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A Reciprocal Space Algorithm For Calculating Molecular Envelope Using The Algorithm Of B.C. Wang

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A suite of programs designed to improve the quality of protein electron density maps has recently been developed and distributed by B.C. Wang and colleagues (Wang, 1985). The basis of their method is to use the electron density map to determine a molecular envelope and then to set the electron density in the solvent region to a constant value (solvent flattening) and apply a positivity constraint to the electron density in the protein region. The modified electron density map is Fourier transformed, and the resulting phases combined with the original m.i.r. (or s.i.r.) phase information. The combined phases are then used to calculate a new electron density map, and the whole procedure is repeated iteratively until there is no further improvement in the quality of the electron density.

Huber's group have successfully used the solvent flattening option in the structure determination of Human alpha-1 proteinase inhibitor (Loebermann et al., 1985) the photo-synthetic reaction centre (Deisenhofer et al., 1984) and a light harvesting biliprotein (Schirmer et al., 1985), all at 3A resolution, and similar but less dramatic results have been obtained by Wang and colleagues in the structure determination of cytochrome c5 at 2.5A resolution (Carter et al., 1985). As expected, the method is most powerful when the solvent content of the crystals is high (70% for the structures from Huber's laboratory).

The concept of using solvent flattening to improve isomorphous replacement phases is not new, and all the necessary programs are available in Bricognes molecular averaging package. Sigler and colleagues in Chicago (Schevitz et al., 1981) used the same approach to produce a dramatic improvement in the electron density map of fMet tRNA at 4A resolution (also 70% solvent). What is novel about Wang's approach is the algorithm that he uses to determine the molecular envelope from the original electron density map. Instead of relying on visual inspection of the map (usually using an interactive graphics display), Wang's procedure has the advantage of being fully automatic. The first step in this procedure is to calculate an "averaged" map from the starting m.i.r. map, by replacing the electron density at each grid point by the weighted average of the electron density at all surrounding grid points within a sphere of radius "R".

The weighting function used is:

$$w(i) = 1 - r(i) / R \qquad \text{for } rho(i) > 0$$

$$= 0 \qquad \text{for } rho(i) < 0$$

where rho(i) is the electron density at grid point "i", at a distance r(i) from the centre of the sphere. It is important to realise that because negative densities are ignored (ie given a weight of zero), the result is NOT the same as simply calculating a map at low resolution. The second step is to compute a histogram of the electron densities in the resulting averaged map, and to choose a "solvent level" so that the number of grid points with density less than this solvent level corresonds to the expected solvent content of the crystal. (The solvent content can be estimated using the formula given by Matthews (1968) based on the unit cell contents and the protein molecular weight.) All grid points in the averaged map with a density less than the solvent level are then considered to be in the solvent, while the remainder define the protein.

The optimum value of the averaging radius "R" depends primarily on the resolution of the map and to a lesser extent on its quality (ie the noise level in the solvent region). Typically a value between 8A and 10A is used to average a 3A resolution m.i.r. map.

The calculation of the average map can be extremely expensive in c.p.u. time, particularly since Wang's distributed programs require that the calculation is done in space group P1. As an example, chloramphenicol acetyl transferase (CAT) crystallises in space group R32 with equivalent hexagonal cell parameters a = 107.6A, c = 123.4A. A 3A resolution map calculated on a 1.1A grid was averaged using a radius R = 10A; this calculation required 35 hours c.p.u. time on a VAX 11/750.

The calculation can be made very much faster by using reciprocal space methods based on the Fast Fourier Transform. Wang's averaging procedure in real space is directly equivalent to convoluting the truncated m.i.r. map (ie the m.i.r. map with all negative electron density values set to zero) with the weighting function w(r) given by:

$$w(r) = 1 - r / R \qquad r < R$$

$$= 0 \qquad r > R$$

This may be written as:

$$rhoav(i,j,k) = rhotr(i,j,k) \text{ ^ } w(r)$$

where rhoav is the averaged map, rhotr is the truncated map and " ^ " denotes convolution.

From the convolution theorem it follows that

$$FT[rhoav(i,j,k)] = FT[rhotr(i,j,k)] * FT[w(r)]$$

where FT[] denotes the Fourier transform. The Fourier transform of the truncated map is readily calculated using the standard FFT program package, and it can be shown that the Fourier transform of w(r) is given by:

$$g(s) = FT[w(r)] = Y(uR) - Z(uR)$$

where:

$$s = 2 * \sin(\theta) / \lambda$$

$$u = 2 * \pi * s$$

$$Y(x) = 3(\sin(x) - x\cos(x)) / x^3$$

$$Z(x) = 3(2x\sin(x)-(x^2-2)\cos(x)-2) / x^4$$

(See James (1948) p466 for a similar example).

Thus to compute the averaged map, the structure factors obtained by back-transforming the truncated map are multiplied by the function g(s) and the modified coefficients are used to calculate a new map which will be identical to that produced by averaging in real space. In the case of CAT, the c.p.u. time was reduced from 35 hours to 40 minutes, even though the calculation was performed in space group P1.

The function g(s) is plotted in Figure 1 for a radius R = 10A. It is similar in form to the transform of a sphere (which would correspond to the weighting function w = 1 for r < R, w = 0 for r > R) but falls off rather less rapidly. The function is less than 0.001 for Bragg spacings less than 5A, and therefore Fourier terms corresponding to spacings less than 5A will make no significant contribution to the averaged map.

The averaging procedure can easily be modified to use different weighting functions w(r), providing that the Fourier transform g(s) can be calculated analytically. Tests using the function:

$$w = 1 - (r/R)^2$$

gave very similar results to the original weighting function, suggesting that the averaged map, and hence the molecular envelope, is rather insensitive to the precise form of the weighting function.

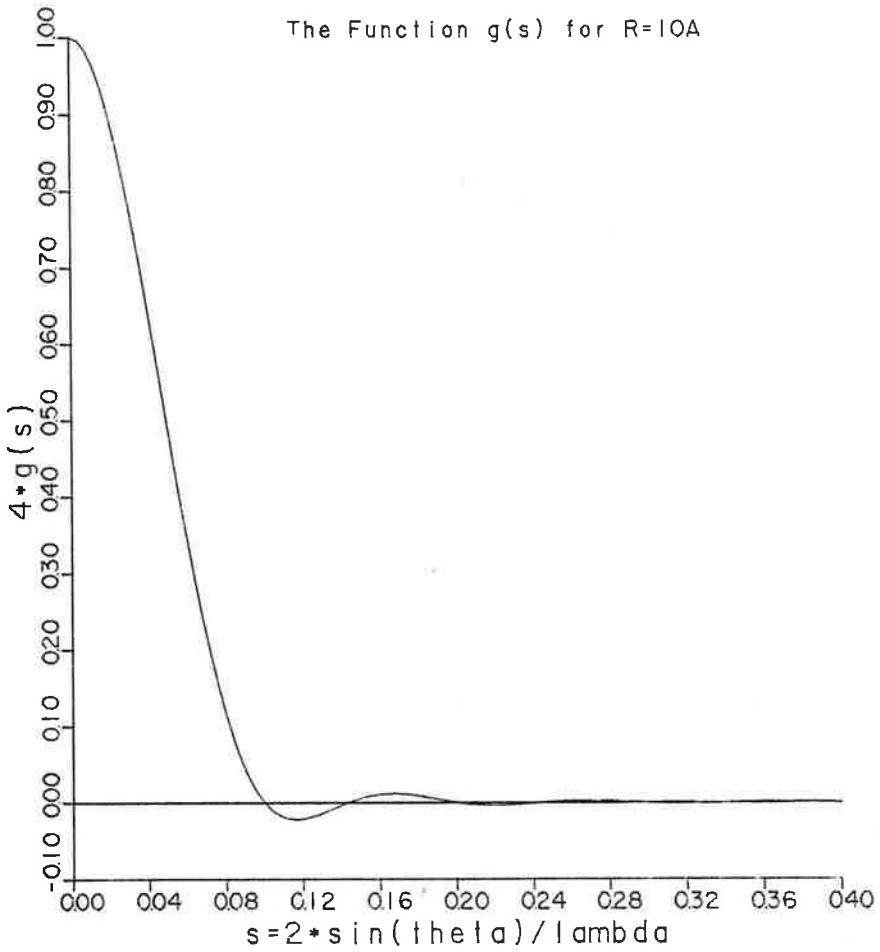


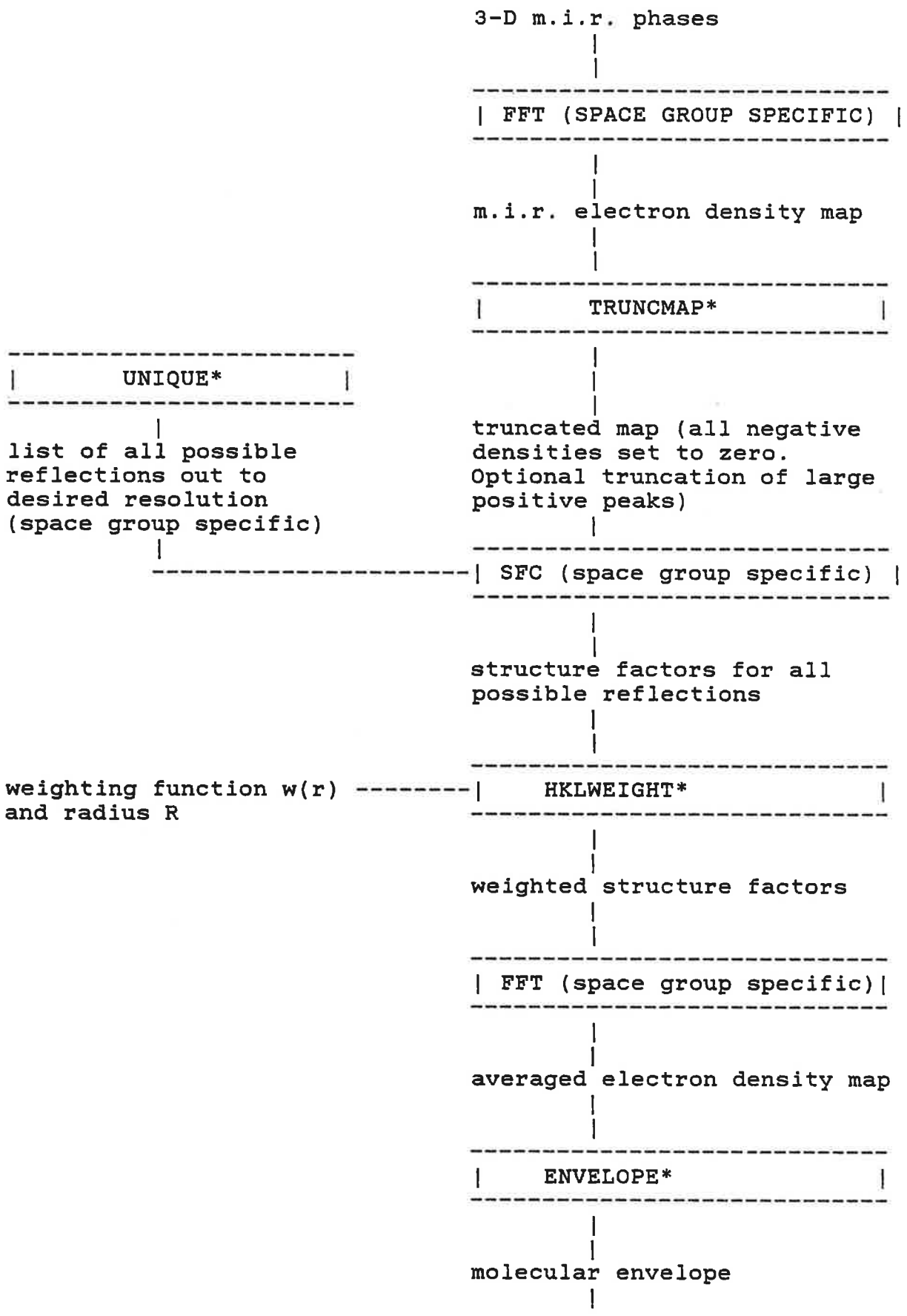
Figure 1

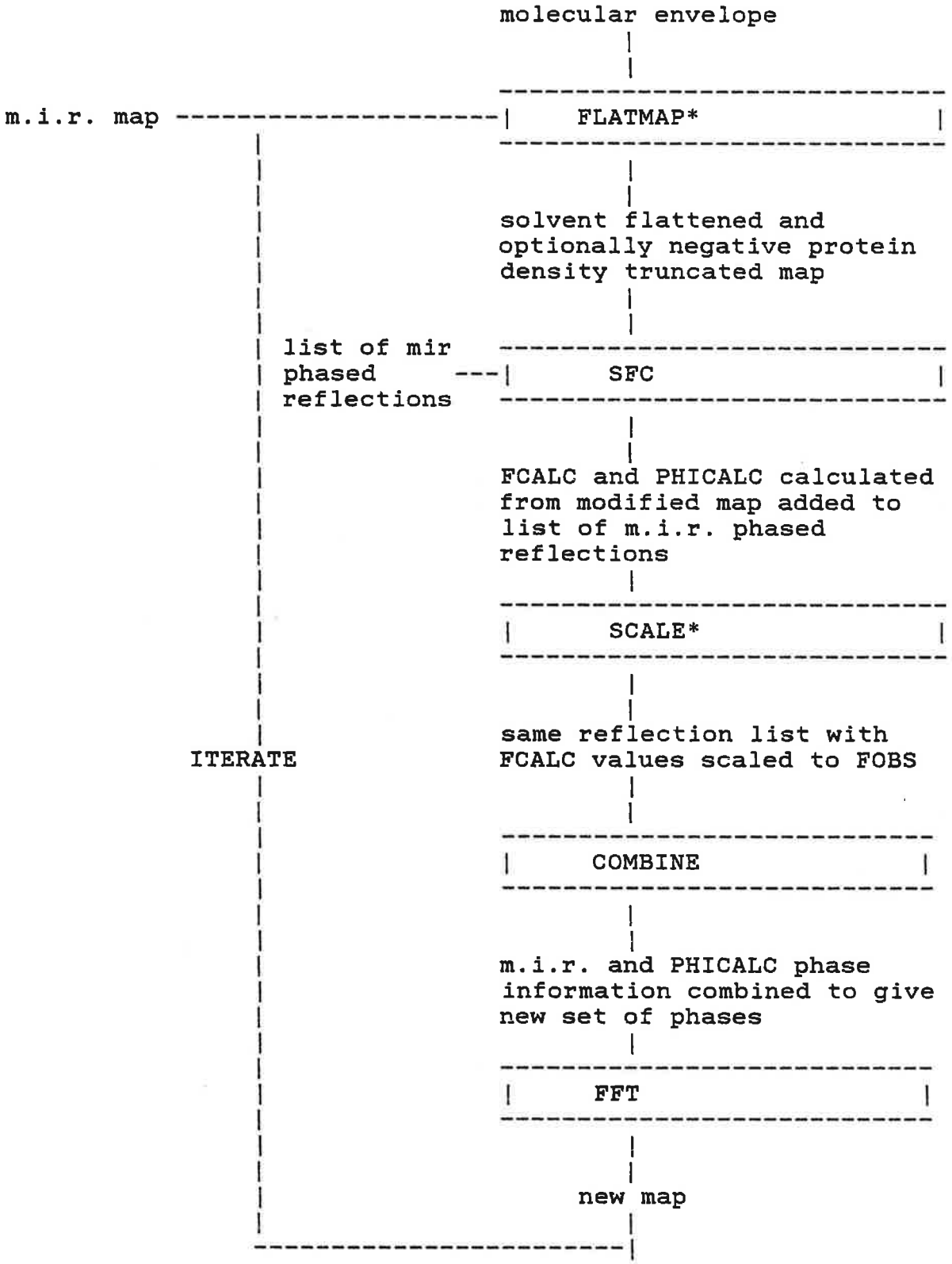
Three practical points are worthy of mention:

1) When using the reciprocal space algorithm, it is essential that all possible structure factors are calculated from the back-transform of the truncated map and included in the calculation of the averaged map. In particular, all low resolution terms must be used, even if those terms were not included in the calculation of the original m.i.r. map. There must be NO low resolution cuttoff in either calculation.

2) Unless there is a version of the back-transform program (SFC) for the correct space group (in which case the program will generate a list of all reflections by default) it will be necessary to provide the program with a list of all possible reflections out to the desired resolution limit. This list is generated by program UNIQUE.

3) The step which truncates the m.i.r. map can also be used to eliminate large positive peaks in the map which could otherwise distort the local molecular boundary. Such peaks can arise from several sources, such as ripples around heavy atom positions, build-up of errors on crystallographic symmetry axes or the presence of metal ions in the protein structure.


3-D m.i.r. phases
FFT (SPACE GROUP SPECIFIC)
m.i.r. electron density map
TRUNCMAP*
UNIQUE*
truncated map (all negative densities set to zero. Optional truncation of large positive peaks)
list of all possible reflections out to desired resolution (space group specific)
SFC (space group specific)
structure factors for all possible reflections
weighting function w(r) and radius R
HKLWEIGHT*
weighted structure factors
FFT (space group specific)
averaged electron density map
ENVELOPE*
molecular envelope



A * denotes programs not currently available in the CCP program package.

Wang's package has been modified to incorporate the reciprocal-space map averaging algorithm and the standard CCP map format and LCF data format. The sequence of steps required to perform one cycle of solvent flattening is given above. Usually the envelope is only determined once, from the original m.i.r. map, so this step need not be repeated in subsequent cycles.

ENVELOPE and FLATMAP are modified versions of Wang's programs ENVELP.FOR and DSFLT.FOR. These are the only programs that have been retained from Wang's distributed package. The changes allow reciprocal space calculation of the envelope and the use of standard CCP map and data file formats. SCALE is the Cambridge program SCALENEW.FOR. UNIQUE, TRUNCMAP, HKLWEIGHT have been written by the author, from whom copies may be obtained.

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DEVELOPMENT OF A VERSION OF THE HENDRICKSON-KONNERT PROGRAM FOR REFINING PROTEINS WITH POLY- OR OLIGO-SACCHARIDE COMPONENTS.

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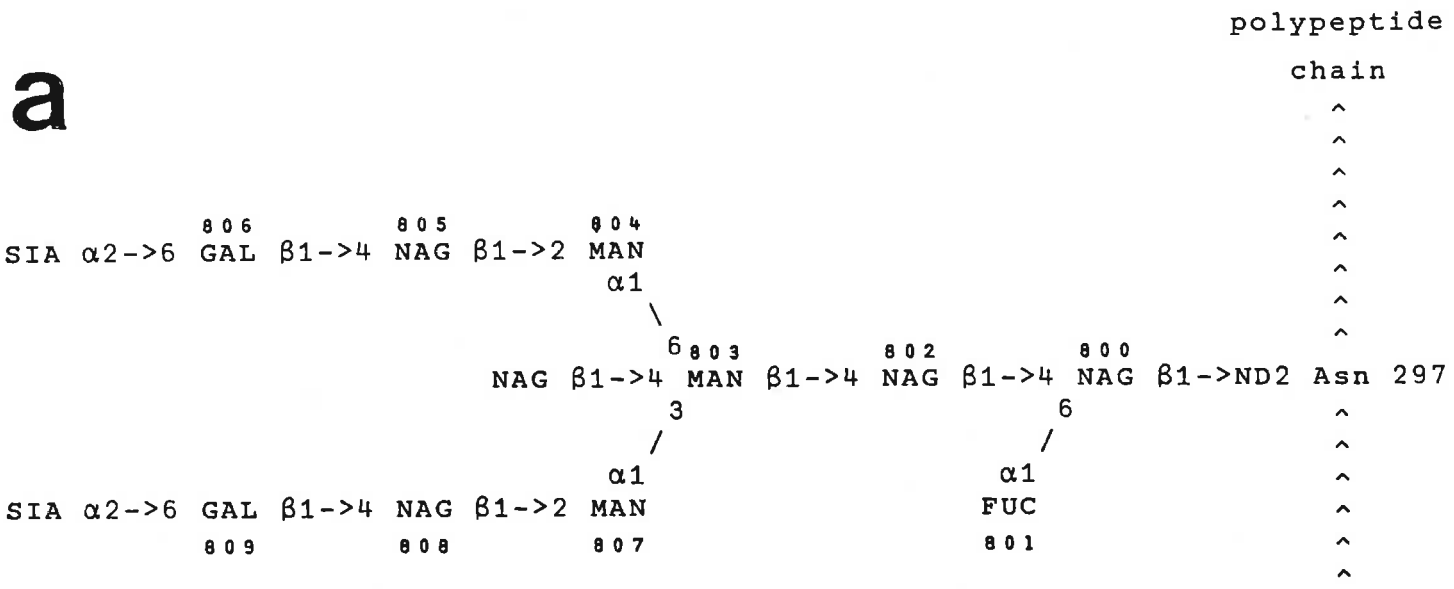
I. INTRODUCTION

The refinement of protein crystal structures using the Hendrickson restrained Structure Factor Least Squares (SFLS) program suite (W.A. Hendrickson & J.H. Konnert in "Biomolecular Structure, Conformation, Function and Evolution", ed: R. Srinivasen, Vol 1, pp 43-57, New York : Pergamon Press) is now a very widely used procedure. There are many reasons for the programs' popularity, but one of them is that the design of the PROTIN program, which is used to set up the coordinates & their restraints for the refinement, makes the incorporation of non-standard residues and small molecules into the list of restrainable entities a very straightforward procedure : it is usually sufficient merely to augment the dictionary of standard groups read by PROTIN, without altering the program itself.

This report concerns a rather more complicated case : the production of a version of PROTIN to restrain the complex carbohydrate component which is encountered covalently bound to the polypeptide chain in glycoproteins. I made this version of the program, which is called PROTFC, while at the Laboratory of Molecular Biophysics, Oxford, in order to refine the structure of the Fc fragment of rabbit IgG at 2.8 Å (B.J. Sutton, P.J. Artymiuk, M. Lewis & D.C. Phillips, unpublished). Each of the two chains of the Fc fragment bears an N-linked complex oligosaccharide of approximate molecular weight 1500 daltons, bound covalently to Asn 297. This is shown in Fig 1.

It was, of course, necessary to include these important oligosaccharide moieties in the refinement in order to obtain as accurate coordinates as possible for them. Not only that, but the omission of such a substantial proportion of the molecule from the model would surely have jeopardised the refinement of the protein part. The geometry of the oligosaccharide therefore had to be restrained in the same manner as the polypeptide chain.

a



b

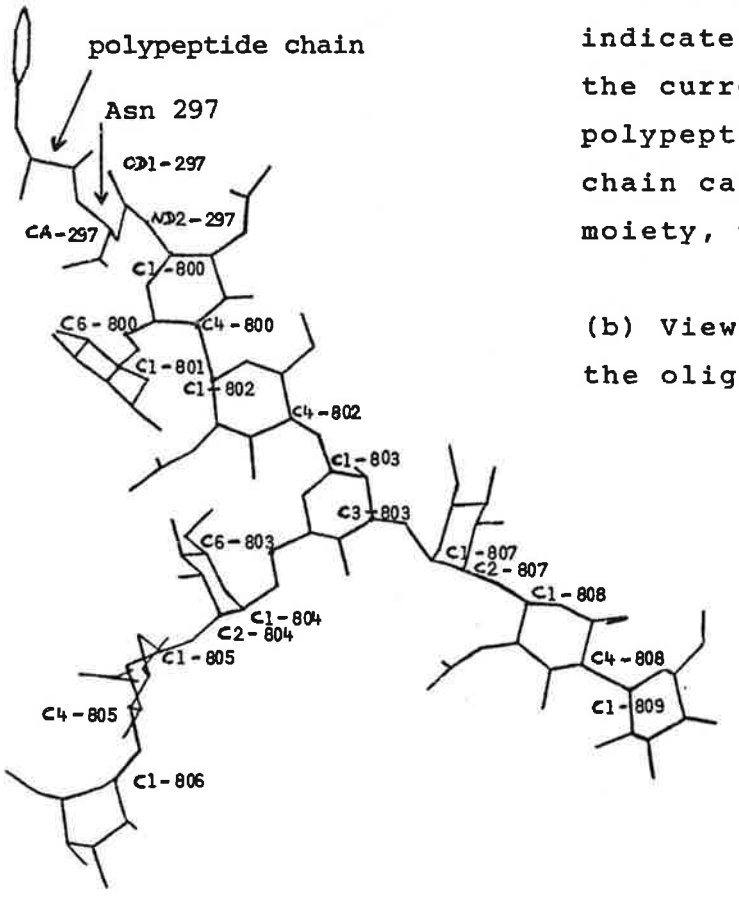


Fig 1 : Diagrams of the oligosaccharide attached to Asn 297 of the Fc fragment of rabbit IgG

(a) Schematic (see Fig 2 for key to 3-letter codes). Residue numbers 800-809 indicate the sugar residues included in the current model attached to one of the polypeptide chains of Fc. The second chain carries a similar oligosaccharide moiety, which is also in the model.

(b) View of the coordinates of one of the oligosaccharides after refinement.

One possible short-term solution might have been to make Asn 297 plus the 120 or so atoms in the attached carbohydrate into one single, extremely large residue. Instead it was decided to create a more generally useful & flexible version of the program which would permit, in principle, the linking together of sugars in any combination, enabling the restrained refinement of many different types of biological polysaccharides. Indeed the program has already been used not only to refine Fc, but also to refine lysozyme with its inhibitor triNAG (ie: D-GlcNAc beta(1->4) D-GlcNAc beta(1->4) D-GlcNAc) at 2.0 Å and, more recently, at 1.7 Å resolution (P.J. Artymiuk, J.C. Cheetham and D.C. Phillips, unpublished).

II. PROBLEMS ENCOUNTERED

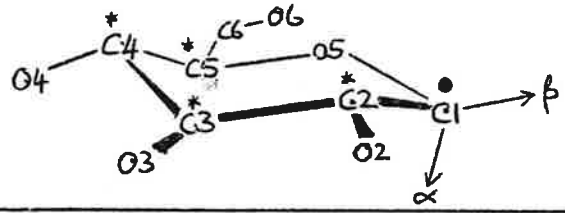
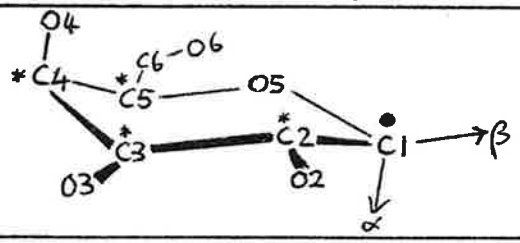
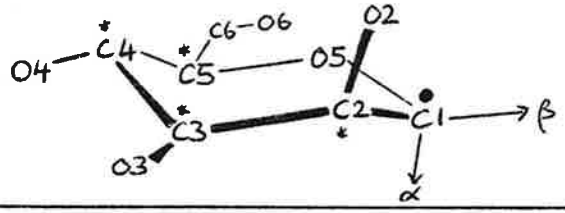
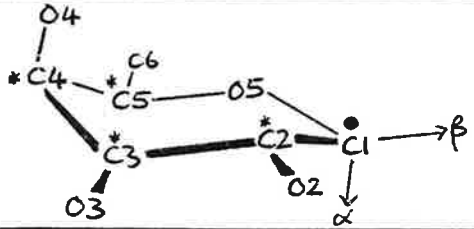
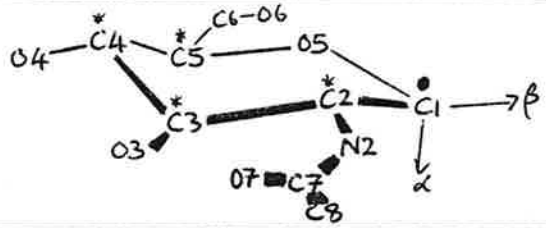
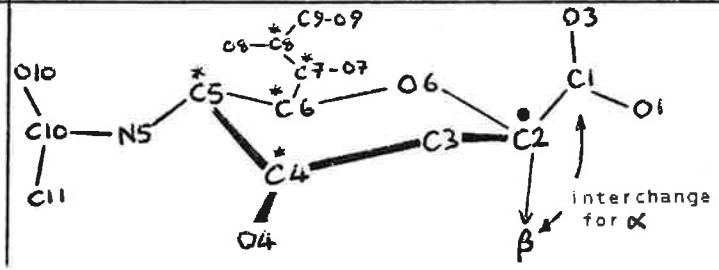
It was necessary to make various modifications and additions to program and dictionary in order to be able to extend the list of restraints to carbohydrates. The problems encountered are now listed, and the way they were overcome indicated :

(a) The inclusion of the 6 sugar residues, detailed in Fig 2. This was reasonably simple, but because protein sidechains have either one or zero chiral centres it was necessary for minor modifications to be made to the program to allow for the restraint of the larger number of chiral centres found in sugars. This is, of course, essential since (for example) glucose, galactose, mannose (and, for that matter, their L-enantiomers and 10 other sugars) have identical covalent connectivity. It is, for example, only the chirality at C2 that makes glucose distinct from mannose from this point of view.

(b) Minor changes were also needed to allow for the multiplanarity of sialic acid which has both an amide and a carboxylic acid sidechain.

(c) Because the sugars are cyclic, there are close 1-4 contacts : across the ring (eg: C2 to C5) and between neighbouring exocyclic substituents (eg: O3 and O4). These are of the order of 2.8 A. To restrain them as fixed 1-4 distances would have been wrong since this would not have allowed for the permitted flexings and puckerings of the saccharide rings. New van der Waals codes needed to be introduced to accommodate these short 1-4 contacts.

FIG 2 : Table of saccharides in new dictionary

3-letter code 1-letter code	ATOM LABELS (* or ● indicates chiral centre)	Common name(s)	No. of chiral centres	No. of planes	Source of coords
GLC (£)		D-glucose	5	0	from: maltose Taksusagania & Jacobson AC B34, p213 (1978)
GAL (*)		D-galactose	5	0	beta-D-galactose Sheldrick AC B32, p1016 (1976)
MAN (?)		D-mannose	5	0	from α-D-Man ρ (1-3)β-D-manρ (1-4) α/β Gl cNAc Warin et al Carbohydr Res 76, p11 (1979)
FUC (#)		D-fucose	5	0	from alpha-L-fucose Longchambon et al AC B31, p2623 (1975)
NAG (!)		D-N-acetyl glucosamine (NAG; GlcNAc)	5	1	α-D-GlcNAc Johnson AC 21, 885 (1966)
SIA (%)		D-sialic acid or D-N-acetyl neuraminic acid (NANA)	6	2	beta-D-NANA Flippen AC 329, p1881

Problems (a), (b) and (c) have been dealt with by making a new dictionary, called CHODICTU, which contains the 6 saccharides. The program PROTFC has been altered to deal with the multiple chiral centres which appear in the new dictionary, and to allow for more than one multiplanar group per polypeptide chain. Also, two new van der Waals repulsion codes have been introduced to deal with the 1-4 contacts.

(d) The most serious problem was the linking of these saccharide residues to form the final oligosaccharide moiety.
This problem falls into 4 parts, of which the third is the worst :

(1) The large number of ways in which two sugars can be covalently bound to each other means that the linkage of sugars is, unlike that of amino-acids, fairly unpredictable. Thus two mannose units can, in principle, be joined in eleven different ways : the glycosidic carbon (C1) of one can be either alpha- or beta-linked (see section (3), below) to O6, O4, O3, O2 or alpha-O1 or beta-O1 of the other.

(2) Unlike a polypeptide chain, an oligosaccharide can be branched (eg: Fig 1).

(3) The link between 2 sugars defines a chirality at the glycosidic carbon (C1 for all of the mono-saccharides in Fig 2, except for sialic acid, where it is C2). This chirality is characterised by the designation alpha- or beta-. Full details of this (and much else) may be found in the IUPAC convention on carbohydrate nomenclature (J.Biol.Chem, vol 247, pp 613-635 (1972)), specifically in section 3.b (ibid. p 614).

The reason this link chirality causes a problem is that the program defines a chirality in terms of the six distances between the four atoms that constitute the chiral centre. In the usual case of an amino-acid alpha carbon, or the beta carbon of Thr or Ile, all these distances lie within the same residue and can be readily defined in the dictionary. But in the case of the link between two sugars some of the distances belong to one sugar residue, some to the other, and some are characterised by the link itself. Also, because of the branched nature of the oligosaccharide, any given sugar residue

can be involved in the definition of several of these chiralities (eg: mannose 803 in Fig 1 is involved in glycosidic linkages to four other sugars; the covalent bond between C1 of NAG 800 and the ND2 of Asn 297 of the Fc polypeptide chain means that the chirality of the beta-1-ND2 link involves three sugar-protein distances (297 ND2 to 800 C1, C2 & O5) and the same NAG is also linked to NAG 802 and to fucose 801). Since the distances defining such a chirality are not all within one residue they cannot be pre-defined in the dictionary. [It doesn't help to rename the O3 of residue 803 (Fig 1) as the O1 of residue 807 - this just makes the chirality of the C3 of 803 equally difficult to define, and ultimately leads to worse complications.]

(4) The restraints are based on atomic coordinates obtained from very high resolution unrestrained small molecule structure determinations of mono-, di- and tri-saccharides. References to the sources of these coordinates are given in the last column of Fig 2. In general the bond lengths and angles found for a given sugar (eg: mannose) in one polysaccharide agree fairly well with the equivalent bond lengths and angles found for the same sugar in a different polysaccharide.

There is, however, one important exception to this : the bond lengths and angles associated with the bridge oxygen in the glycosidic link are known to be subject to considerable variation. Values for the C-O-C bridge angle of between 111 and 122 degrees have been reported. The value of this angle seems to depend on a large number of factors (see, for example, the discussion in Kanters et al : Acta Cryst, vol B32, p2834), and until more small molecule polysaccharide structures have been determined it will be difficult to rationalise.

The best way to specify the large number of arbitrary links between sugars and to allow the user to select the value for the angle at the bridge oxygen seemed to be through the use of a variant on the existing special distance restraint card in the PROTIN parameter file. This is most often used to define S-S bond distances when refining an ordinary protein structure.

In PROTFC this card can still be used in the standard way. However, if the distance between a glycosidic C (C1, usually) in one sugar residue and a bridge O in another residue (say, On) is specified, and if a value for the bridge angle C1-On-Cn is also supplied by the user, then a new subroutine, named SUGLINC, is invoked. This sets up all the restraints necessary to specify all the covalent bond distances, bond angles (O5-C1-On and C2-C1-On [though these two can alternatively be set to user-defined values]), and the C1 chirality in each link. The van der Waals repulsion restraints which would otherwise act between the atoms of the two sugars are turned off near the link. Whether the link is alpha- or beta- is specified by the user : if the bridge angle is set to a positive value a chirality restraint corresponding to an alpha link is imposed; if it is set negative then the beta configuration is restrained.

III. CONCLUSION

The refinements of Fc and of hen lysozyme + triNAG, which were mentioned in the introduction, were successfully performed from Oxford on the Cray 1 computer which was originally at SERC Daresbury and later (lamentably) at the University of London Computing Centre. PROTFC was used in conjunction with Bill Pulford's Cray version of the PROLSQ program. Minor modifications have since been made to the program to run with the Perkin-Elmer FORTRAN 77 compiler at Sheffield.

In its present state the program can be used to set up the list of restraints for the Hendrickson SFLS program for any protein with associated polysaccharide, whether branched or straight, which contains glucose, galactose, mannose, fucose, N-acetyl glucosamine and/or sialic acid. These include, for example, small polysaccharides like triNAG, lactose & sucrose or large branched moieties like glycogen or N-linked complex oligosaccharide. Moreover, the dictionary can now easily be extended to include other sugars, and to compounds related to sugars, such as nucleotides.

FIRST ELECTRON DENSITY MAPS FROM LAUE PHOTOGRAPHS OF PHOSPHORYLASE b CRYSTALS

Janos Hajdu (Laboratory of Molecular Biophysics, Oxford)
Pella Machin, John W. Campbell, Ian Clifton & Sebastian Zurek (Daresbury)
Sheila Gover & Louise N. Johnson (Laboratory of Molecular Biophysics, Oxford)

The use of the Laue technique (stationary crystal and white radiation) opens up completely new territories in the study of structural changes, catalysis or phase transitions in protein crystals. The extremely high intensity of the white X-radiation generated on the Wiggler magnet at the S.R.S. Daresbury allows the recording of a Laue photograph from a protein crystal on a millisecond time scale. Moreover, under favourable conditions and with a high crystal symmetry, a high proportion of data to a certain resolution (say ~3Å) can be recorded at a single exposure. In the case of phosphorylase b crystals ($P4_32_12$), we hoped to record about 70% of the unique data set to 3Å on one photograph.

Last July we reported on our Laue experiments aimed to study catalysis and structural transitions in crystals of the enzyme (Hajdu & Stuart (1985) : Information Quarterly 15, pp 17-18). Here we describe the calculation of electron density maps from some of the experiments and discuss possibilities of improving the procedure. In all of the experiments, a starting "native" photograph was taken before the onset of the structural change, reaction etc. Changes relative to this picture were monitored by taking subsequent photographs along the reaction coordinate after triggering the process. We did not contemplate solving completely new structures with the white radiation technique but concentrated on studying structural alterations in molecules of already known structure. We aimed to combine the phase information from the existing monochromatic data set with the intensity changes measured in the sequence of Laue photographs. Thus, exploiting the extreme data rate available with the Laue technique (well over 100,000 reflections/second can be recorded), we hoped to gain insights into transient phenomena previously inaccessible to crystallography.

A crystal of phosphorylase b was mounted in a thermostated flow cell (Hajdu et al. (1985) : J. Appl. Cryst. 18, 528-532) with its c axis normal to the beam and rotated by ϕ=22.5° from the position with a antiparallel to the beam. This position allows us to sample most of the unique portion of the reciprocal lattice (cf. Information Quarterly 15, 17-18). One of the substrates, maltoheptaose, was allowed to diffuse into the crystal; followed by the other substrate, phosphate, to trigger the reaction. Conditions were chosen to ensure about 90% saturation of the binding sites for maltoheptaose prior to the addition of phosphate. Laue photographs were taken before, during and after the addition of substrates. The crystal was kept in the same orientation throughout, except for frequent translation along the axis (c) normal to the beam (about 40 photographs can be recorded from an average phosphorylase b crystal using a 200μm collimator). The crystal-to-film distance was 128mm. The exposure times with the synchrotron operating at 2 Gev (Wiggler at 5T) with a circulating current of 130-100 mA were 250 msec. The effective wavelength range at station 9.6 was 0.45-2.5Å. With this experimental set-up we hoped to study the time course of the binding of substrates, the transient accumulation of intermediates in the crystal, and the formation of the end products. However, an unexpected order-disorder transition was observed on the addition of phosphate, temporarily affecting the crystal lattice. At the present time we have processed the first 4 photographs only, showing the binding of the oligosaccharide, maltoheptaose, to the enzyme.*

The orientation of the crystal was determined prior to the experiment by taking monochromatic stills at ϕ=0° and ϕ=90° and using the standard IDXREF procedure. This first orientation matrix was used to generate a starting Laue pattern by the program LGEN on the PERQ computer. Programs developed at the Daresbury Laboratory were used throughout. The match between the predicted and observed patterns was checked and, where necessary, was improved using the "spotin" option in LGEN on the PERQ or DISPLAUE on the VAX, followed by refinement of the orientation with REFLAUE and prediction of the new pattern with LGEN.

Next, a simple box-type integration was performed (MOSLAUEN) with no profile fitting. This limited the number of reflections which could be successfully integrated to less than the total on a film. A spot separation limit of 0.3mm *was imposed. In all,* 22449 *spots were generated to* 3Å *resolution :* 5458 *of these were wavelength overlaps,* 5997 *were spatial overlaps and* 2451 *were overlaps of both types.* 15538 *reflections were output for integration, of which* 11639 *were singlets and the rest multiplets. Films were scanned on a* 50μm *raster. Each film pack consisted of* 6 *films interleaved with* 3 *Al foils and* 1 *Cu foil to increase the dynamic range. Care was taken to get the best possible set of camera constants, etc. in the refinement routine of MOSLAUEN before starting the integration. Background residuals for the "A" films were in the range* 1.4-2.8, *for the "B" films* 2.5-3.8, *for the "C" films* 3.7-4.8, *for the "D" films* 4.4-5.0, *for the "E" films* 7.8-15.9, *and for the "F" films* 29.4-40.2. *This drastic increase in the background residual with the "E" and "F" films could be due to the fact that only these were interleaved with a Cu foil. The fluorescence of Cu could have had this adverse effect. No such increase was found with films interleaved with Al foils only. Fortunately, the number of reflections recorded on the "E" and "F" films did not exceed* 800. *Inter film scaling with AFSCALE showed that the agreement between symmetry-related reflections which appeared within* $\Delta\lambda$=0.05Å *was better than* 5%; *this analysis can't be performed reliably with* $\Delta\lambda$ *values greater than about* 0.08Å *as the orientation of the crystal plays a rôle. Deconvoluting the harmonics was performed using the program UNSCRAM, but these reflections have not been included in later stages of the processing.*

Phases from a monochromatic reference data set were used. The structure of phosphorylase b has been solved using conventional techniques and monochromatic X-radiation (Weber et al. (1978) : Nature 274, 433-437), the refinement to 1.9Å *is almost completed and the current crystallographic R factor for some* 60000 *reflections with intensities greater than* 3σ *is* 19.0% *in the* ∞-1.9Å *resolution range. Phases were combined with the intensities measured on the Laue photographs and maps calculated in* 3 *different ways :*

1) *Scaling the Laue intensities to the monochromatic reference intensities using LAUESCALE and then calculating difference Fouriers between the monochromatic and the Laue data;*

2) *Scaling the Laue intensities to the monochromatic reference set using LAUESCALE and calculating difference Fouriers between the Laue data scaled in this way;*

and 3) *Using the first Laue photograph as the reference data set, scaling the subsequent Laue photographs to it using an anisotropic temperature factor and calculating the coefficients*

$$\frac{F_{LAUEn} - F_{LAUE1}}{F_{LAUE1}} * F_{MONOCHROMATIC}$$

for input to difference Fourier maps.

After the various scaling procedures, we were left with a unique set of 5261 *reflections, about* 30% *of all data to* 3Å *resolution. The difference maps obtained with this set were fairly noisy, but showed the bound oligosaccharide at the glycogen storage site. The noisiness could be attributed to two factors :* 1) *genuine errors in the Laue data (recording, processing, etc.); and* 2) *the fact that only about* 30% *of the complete data to* 3Å *resolution was available for the calculations. To test this, a reference map was calculated from monochromatic data using only those reflections which survived the Laue processing. The resulting map was fairly similar to the maps calculated from the Laue data, but the Laue maps still seemed to be more noisy. Clearly, there is room for improvement and we hope to achieve it in three ways :*

1) *Our first concern is to increase the number of reflections which can be integrated on a Laue photograph. A suitable profile fitting program could give us the desired increase and including unscrambled harmonics will bring the figure even higher. Then the optimisation of the crystal-to-film distance and the use of a larger film at longer crystal-to-film distances should be considered.*

2) *Reflections with longer wavelength X-radiation suffer from extensive absorption, both within the crystal and on their way to the film. Absorption by the air could be decreased by using a helium cone. Small alterations in the crystal orientation (e.g. during translation) could still severely affect the absorption of these reflections in subsequent photographs. A possible way around this problem is to predict the Laue pattern of the longer wavelength reflections and then, after integration, remove these from further processing. Because long wavelength reflections appear at higher θ values, they represent only a small fraction of the data recorded on a flat-plate cassette at usual crystal-to-film distances.*

3) *Imposing any resolution limit (3Å in this case) when predicting the Laue pattern is clearly arbitrary. The crystal may diffract well beyond this limit, resulting in wrongly-predicted singlets which in reality are multiplets and should be treated accordingly. This can be corrected for by predicting the pattern to higher resolution and reprocessing the data. The use of a wrong, too narrow, wavelength range has similar effect.*

In principle, perhaps the best way to proceed is to predict for all possibilities (higher resolution, wider λ range) and then pick up a reliable set of data by using various filters (λ cut-offs, intensity cut-offs, σ cut-offs, resolution cut-offs). Reprocessing is well under way and we hope this will give us room to "play" with the data, to assess quality and to calculate better maps. Further development of the method will be pursued with urgency.

We would like to acknowledge the continuing assistance in this work from Dave Stuart and John Helliwell.

The development of the Leeds Liquid Crystal Stereo Viewer.

A personal account by Sandy Geddes (Astbury Department of Biophysics)

The ability to see pictures in 3-d on a computer display screen is invaluable in fitting electron density maps, comparing molecular structures, docking substrates and inhibitors, and generally manipulating molecular models.

Until about three years ago we used the Ortony viewing system for our vector display system. Here, two images of the structure are displayed one above the other on the screen. The coordinates for the upper image are generated from the lower by a rotation of 5 or 6° about a vertical axis more or less through the centre of the molecule followed by a reflection through a horizontal plane half-way up the screen. A horizontal transparent sheet abuts the screen mid-way between the two displayed images. The observer wears polarising spectacles in which the directions of polarisation of the right and left hand lenses are crossed and looks down through the transparent sheet at the lower image; the upper image is seen superimposed on the lower by reflection off the top surface of this sheet. Light from the upper picture is polarised by the reflection and is seen through only one of the lenses; the other lens cuts off this light, but allows transmission of light from the lower picture. The two different images are combined by the brain and the observer perceives a 3-d picture.

One of the advantages of this system over the commonly used sideways separated "stereo pairs" is that the viewing distance is not critical and, in principle, two or more observers can view the picture at the same time. In practice, 3-d viewing was only truly satisfactory for one person at a time and it was frequently troublesome to get the images properly superimposed. More often than not, users preferred to employ real-time rotation of the image to achieve a stereo effect, and this had the advantage that the whole screen could be used to display a single image, with a consequent gain in the amount of detail which could be clearly resolved.

In 1983, Andy Morffew was kind enough to arrange for us to borrow a Bausch and Lomb viewer from IBM Winchester. This device uses the "tachistoscopic" method of stereo-viewing in which the left and right eye views of the structure are displayed on the screen alternately and in rapid succession - each view is on the screen for about 20 milliseconds, corresponding to the 50Hz refresh rate of most display tubes. The screen is viewed through narrow horizontal slits at the front and back of a horizontal cylinder of about 5cms diameter. Inside this cylinder is another co-axial, opaque, cylinder which rotates about the common axis. Parts of this inner cylinder are cut away in such a manner that when the left eye can see through both the front and back slits of the outer cylinder the view of the right eye is obscured. When the inner cylinder is rotated through 90°, the right eye can now see through both slits in the outer cylinder and the view of the left eye is obscured. The speed of rotation of the inner cylinder is adjusted so that the left eye has a clear view of the screen when the left hand view of the structure is being displayed and the right eye sees the screen only when the right hand view of the structure is being displayed. The phenomenon of persistence of vision

allows the brain to merge the two different views and the picture is perceived in 3-d.

We were impressed by the quality of the stereo perception afforded by this device; but it was expensive to purchase since a costly computer interface is required to surmount the inherent difficulty of achieving stable synchronisation between the refresh cycle of the display, which may not be constant, with a mechanical shutter having a relatively high inertia. Mark Harris tried to build a cheaper version made from a plastic lemonade bottle (the outer cylinder) and the centre of a toilet roll (the inner cylinder) but despite the delightful ingenuity of the assembly we could never quite persuade ourselves that the stereo perception was anything other than imagination.

The problem was much discussed over coffee. On the one hand we had a cheap system involving polarising spectacles, and on the other hand there was a more effective system employing a mechanical shutter which we could not afford. A group which included Tony North, Mark Harris and John Lydon considered the possibility of a tachistoscopic device using electro-optic rather than mechanical shutters. John Lydon had a consuming interest in liquid crystalline materials and was able to describe how these could be used as shutters, although at that time a liquid crystal acting as a fast switching shutter in a tachistoscopic device was not available. The most promising liquid crystal seemed to be the nematic type, which for the purposes of discussion can be assumed to comprise long thin molecules which exhibit long range order and line up with their long axes parallel, although they are otherwise randomly distributed. If such a material is placed in a thin glass cell whose inner surfaces are etched to produce fine parallel grooves then the molecules tend to line up along the direction of the grooves. If the grooves on one side of the cell are at right angles to the grooves on the other then the molecules line up with the grooves on one surface and gradually spiral round to line up with the grooves on the other surface. This "twisted nematic" cell has the property of rotating incident light by 90° on its passage through the cell. If polarising films are placed on the front and back surfaces of the cell, with their directions of polarisation crossed, then the first film polarises the incident light, the liquid crystal rotates the direction of polarisation by 90°, and this polarised light is transmitted by the second film. It also happens that the application of a suitable voltage between the front and back of the cell (via transparent metallic electrodes) causes the molecules to line up with the direction of the applied electric field and the liquid crystal no longer rotates incident polarised light. An electro-optic shutter is made simply by placing a twisted nematic cell between crossed polars and switching the voltage on and off (in practice, a low frequency pulse is used to stop the cell discharging). However, it turns out that the reorientation of the molecules which takes place when the voltage is switched off is a relatively slow process and not nearly fast enough for a tachistoscopic stereo viewer.

John Lydon introduced us to Mike Clark at the Royal Signals and Radar Establishment at Malvern, who had recently devised a way of making these cells switch more quickly by the application of a high frequency voltage pulse soon after the low frequency pulse ended. The dielectric behaviour of the molecules is a function of the frequency of the applied field and above a certain frequency the molecules are driven to lie perpendicular to the applied field rather than parallel to it as they do at lower frequencies. The problem remained to find values for the low and high pulse frequencies, amplitudes and

durations, and the interval between the low and high frequency pulses, which would give a shutter of sufficiently short transition times between the clear and opaque states - a simple 7 parameter problem! Fortunately, Mike Clark was able to lend us a marvellous black box, about the size of a small suitcase, which could deliver pulses with any chosen parameters and it was not too long before Mark Harris had sorted out the optimum parameters for this "dual-frequency-addressed" liquid crystal shutter.

The next problem was to reduce the suitcase full of electronics to more portable dimensions. Now, I don't consider that it was luck that a liquid crystal expert worked in a department where there was a need for 3-d molecular graphics (after all, biophysicists are always preaching the unification of disciplines); but there was a touch of serendipity about the fact that Mark, a graduate chemist, was also an electronics hobbyist. In a few months he had assembled the appropriate circuitry. In the beginning there were wires everywhere and owing to lack of bench space various parts of the circuit were kept in separate drawers of a small office-type chest of drawers. This became known affectionately as the "Tower" and lacked only an anodised surface to compete favourably with similarly named objects in the local hi-fi supermarkets.

The only problem that remained was to synchronise the liquid crystal shutters with the alternating images on the screen. Plunging into the back of the Evans and Sutherland display system in order to tap into the refresh circuitry is not something to be undertaken lightly. Mark and Tony North independently arrived at another possible solution - why not take synchronisation signals optically from the screen? This could be done by displaying small symbols alternately in a corner of the screen at the same time as the left hand and right hand images are displayed and sensing these symbols with photodetectors coupled to the appropriate liquid crystal cells. The advantage of having two sensors rather than one is that it is possible to maintain the correct chirality of the perceived 3-d image at all times. The screen refresh rate itself can be detected with a single sensor, but whether or not the correct image, or a back to front one, is seen would be a matter of chance. So, our prototype system had the electronic components jostling in wooden drawers, the photodetectors stuck to the screen with insulation tape (later to be replaced with corn plasters) and the liquid crystal cells mounted on a rectangular piece of perspex with paper clips (to be held à la lorgnette); but it worked!

A few "pre-production" models were deliverd in 1984 to a few other laboratories (York, Birkbeck, Oxford, and MRC Cambridge) for field testing and as far as we know these are still working well. We wanted to make sufficient for all UK protein crystallography groups but were advised that this would affect any patent applications. Anyway, a much improved commercial version went on sale at the beginning of this year and I hope the wait has not been too long.

This ends the story as far as molecular graphics research is concerned, but it is just the beginning when the wider applications are considered.

During all this development we had at the back of our minds the potential application to television. Television signals are broadcast as two interlaced frames; during the first frame, lines 1,3,5... are drawn on the screen, and in the second frame, lines 2,4,6... are drawn. The idea was to put the left eye view of an object or scene on

one frame and the right eye view on the other, interlaced, frame. Now, for each frame displayed on the TV screen there is a corresponding scan of the image on the TV camera tube itself. We had to arrange for a left eye view of the object to be on the camera tube during one frame scan, and a right eye view during the following scan. The first system tried involved a single television camera with liquid crystal "spectacles" placed a few inches in front of the camera lens and a beam-splitting device placed between the camera lens and the spectacles. Light from the object passing through one of the liquid crystal cells went directly to the camera lens after transmission through a semi-silvered mirror inclined at 45° to the light path; light from the other cell was reflected first from an ordinary mirror parallel to the semi-silvered mirror and then from the back surface of the semi-silvered mirror onto the camera lens. The immediate problem was a lack of intensity balance between the two images because our semi-silvered mirror reflected rather more than 50% of incident light. We could not complain; the mirror had been kindly donated by a local manufacturer whose major products were glass pelmets for gypsy caravans and bevelled glass decorations for fruit machines in Las Vegas. An attempt was made to reduce the light imbalance using neutral density filters at appropriate positions in the light path. Light from the object now had to pass through the polarising films, the liquid crystal cells, and then suffer imperfect reflections or transmissions before it ever reached the camera tube. Actually, it was quite difficult to see anything at all on the television screen and we had to use arc-lamps placed close to the object to provide sufficient illumination. These tended to melt the molecular models being televised. Nevertheless, Mark was convinced that he could perceive a 3-d image for brief periods, although others were less enthusiastic.

We debated whether or not to spend some money on a proper beam-splitter, but decided instead to try an alternative approach of using two synchronised television cameras mounted side-by-side, the University Audio Visual Unit having offered to loan us the equipment free of charge. The camera lenses looked just like a pair of eyes viewing the object. A liquid crystal cell was mounted on each camera in the position normally occupied by the lens cap. Each cell opened and closed in synchrony with the camera frame scans, but when one cell was open the other was closed. The output signals from the two cameras were merged using a conventional studio mixer so that the signal reaching the television monitor comprised one frame showing the view seen by the left hand camera followed by a frame showing the view seen by the right hand camera. This system worked quite well and everybody who tried it could perceive a 3-d image. However, the picture quality was somewhat degraded (probably because the optics of the liquid crystal cells were not as good as those of the camera lenses) and, unexpectedly, there was a certain amount of ghosting. We thought this must be due to the presence of an after-image left on the camera tube and this was being scanned when the shutter was closed so that in any one frame a faint image from one camera was being merged with a bright image from the other camera.

The final stage of the development was to remove the liquid crystal shutters so that the cameras had an unimpeded view of the object and to use electronic means to blank-out alternate frames from each camera. This "video processor" was built by Don Peel in the Audio Visual Unit and it worked splendidly at first use, both restoring the picture quality and eliminating the ghosting. For the initial video recordings that we made, synchronisation of the alternating images on the television screen with the switching of the liquid crystal

shutters worn by the observer was achieved with a small device built by Martin Leybourne in our electronics workshop. This separated the frame synchronisation pulses from the composite video output of the recorder and sent them to the logic circuitry driving the shutters. For broadcast transmission, however, we were concerned that the frame synchronisation pulses would have to be extracted from the demodulated RF signal at the receiving monitor and so a method was devised to allow the use of Mark's optical sensors. Again, we were lucky to have the considerable resources of the Audio Visual Unit at our disposal. While a scene is being televised, a device called a "rectangle generator" can create a black area on the screen into which other pictures can be inserted. A BBC microcomputer was synchronised with the television cameras and a program was written to draw symbols in such a small black area at the corner of the screen. One of the "colours" available to the BBC micro is flashing black/white and the rate of flashing can be adjusted using simple commands. We have made a demonstration video recording using this system and it works perfectly well. However, it is not suitable for televising scenes remote from studio facilities and the next step is to modify the "video processor" so that it can provide on-screen synchronisation signals.

There is an obvious use for stereo TV in a teaching and conference environment; lectures can be televised and any molecular models used for demonstration purposes can be shown in 3-d. In addition, 3-d video recordings made from computer graphics displays can be edited into the televised lecture. However, it is likely that the major applications will be outside our field; for example, in the remote handling of radio-active materials, in the remote inspection of welds in the legs of North Sea oil rigs, and so on. Domestic stereo TV is now certain to come withn a few years. Our system, or a development from it, may not be the one eventually chosen, but it could be. The demands of the home viewer will be far more exacting that those of a protein crystallographer who has spent years trying to fit electron density maps with two dimensional images. It will probably be necessary to move to larger flat screens with dark surroundings since the brain sometimes objects to images protruding from a small screen at the viewing distances normally encountered in the living-room. The use of interlaced frames reduces the resolution of the image, so high-line-rate non-interlaced systems would be preferable and, interestingly, such systems are now about to enter the domestic arena.

In summary, I would like to make 3 points. Firstly, The demands of protein crystallography over the years have led to numerous technological spin-offs, particularly in the applications of computational methods and computer graphics. Secondly, I am more than ever convinced of the value of interdisciplinary science, as exemplified by molecular biophysics. Thirdly, the development described above would have proceeded much more quickly if the other demands on our time had not been so great. I am far from alone in pleading for more "thinking time" for scientists in UK universities. Finally, I must comment on the tremendous amount of fun that we had during this development; due in large part to Mark's keen sense of the ridiculous and his desire to out-perform Heath-Robinson whenever possible. Of course, it is all much more serious now that a commercial product has gone on sale; I'm sure that we should have had less fun if the prospect of commercial gain had been a major driving force at the outset. This is something to be borne in mind in the current drive to commercialise university research. I would like to end with the following lines written by Henry More about 300 years ago. I'm not really sure if they are apposite, but I like the sentiment.

I hate and scorn that Kestrell kind
Of bastard scholars that subordinate
The precious choice inducements of the mind
To wealth or wordly good. Adulterate
And cursed brood! Your wit and will are born
Of the earth and circling thither do return.

Profit and honour be those measures scant
Of your slight studies and endeavours vain,
And when you once have got what you did want
You leave your learning to enjoy your gain.
Your brains grow low, your bellies swell up high,
Foul sluggish fat ditts up your dulled eye.

Sandy Geddes