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APPENDIX

Application of Ring-Current Theory Based on the Johnson–Bovey Equation to the Aromatic Amino Acids

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The calculation of the ring-current shifts was by the semi-classical Johnson–Bovey (1958) equation:

$$\delta' \times 10^{-6} = \left(\frac{ne^2}{6\pi mc^2 a}\right)\left\{\frac{1}{[(1+\rho)^2+z^2)^{\frac{1}{2}}]}\right\}\left\{\mathrm{K}+\left[\frac{(1-\rho^2-z^2)}{(1-\rho)^2+z^2}\right]\mathrm{E}\right\} \quad (1)$$

in e.s.u., where ρ is the chemical-shift difference in p.p.m. between the benzene signal and a close olefinic analogue, n is the number of electrons, a is the current-ring radius, e, m and c are the standard constants, K and E are the first and second complete elliptic integrals, and are a function of ρ, z and q, where q is the separation of the aromatic ring from the π-electron cloud and ρ and z are the radial and elevational cylindrical co-ordinates respectively (all three being in units of a).

The equation is derived by considering the secondary magnetic field produced by two currents of electrons, at distances $\pm q$ above and below the aromatic-ring atomic plane. The classical equations for the current and for the magnetic field are used.

When the biochemical application of the Johnson–Bovey (1958) equation is considered, due account must be made of the arbitrary aspects that arise from the theory's semi-classical nature. Accordingly, in its application to tryptophan, phenylalanine, tyrosine

and histidine, the analogies with the treatment of ring-current shifts by quantum mechanics are used where possible as a semi-theoretical justification. The geometries of the rings are taken from standard crystallographic structures. Three rules are used. (*a*) The ring-current shift is multiplied by a factor corresponding to the ring current as calculated by self-consistent wave theory (Table 1) (Giessner-Prettre & Pullman, 1969). (*b*) The separation of the two current loops above and below the aromatic ring is held constant at $2q = 0.128$ nm as the $2p$ orbital is assumed to be the same in all systems. The radius of the ring is, however, set at 0.1182 nm or 0.139 nm to correspond to a five-membered or six-membered ring. Therefore the geometrical dependence of E (ρ, z, q) will be different for the five-membered or six-membered rings (Perkins, 1977). (*c*) The contributions from the five-membered and the six-membered aromatic rings are added to determine the shifts for the tryptophan case (Haigh & Mallion, 1971).

A FORTRAN program JBTAB was thus written and implemented on the Oxford ICL 1906A computer to prepare tables of ring-current shifts for the aromatic amino acids tryptophan, phenylalanine, tyrosine and histidine by the Johnson–Bovey (1958) equation. Microfiche copies of the tables are available on request.

It should be noted that quantum-mechanical tables of ring-current shifts may also be calculated by the Haigh–Mallion (1971) method. This, in fact, was done and these are also available. However, the disadvantage of these tables is that the London approximation is crucial to their success. This approximation breaks down when the parts of interest are near the π-electron clouds, i.e. above and below the aromatic ring (Haigh & Mallion, 1971). Because only upfield-shifted protons are studied, which must therefore be above and below the ring, only the Johnson–Bovey (1958) equation was used in the work described above and it is therefore the only one discussed.

This equation is adequate for six-membered aromatic rings, such as phenylalanine and tyrosine, but it is more difficult to apply this treatment to tryptophan. Giessner-Prettre & Pullman (1971), in their variation of the method, have demonstrated that the effect of neglecting the five-membered ring is to overestimate by 30% the calculated ring shifts in a plane 0.35 nm above the plane of the aromatic ring (e.g. by assuming two fused six-membered rings to represent tryptophan) (Giessner-Prettre & Pullman, 1971). From our tables, as calculated above, the overestimate made by assuming a six-membered ring instead of a five-membered ring is again about 30% for $\rho = 0.0$ nm, rising to about 40% for $\rho = 0.20$ nm (for $z = 0.34$ nm). This suggests that the two different initial assumptions as used here and by Giessner-Prettre & Pullman (1971) about q are not quantitatively crucial in this application, given the reliability of the Johnson–Bovey (1958) equation.

Manual and computer-search approaches were used in determining the structure of the interaction with the ring-current shifts of a neighbouring aromatic ring. Some generalizations are first noted.

(*a*) All the aromatic rings by this method have axial symmetry and not hexagonal, except for the tryptophan indole, where there is a mirror plane perpendicular to the ring plane and passing through the centres of the two rings. For any one geometrical solution involving a six-membered ring and a tryptophan residue, an infinite number of other similar solutions can be generated by using only the shifts from an aromatic ring with axial symmetry. However, by using the shifts from the tryptophan residue, only three other solutions are obtained (two above and two below the ring). If the six-membered aromatic compound is asymmetrical, and cuts the mirror plane of the tryptophan, only two solutions will exist.

(*b*) A complete specification for geometrical fitting requires five parameters, i.e. the two angles for the symmetry axis of the ring, and three Cartesian or cylindrical co-ordinates to specify the relative position of the ring centre. These are simplified as follows. All the atoms of the aromatic ring(s) are assumed to lie in a common plane in a rigid frame. From there, the separation of the aromatic rings in a stacking interaction is taken to be 0.33 nm, the closest distance of approach (Hanson, 1964). From the van der Waals radius of hydrogen and the half-thickness of an aromatic ring (0.115 and 0.165 nm respectively), the distance of closest approach of a proton to an aromatic ring is 0.28 nm perpendicular to the ring plane.

The manual method involved drawing contour diagrams of the ring-current shifts on transparencies, by using the data in the tables (see above). These are used in superposition with a scale drawing of the ligand to evaluate rapidly a series of geometries for which good fits with the experimental data exist. The co-ordinates used for the dinitrophenyl ring are an average of four structures (Galigné & Falgueirettes, 1969, 1970; Gartland *et al.*, 1974; Harlow *et al.*, 1974; Ottersen & Seff, 1974). For the analysis of the ring-current shifts in the Fv fragment–hapten complex, the type of dinitrophenyl–tryptophan structure found for the binary complex is used as a starting point. This assumes that the nature of the stacking interaction is unchanged in the Fv fragment, as suggested by the absorbance (Eisen *et al.*, 1968) and circular-dichroism studies (Freed *et al.*, 1976).

The computer search was done with a FORTRAN program RSEARCH written for the Oxford ICL 1906A computer (Perkins, 1977). This tested all the possible geometries of a stacking arrangement of substrate and ring in order to determine the number of solutions in a given error range. The shifts are

calculated by using the Johnson–Bovey (1958) equation for all the protons of the molecule in each geometry. These are tested against the experimental value of the shift. The geometry is rejected if the difference between experimental and calculated values is larger than a specified tolerance, otherwise the solution is printed out in full. This systematic search involves the three Cartesian co-ordinates x, y and z, and one rotation angle to specify the relative orientation of the two molecules. There are also facilities for printing out the shifts without testing them for a geometry, for the averaging of the shift data for methyl groups, where an average of 12 points around the circumference of the proton rotation is used (Ford, 1974), and for the use of shift ratios rather than calculated shifts in the search. The advantages of shift ratios lie in minimizing the uncertainties of an unknown ring-current strength (as in the dinitrophenyl ring), those resulting from the existence of binary and ternary complexes (in the cases where the shift ratios are constant over the titration ranges) and those arising from experimental errors in the binding constant. This is at the expense of losing one independent variable in a set of experimental measurements. Thus the study of the Dnp-aspartate/tryptophan model compound was based on three shifts of the dinitrophenyl ring protons and five on the indole ring, which reduce to two and four independent observations on using shift ratios. In principle, there are sufficient experimental observations to fix the three parameters needed to define the structure of the model compound on assuming a stacking geometry at a fixed separation.

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