

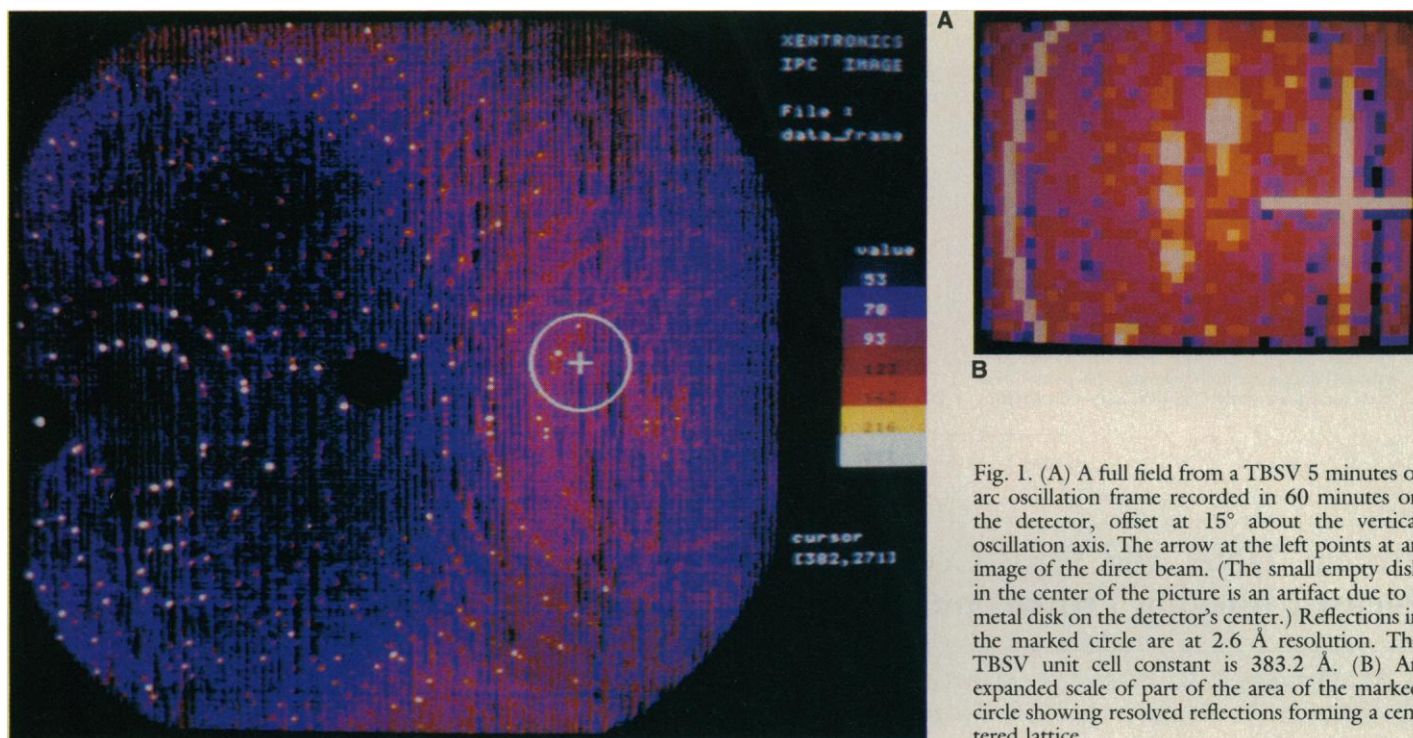


Fig. 1. (A) A full field from a TBSV 5 minutes of arc oscillation frame recorded in 60 minutes on the detector, offset at 15° about the vertical oscillation axis. The arrow at the left points at an image of the direct beam. (The small empty disk in the center of the picture is an artifact due to a metal disk on the detector's center.) Reflections in the marked circle are at 2.6 Å resolution. The TBSV unit cell constant is 383.2 Å. (B) An expanded scale of part of the area of the marked circle showing regular resolved reflections forming a centered lattice.

rotating anode, 100- μ m focusing cup, Franks double mirror collimation optics). During 12 months of operation, the sealed detector has never failed, and no dead spots have appeared. The detector is used on a rotation oscillation camera (Supper, Natick, MA) with a vertical rotation axis. The detector can therefore be swung around in the horizontal plane to collect high angle data. Crystal to detector distance is variable: 12 cm was used for lysozyme, variable surface glycoprotein (VSG), influenza virus hemagglutinin (HA), and repressor-DNA, and 18 cm for TBSV (8). The data acquisition computer (CADMUS 9730) includes 1.5 megabytes of memory, a 65-megabyte Winchester disk, and a nine-track tape drive. Output is displayed on a color raster graphics terminal.

The direct output from the electronic detector is an x and y coordinate of the position of incidence of each photon. Several factors distort this output. The two most significant are anode modulation, caused by the presence every 1 mm of anode wires, which locally attract more charge, and pincushion distortion, created by the curvature of the detector face. The first of these is corrected at the time of data collection by a mapping function from the detector position outputs (X_D , Y_D) to the 512 by 512 pixels (X_p , Y_p) in the computer memory image array. The mapping function is produced with an ^{55}Fe source to create an even flood field. The pixel edges and size are defined [in detector output coordinates (X_D , Y_D)] so that each pixel contains 1/512 of the

total intensity when the detector is evenly illuminated. Residual effects of anode modulation not equalized by this approach are compensated by taking background from the same pixels as spot intensities (see below). This correction is sufficient for the accurate integration of reflections, which is a local operation. To locate reflections accurately, we have introduced a correction for positional "pincushion" distortion. This correction is an interpolation between curved "detector space" and flat "true space," using data from the image of a brass calibration plate with a regular array of holes that fixes to the front of the detector. This two-stage correction preserves the maximal amount of positional information by dividing the axes into bins that each have an equal probability of acquiring a positionally random event. These corrections are required for the very high spatial resolution and require about 1 to 2 hours of detector time every week or two.

The crystal is first approximately aligned by a series (two to four) of "still" frames collected by the detector and analyzed on the detector graphics terminal by an interactive program developed by one of us (P.M.). This requires 5 to 10 minutes, during which crystal quality can also be assessed visually from the data displayed on the computer screen. Data are collected as a series of consecutive oscillation "frames" (a 5 minutes of arc angle oscillation exposure in our current practice). Each frame, a 512 by 512 pixel image, is written to magnetic tape after it is collected. A 2400-foot tape holds just

over 100 frames at 1600 bytes per inch. Our strategy is to determine the precise crystal setting parameters directly from an analysis of the same data frames that are used to integrate the reflections. The method is based on techniques for oscillation-film data processing (9). This differs from the "electronic stationary picture method" (3) in which crystals are carefully aligned first and frames processed as they are collected.

Consecutive small oscillation (5 minutes of arc) frames and a film-scanning-like strategy offer several advantages:

1) The signal-to-noise ratio is increased relative to oscillation film methods, because spots only need to be integrated on frames in which they appear. (A typical spot may span two to five frames depending upon beam divergence, crystal mosaicity, and position relative to the oscillation axis). On an oscillation photograph, a spot is often recorded only during 1/10 of the oscillation range, while 9/10 of the time that region of the film records background scatter.

2) Crystal exposure time is not wasted in finding accurate setting parameters, since the same data are used both for setting information and intensity data.

3) The reflections are examined in three dimensions (x , y on the detector face and frame number, which indexes the oscillation angle ϕ), because a series of frames are analyzed at one time. Examination of a three-dimensional box (x , y , ϕ) around a reflection allows its x , y , ϕ centroid and its rocking curve width, γ , to be determined. These are equivalent to knowing partial-spot

intensity ratios in oscillation film post-refinement, from which precise crystal alignment parameters and spot shapes can be calculated by established methods (9).

4) A knowledge of the spot position and width in three dimensions allows integration to be limited to pixels on frames containing the spot and allows background to be collected from corresponding pixels on frames "before" and "after" the reflection itself. This method of background subtraction

minimizes systematic errors due to pixel size variation and other nonuniformities due to anode modulation.

Our program to process the detector output is written in Fortran for a VAX 11/780 with a VMS operating system (Fig. 2). To initiate processing, a small sample (50 to 100) of intense reflections is located, usually from frames at the beginning and end of the ϕ range on the tape. The centroids (x, y, ϕ) and ϕ widths of the spots are determined by

examining the reflections in three dimensions. These observations, along with estimates of the unit cell parameters, camera parameters, and approximate crystal setting angles, are used to predict the indices of this sample of reflections.

Three groups of parameters are then refined based on the observations and on the condition that indices be integral. (i) The unit cell dimensions and crystal alignment angles (together with the wavelength, which is known) determine the rotation angle ϕ (or frame number) at which a reflection appears. (ii) These, together with camera parameters (crystal to detector distance, camera tilt, and rotation), determine where on the image plane a reflection appears. (iii) Finally, the width of the rotation profile is determined by the beam divergence, dispersion, and crystal mosaicity.

The first two groups are refined together by a modified least-squares system. The errors in frame number (rotation angle) are weighted at least a thousand times more strongly than those in the detector face x, y position. This weighting ensures that the crystal parameters, which affect the rotation angle, effectively refine independently of the camera parameters, which do not. Ill determinacy of the least-squares equations due to interactions between crystal and camera parameters is thus prevented. The weighting is justified because the ϕ angle for spots is well measured and independent of residual distortions that may affect x, y position on the detector face. In detail, we proceed as follows. Profiles of the intense reflections chosen for the refinement are first used to refine the rocking curve and to improve the accuracy of ϕ (centroid) for each spot. A rocking curve of the form

$$I = I_0 \{1 - \sin[(\pi/2)(\beta - \beta_0)/\gamma]\}$$

is fit by a least-squares calculation, minimizing

$$\sum_i \text{spots} \sum_f \text{frames} (I_{fi}^o - I_{fi}^c)^2$$

Here, I is intensity, integrated on the (x, y) plane, a function of ϕ , I_0 is a scale factor, I_{fi}^o is the integrated intensity on frame f from spot i , I_{fi}^c is the calculated intensity on frame f from spot i , and γ is the half width of the rocking curve.

The definition of the angle β can be found in (9). The parameter γ is assumed to have a form (9)

$$\gamma = \gamma_0 + \gamma_1 \tan \theta_i$$

where θ_i is the Bragg angle for reflection i , γ_0 is a parameter to be refined, and γ_1 is the dispersion of γ , given by $\Delta\lambda/2\lambda = 0.0013$ for the $\text{CuK}\alpha$ doublet.

The rocking curve is used at this point to obtain a better estimate for the ϕ angle of

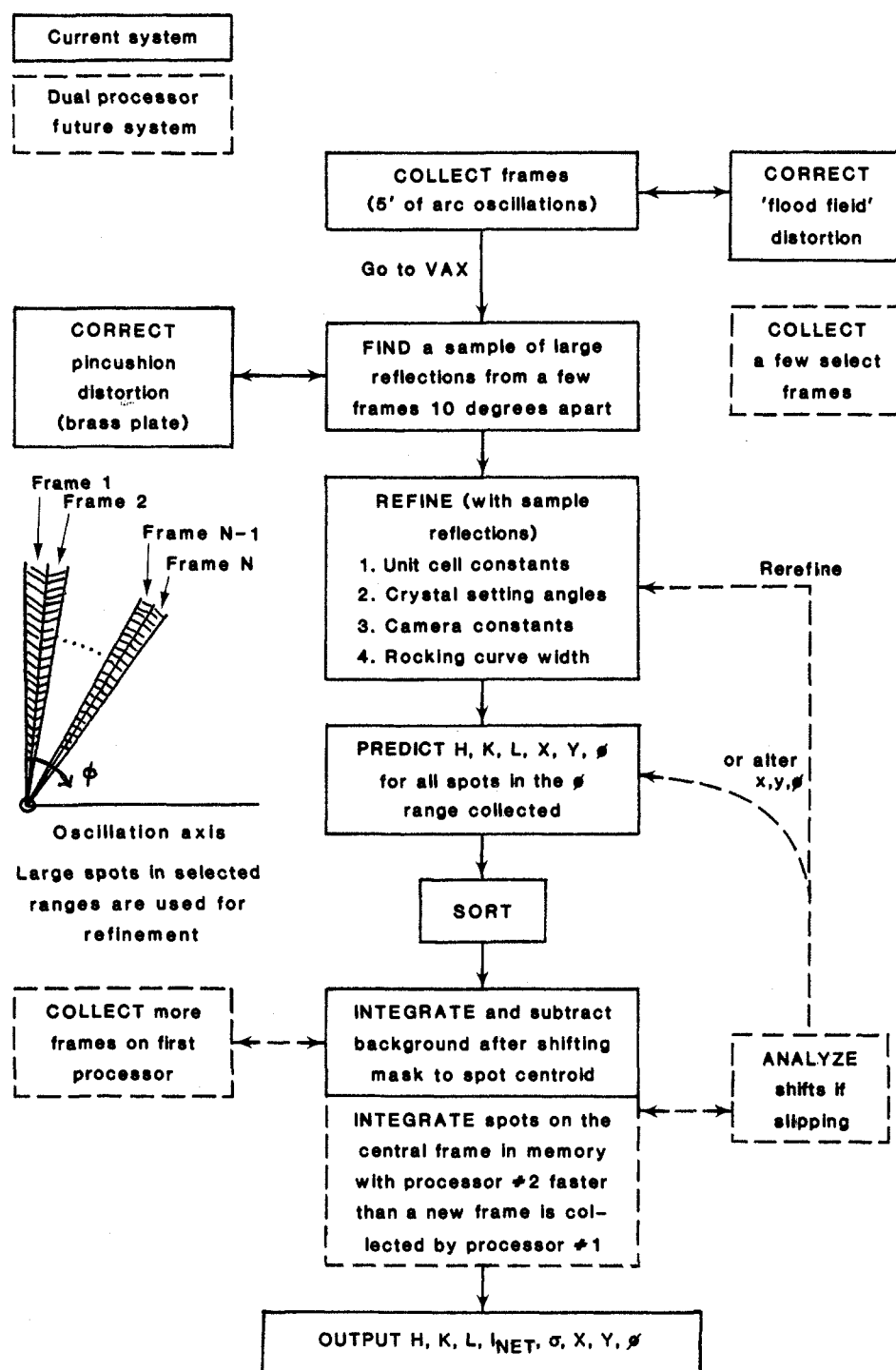


Fig. 2. A flow chart of the current processing programs which operate on a VAX 11/780. The additions for a dual-processor system are shown in dashed lines.

unit (cpu) time per 1000 reflections. Integration of those spots required 101 cpu seconds per 1000 reflections.

A major advantage of area detector data collection is the increased speed and ease of data collection. Data collection rates can be 100 times faster than conventional diffractometry (10) because many of the simultaneously occurring reflections can be collected at one time. Data rates are not as dramatically increased over oscillation photography, because oscillation photography already records the simultaneously occurring reflections. For identical beam geometries and crystal to detector (film) distances, there is only one essential difference between oscillation photography and area detector methods: background scatter adds to intensity within the area of a spot during the entire oscillation range of film, whereas it adds to spot intensity only on those detector frames included in the integration (that is, only for the ϕ range of the rocking curve). As pointed out above, the ratio of these two ranges is typically about 10:1, giving an increase in speed for comparable counting statistics of $\sqrt{10} = 3.2$. Contributions from chemical fog and film scanning errors make the comparison even more favorable. For a particular choice of counting time, the detector also has the advantage of large dynamic range. Film to film scaling errors within a pack are avoided—an especially significant contribution for strong reflections.

Data collection from crystals unsuitable for diffractometry because of large unit cell sizes and weak diffraction is a particularly stringent test of an area detector. Such crystals require very long exposures. For example, a one-degree oscillation photograph of the influenza HA crystals requires a 12-hour exposure (Franks focusing optics, 100- μ m focus, 40 kV, 20 mA, Elliot GX-6); since each reflection is 6 to 10 minutes of arc wide, individual reflections are exposed for two hours each, in contrast to the 1- to 2-minute "exposure" time for reflections from protein crystals suitable for conventional diffractometry.

The current data collection system was tested on five weakly diffracting protein crystals for which high resolution data were uncollectible on a conventional diffractometer (Table 1). The unit cell dimensions range from 96.3 to 177.4 Å and the daltons per asymmetric unit from 35,000 to 200,000. *R* factors were calculated by comparing intensities of symmetry-related reflections. In the two cases where oscillation film data sets were available on the same proteins (influenza HA and 434 repressor-DNA complex), data measured on the detector system were of better quality than those measured by film. Low resolution data on

the VSG had Friedel *R* factors comparable to 6 Å resolution data collected by automated diffractometry. In the high resolution (3 Å) data set of VSG (see Table 1), 3968 reflections (2106 unique) were collected from a typical crystal of the 13 used in the complete data set in the resolution range from 15 to 2.7 Å, with a symmetry *R* factor on intensities of 4.2% when 22 reflections were rejected.

Data from native VSG and from a mercuric iodide isomorphous derivative were measured to 6 Å resolution on the detector system, allowing a difference Patterson to be calculated. Figure 3A shows a comparison of the two Harker sections at $z = 1/4$ and $z = 1/2$ ($P4_12_12$) of this difference Patterson from data sets collected on the detector and on an automated diffractometer (Nicolet P3). The difference Patterson from the de-

tector data sets is readily interpretable in terms of a single heavy atom site and is approximately comparable in signal-to-noise ratio to an identical difference Patterson calculated from diffractometer data. Anomalous differences at 6 Å resolution between Friedel pairs of reflections were also measured both by automated diffractometry and by the detector and found to correlate with a coefficient (11) of 0.54, indicating significant agreement (12). An anomalous difference Patterson (Fig. 3B) calculated from data collected on the detector in the resolution range of 6 to 4 Å showed major peaks corresponding to those in the isomorphous difference Patterson (Fig. 3A), indicating that high resolution anomalous scattering differences were detected accurately.

Data were collected to 7 Å from crystals of the complex of the DNA-binding domain from bacteriophage 434 repressor with a 14-bp DNA operator (13), as well as from isomorphous crystals of such a complex with DNA substituted at one position with 5-BrdU (for T). The intensity differences were very small, but threefold noncrystallographic symmetry averaging produced an interpretable difference Patterson and led to a successful solution of the crystal structure (14). The noncrystallographic Harker section is shown in Fig. 4.

Data at 2 Å resolution have also been collected from more conventional, strongly diffracting protein crystals of a chemically cross linked ribonuclease, with the use of a similar detector at Genex (15).

One of us (M.B.) has recently developed an on-line integration system that allows automatic re-refinement of setting parameters during the data collection (16). The program runs on a second microprocessor.

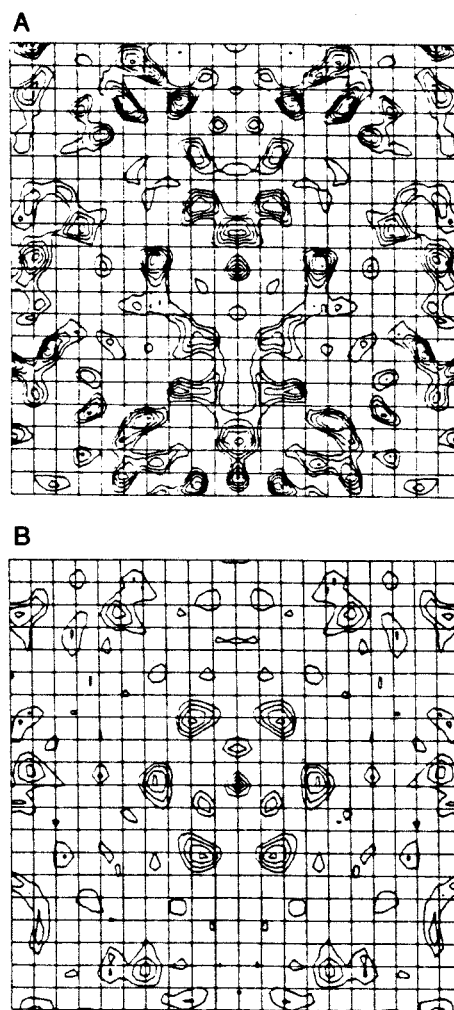


Fig. 4. A difference Patterson section perpendicular to the body diagonal [which is parallel to a noncrystallographic axis of threefold skew symmetry (13)] at $Z_{\text{skew}} = 1/3$ from the I434 repressor-DNA (see text for details) cocrystal difference Patterson. (A) Unaveraged; (B) averaged about the body diagonal of the Patterson unit cell, which is a noncrystallographic threefold rotation axis.

REFERENCES AND NOTES

1. U. Arndt and A. J. Wonacott, *The Rotation Method in Crystallography* (North-Holland, Amsterdam, 1977).
2. C. Cork et al., *J. Appl. Cryst.* 7, 319 (1974).
3. N.-H. Xuong, S. T. Freer, R. Hamlin, C. Nielsen, W. Vernon, *Acta Cryst.* A34, 289 (1978).
4. U. Arndt, *Nucl. Instrum. Methods* 201, 21 (1982).
5. R. Hamlin et al., *Acta Cryst.* 14, 85 (1981).
6. W. A. Wooster, *ibid.* 17, 878 (1964).
7. S. C. Harrison, *J. Appl. Cryst.* 1, 84 (1968).
8. Collimation optima and corresponding specimen-detector distances are not straightforward to calculate. With a nondivergent beam, such as we can achieve in principle with mirrors, there is an advantage to placing the detector as far from the crystal as is consistent with its aperture, the desired resolution, and so forth, since the background will fall off as $1/d^2$, while the diffracted beam will hardly fall off at all. In practice, mirror aberration gives some effective divergence and less gain in signal-to-noise than expected.
9. F. K. Winkler, C. E. Schutt, S. C. Harrison, *Acta Cryst.* A35, 901 (1979).
10. N.-H. Xuong, D. Sullivan, C. Nielsen, R. Hamlin, *ibid.* B41, 267 (1985).
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$$\text{Correlation} = \frac{\sum (\Delta_{\text{xen}} \times \Delta_{\text{dif}})}{\sum (\Delta_{\text{xen}}^2 \times \Delta_{\text{dif}}^2)^{1/2}}$$

- where Δ_{Xen} is the Bijvoet difference ($|F^+| - |F^-|$).
12. P. M. Colman, J. N. Jansonius, B. W. Matthews, *J. Mol. Biol.* **70**, 701 (1972).
 13. J. Anderson, M. Ptashne, S. C. Harrison, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1307 (1984).
 14. J. Anderson, M. Ptashne, S. C. Harrison, *Nature (London)* **316**, 596 (1985).
 15. P. C. Weber, S. Sheriff, D. H. Ohlendorf, B. C. Finzel, F. R. Salemme, *Proc. Natl. Acad. Sci. U.S.A.*, in press.

16. M. Blum, P. Metcalf, S. C. Harrison, D. C. Wiley, in preparation.
17. C. Katayama, N. Sakabe, K. Sakabe, *Acta Crystallogr.* **A28**, 293 (1972).
18. C. E. Schutt and P. R. Evans, *ibid.* **A41**, 568 (1985).
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Separation of Drug Stereoisomers by the Formation of β -Cyclodextrin Inclusion Complexes

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For many drugs, only racemic mixtures are available for clinical use. Because different stereoisomers of drugs often cause different physiological responses, the use of pure isomers could elicit more exact therapeutic effects. Differential complexation of a variety of drug stereoisomers by immobilized β -cyclodextrin was investigated. Chiral recognition and racemic resolution were observed with a number of compounds from such clinically useful classes as β -blockers, calcium-channel blockers, sedative hypnotics, antihistamines, anticonvulsants, diuretics, and synthetic opiates. Separation of the diastereomers of the cardioactive and antimalarial cinchona alkaloids and of two antiestrogens was demonstrated as well. Three dimensional projections of β -cyclodextrin complexes of propranolol, which is resolved by this technique, and warfarin, which is not, are compared. These studies have improved the understanding and application of the chiral interactions of β -cyclodextrin, and they have demonstrated a means to measure optical purity and to isolate or produce pure enantiomers of drugs. In addition, this highly specific technique could also be used in the pharmacological evaluation of enantiomeric drugs.

CHIRAL DISCRIMINATION HAS BEEN a long-standing problem in the development, use, and action of pharmaceutical agents. Numerous examples exist where the undesired effects of one isomer limit the overall effectiveness of the active species because of host toxicities, biodistribution problems, altered metabolism, and unwanted drug interactions. This problem is illustrated by the prototype β -blocker pro-

pranolol. *d*-Propranolol is approximately 40 times more potent than *l*-propranolol and appears to mediate the antiarrhythmic and antihypertensive activity of the racemic mixture, whereas only *l*-propranolol appears to be beneficial in treating angina pectoris (1). A similar situation occurs with synthetic opiates such as methadone, for which there may be three to five stereoselective opiate receptors, each of which triggers a different

physiological response (2). The use of specific isomers could allow one to elicit more exact therapeutic effects. Unfortunately, as is true for approximately 25 percent of pharmaceutical products, only racemic mixtures of propranolol and methadone are available for clinical use. This is a direct result of the difficult (and thus expensive) traditional methods for resolving enantiomers or for completing stereoselective syntheses.

Recently, a number of highly specific liquid chromatographic techniques were developed to separate certain enantiomers (3–7). However, only a few compounds of clinical interest were resolved (5, 8–13), and these were restricted to a few pharmaceutical classes. There was evidence that stable, high-coverage, β -cyclodextrin-bonded media could be employed for stereoselective drug separations (5, 8, 14–17). Indeed, it had been shown that cyclodextrins are useful as biomimetic models in studies of substrate binding (18–20), enzymatic catalysis (18–22), and membrane transport (19) and as novel reaction media (23–25). We now show that β -cyclodextrin-bonded media separate stereoisomers of a wide variety of clinically relevant cyclic and heterocyclic drugs.

The two types of separations investigated included that of enantiomeric drugs (Table 1) and of diastereomeric drugs (Table 2). Although no chiral stationary phase could be universally effective for the resolution of enantiomers, the variety of compounds resolved by inclusion-complex formation was encouraging. Resolution values (R_S) greater than 1.0 were obtained for the drugs propranolol, chlorthalidone, mephenytoin, phenoximide, nimodipene, triazoline, ketoprofen, chlorpheniramine, methylphenidate and the barbiturates hexobarbital and mephobarbital. Slightly lower, but satisfactory, resolution was obtained for methadone, verapamil, metoprolol, aminoglutethamide, and nisolidipene. These compounds were

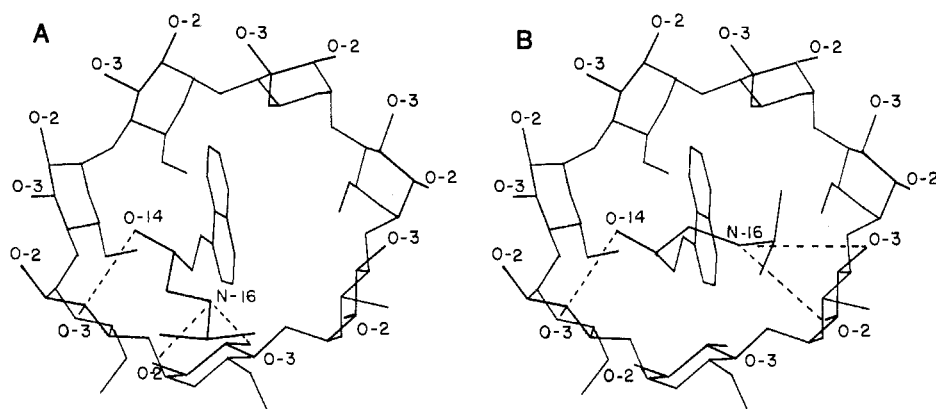


Fig. 1. Computer projections of inclusion complexes of (A) *d*-propranolol and (B) *l*-propranolol in β -cyclodextrin, from x-ray crystallographic data. Dotted lines represent potential hydrogen bonds (distances noted in the text). The configurations shown represent the optimal orientation of each isomer on the basis of the highest degree of hydrogen bonding and complexation.

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