral range around 3000 cm^{-1} (C–H stretch region), where solvent lines are exclusively detected.

The vibrational frequencies of the observed lines are listed in Table I, in which we also list the depolarization ratios (DR) of the more intense lines.[28] [The DR is obtained by dividing the peak intensity of the light scattered perpendicular to the plane of polarization of the incident beam ($I_\perp$) by the peak intensity of the light scattered parallel to that plane ($I_\parallel$).] Most of the lines observed are polarized, including the weaker lines, the DR's of which are not listed in Table I. The recorded (Figures 1–3) and tabulated data (Table I) indicate that the terminal groups in retinol, retinal, and retinoic acid can be identified by the frequency of the Raman line at 1580–1590 cm^{-1} and, moreover, that steroisomers of the aforementioned compounds can be clearly distinguished. The line at 1580–1590 cm^{-1} is broad at room temperature but reveals a structure at low temperature. The influence of stereoisomerism is particularly marked within the group of lines between 1100 and 1400 cm^{-1}, which does not alter upon cooling. The temperature-dependent spectra (Figure 3) were obtained from a sample of octanol solution adsorbed on filter paper.

III. Discussion

The most significant result of this work is the empirical demonstration that Raman spectra are very sensitive not only to the nature of the terminal group of vitamin A type molecules but even more so to the particular isomer configuration. The features identifying the isomers in the Raman spectra are more apparent than those of the respective absorption,[15] fluorescence,[29,30] or ir[22] spectra.

The molecules under study are rather large for vibrational analysis. Nevertheless, qualitative conclusions may be drawn from a simplified model, which would eventually be incorporated into a more complete picture.

For this discussion we refer to the literature on the ir and Raman spectra of conjugated polyenes up to hexatriene,[1,4–6,26] ir absorption in carotenoids,[22] and the ir spectra of the trans isomers of vitamin A.[31]

In the first place, the absence of the usually very strong C–H stretch Raman lines indicates that in the dilute solutions we are observing only the lines that are resonance enhanced due to their coupling with the conjugated π-electron system. The *internal* coordinates most strongly coupled to the linear π system are the stretching deformations of the skeletal C=C and C—C bonds in the conjugated chain.[32] Hence, the extent of resonance enhancement of any Raman-active *normal* coordinate depends on the contributions of the aforementioned *internal* coordinates to it.[33] We conclude that vibrations pertaining to sections of the molecule well decoupled from the conjugated chain do not contribute to the observed spectra. The observed

(31) Stadler Standard Spectra (Infrared), Spectra No. 11591, 14672–14679, 16100–16102, 16105–16106; also see O. Isler, H. Lindlar, M. Montavon, R. Rüegg, and P. Zeeler, *Helv. Chim. Acta*, 39, 249, 274 (1956).

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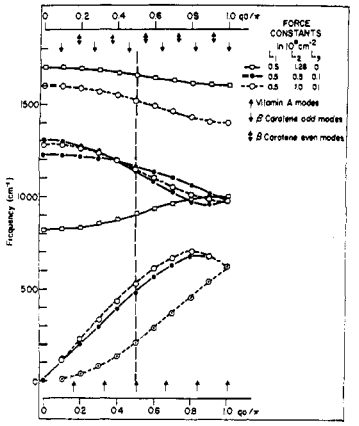

Figure 4. Dispersion curves (frequency *vs.* wavenumber) of the in-plane lattice modes computed for an infinitely long, 120° zigzag chain consisting of equal masses M bonded by unequal alternating bonds.[35] $L_1 = \Lambda_d/M$, $L_2 = \Lambda_s/M$, and $L_3 = A/M$, where Λ_d and Λ_s are the stretching force constants of the C=C and C—C bonds, respectively, and A is the force constant for the in-plane deformation of the angle between two consecutive bonds. The arrows indicate the possible values of qa/π of the standing wave modes in the infinite chain which are congruent with the lattice modes of the finite molecules pertinent to this work. When $L_3 = 0$ the two acoustic-like branches (zero frequency at $qa/\pi = 0$) are identically zero.

vibrations are then subdivided into "*lattice*" *modes* and *group modes* which differ in that the lattice modes depend on the length of the conjugated molecule, whereas the group modes do not.

The group modes involve the groups distributed along the conjugated chain, *i.e.*, the protons and the isoprene-derived methyls. We tentatively assign the weak lines at 963–965, 1005, and 1443 cm^{-1} to the group modes because they are ubiquitous in carotenoids and vitamins A, and yet are rather independent of the chain length and the terminal groups or the isomer configuration. The relatively intense polarized line at 1005 cm^{-1} is not present in the polyene spectra and is therefore typical of isoprenoids. We tentatively assign it to the C–C stretching vibration of the side methyl relative to the conjugated chain. The weak line at 1443 cm^{-1} may correspond to asymmetric C–H bending within the methyl group. The line at 965 cm^{-1} is coincident with a very intense ir band which is assigned to the trans-ethylenic out-of-plane C–H bending[19–26] of the chain protons. The Raman line is markedly depolarized (A_2 or $B_{1,2}$ symmetry). We believe that this mode, by deforming the 120° sp^2 bond angle on the chain, perturbs the π-electron system more than the corresponding C–H stretch, which indeed is not detected in the spectra. The ir-active band has a structure responsive to isomerization, whereas the coincident Raman line is rather structureless. Other group modes, such as in-plane C–H bending of the chain protons in the 1100–1400-cm^{-1} region, are hard to identify among the conjugated C–C stretching modes (which strongly contribute to the "lattice" middle band).

The vibration at 1645–1650 in the retinals is characteristic of the carbonyl terminal group. Its marked loss of intensity upon cooling probably indicates enhanced hydrogen bonding to the alcohol solvent at low temperature.

The lattice modes, derived from C=C and C—C stretching and C=C—C bending, are most conspicuous in the 1100–1400 and 1500–1600 regions and are polarized, as expected of A_1 modes in the approximate C_{2v} symmetry of the backbone chain of the conjugated isoprenoids. (In β-carotene, the inclusion of inversion in the symmetry group makes the selection rules for Raman-active modes more restrictive.) Ir-active lattice modes coincident with Raman modes, which would be permitted by the C_{2v} selection rules, are weak. Indeed one expects very small dipole changes caused by carbon–carbon displacements within the conjugated skeleton.

The in-plane lattice modes of the conjugated skeleton of trans isomers can be referred to the lattice dynamics model which was applied by Kirkwood[34] to alkanes and extended by Tric[35] to conjugated polyenes. Following Tric, we have computed (Figure 4) the dispersion curves of the in-plane skeletal vibrations of an infinitely long polyene chain. Nearest neighbor interactions are expressed in terms of three elastic force constants: Λ_s (or L_1), single-bond stretch; Λ_d (L_2), double-bond stretch; and Λ_A (L_3), C=C—C in-plane bending. We did not attempt to fit the curves to measured spectra, but rather picked parameters yielding eigenfrequencies in the range where the lattice modes are encountered. The unit cell of a linear *polyene* has two carbon atoms, one single bond, and one double bond, thus accounting for the four continuous branches in the dispersion curve. The allowed wave vectors $q = 2\pi/\lambda$ (where λ is the wavelength of the vibration measured along the extended dimension of the chain) range from zero to $\pm \pi/a$, where a is the "lattice constant" (minimum distance between two translationally equivalent atoms along the chain). The lattice constant of the conjugated *isoprenoid* chain is twice that of the polyene because of the side methyl near every fourth carbon atom. Accordingly the "Brillouin Zone" of the isoprenoid chain is derived from that of the polyene chain by folding at $qa = \pi/2$.

In order to simulate molecular eigenmodes (in which all the atoms vibrate in phase) on the infinite chain model, clamped boundary conditions have to be imposed. The modes are standing sine waves which have zero amplitude at the boundaries. Thus, from the continuum of q values we select those which fulfill $qa = j\pi/2N$ in the folded zone and $qa = j\pi/N$ in the unfolded zone, where N is the number of conjugated double bonds and j is an integer. $N = 11$ in β-carotene and $N = 6$ in the vitamins. β-carotene has inversion symmetry, so that only the modes with even j are Raman active.

Using this model, we associate the observed lines between 1500 and 1600 cm^{-1} with the upper branch (Figure 4), the series of lines starting at 1145–1160 and going up to 1400 cm^{-1} with the middle branch, and the lines around 700–800 cm^{-1} with the top of the lowest (acoustic-like) branches.

The various modes of the nontrans isomers can be accounted for by the model, in which the perturbations of frequency and Raman intensity depend on the location of the cis bond relative to the maxima of the standing waves in the trans model. Thus we expect the lines least affected by isomerism to correspond to the modes having the shortest wavelengths (largest q) in

(34) J. G. Kirkwood, *J. Chem. Phys.*, 7, 506 (1939).
(35) C. Tric, *ibid.*, 51, 4778 (1969).

the unfolded zone. In the context of the model we would assign the modes at 1115–1119 and 1145–1160 to the largest q values of the middle branch. The mode at the band edge ($qa = \pi$) is odd for β-carotene and should be absent from its Raman spectrum.

The lines corresponding to the higher branch are much broader than the others and this very intense section of the spectrum exhibits at room temperature a less detailed structure than the lower frequency section. The low-temperature spectrum, however, does reveal a structure: two distinct main peaks appear in the spectrum of the trans isomer (Figure 3), indicating that the broad shoulder observed at room temperature on the low-frequency side of the 1570–1590 line of the vitamins is indeed a separate mode. In attempting to identify this mode on the upper branch of the dispersion curve, we noticed that the slope of the branch in the unfolded zone, in which the frequency decreases with increasing q, is the same for any reasonable combination of three force constants. We therefore concluded that, in the unfolded zone, the lower of the two lines corresponds to the higher q value. Its frequency (1538 cm^{-1}) is above that of the upper band line in β-carotene.

The model encounters difficulty when applied to series of polyene molecules of increasing lengths. The *fundamental* "lattice mode" of each short molecule is modeled by a standing wave in the infinite chain, chosen so that the wavelength equals twice the length of the molecule.[34] The locus of the points representing the measured frequency of the strong Raman line at 1500–1650 cm^{-1} *vs.* the inverse length of the molecule, taken for a series of polyenes,[1,4–5,26] should correspond to the upper branch of Figure 3. In reality the *slope* of the calculated upper branch opposes the experimentally observed trend of a *diminishing* frequency for an *increase* in chain length.

The contradiction could be explained with the following argument. The computation of the dispersion curves (Figure 4) was based on the assumptions that three force constants were adequate for characterizing the system and that the numerical values of these force constants were applicable to polyene molecules of any length. Any one or both of these assumptions may have to be modified. (a) If the assumed length independence (*i.e.*, constancy) of the force constants is maintained, the number of force constants would have to be increased so as to include additional interactions. Then the slope of the upper dispersion branch could really be reversed. A meaningful multiparameter model of this kind was reported by Popov and Kogan.[26] (b) If the spring constants are permitted to depend on the molecular length, so that increased length would partly equalize single and double carbon–carbon bonds by virtue of enhanced π conjugation,[36–39] then, obviously, molecules of different lengths cannot be referred to a single dispersion curve. The process of bond equalization reaches a limit in long polyene chains, with residual localization of double bonds still present.[40–42] It is doubted, however, whether increased conjugation alone could account for the drop in the upper-band fundamental from 1652 cm^{-1} in ethylene to 1525 cm^{-1} in β-carotene. (A detailed study of the effect of conjugation on the vibrational frequencies is in progress.)

Shifts of vibrational frequencies can be readily ascribed to changes in the degree of conjugation when molecules of equal lengths are compared and when different terminal groups are the cause of the change.[39] The vibrational mode most responsive to the nature of the terminal group in the vitamins A is the one at 1580–1590 cm^{-1} (Table I). We assume (a) the contribution of the C=C internal coordinates to the mode is larger than that of the C—C coordinates,[26] (b) increased conjugation reduces the C=C force constant and enhances the C—C constant,[39] (c) conjugation increases with the electron affinity of the end group in the order $-CH_2OH$, $-COOH$, $-CHO$.[39] Then one predicts $\bar{\nu}$(retinol) > $\bar{\nu}$(retinoic acid) > $\bar{\nu}$(retinal). Indeed $\bar{\nu}$(retinol) = 1588–1592 cm^{-1}, $\bar{\nu}$(retinoic acid) = 1583 cm^{-1}, and $\bar{\nu}$(retinal) = 1570–1579 cm^{-1}, as expected. One is tempted to extend the argument and claim that $\bar{\nu}$(*trans*-retinal) = 1570 cm^{-1} is lower than $\bar{\nu}$ of the *cis*-retinals because the path of conjugation is interrupted in the cis isomers. The argument does not hold, however, for the retinols.

IV. Concluding Remarks

In the present work we have demonstrated a correlation between the isomer configuration and the resonance-enhanced part of the Raman spectrum in a series of vitamin A molecules. It was shown that such spectra are readily obtained in rather dilute solutions even in minute quantity adsorbed as a small spot on filter paper (the diameter of the area illuminated by the laser could be reduced to $\sim$30 μ). These results suggest the usefulness of a tandem combination of chromatography and Raman spectroscopy to the simultaneous separation and analysis of vitamins A, carotenoids and, hopefully, other biochemicals.

Acknowledgments. We are indebted to Mr. R. G. Kilponen and Dr. T. Cole of this laboratory and to Professor B. Rosenberg for their help and advice.

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