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The X-ray Analysis of the Structure of Cephalosporin C

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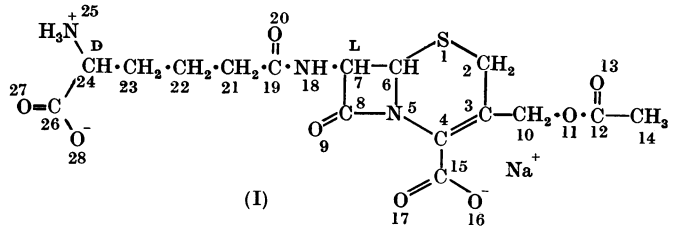
Crystallographic measurements on the sodium salt of cephalosporin C were first made in order to determine the molecular weight of this compound soon after it was isolated by Newton & Abraham (1955). The crystals were then too small for detailed work, but the measurements showed that the lattice had one very short unit-cell dimension of 4·9 Å, a feature that suggested that it might be possible to solve the crystal structure by direct methods, without the introduction of atoms heavier than those already present. As more material was obtained and larger crystals grown, additional unsuspected complications in the crystal lattice were observed and these have hindered the precise solution of the structure. The positions now found for the atoms in the crystal give good general agreement with the observed X-ray intensities but are not defined with great accuracy;



(I)

they correspond with the structural formula (I) described by Abraham & Newton (1961) in the preceding paper.

Preliminary crystallographic examination

Crystalline samples of the ammonium, barium, calcium, potassium and sodium salts of cephalosporin C were prepared by Abraham & Newton; those of the sodium salt were most promising, the ammonium salt rather less so, the rest microcrystalline. Experiments on growing good crystals were only successful for the sodium salt. The method which gave the best results was as follows:

Samples ranging from 1 to 5 mg. of the salt were dissolved in one drop more than the minimum of water. Ethanol was then added in excess. In the intermediate concentrations crystals grew slowly over a period of some weeks and well-ordered crystals up to $1{\cdot}5 \times 0{\cdot}25 \times 0{\cdot}1$ mm.^{3} were obtained. They were easily removed after replacement of the mother liquor with absolute ethanol or after suction filtration.

Even the best crystals were of low reflecting power and deteriorated noticeably in the X-ray beam; no reflexions were observed beyond a spacing limit of 1·04 Å. Many crystals, particularly those which had grown rapidly, showed serious distortions; some of the earlier recrystallized specimens gave reflexions over 1 cm. long on Weissenberg photographs.

The crystals were laths on (001), elongated along the b axis. The crystal data are as follows:

$$a = 38{\cdot}88 \pm 0{\cdot}15 \text{ Å}, \quad b = 4{\cdot}99 \pm 0{\cdot}04 \text{ Å},$$
$$c = 25{\cdot}65 \pm 0{\cdot}10 \text{ Å}, \quad \beta = 115° 25 \pm 15',$$

space group, C2, for which Z is 4. From the earlier measured density, $D_m = 1{\cdot}38$, the molecular weight of the asymmetric unit is calculated to be twice the figure 467 ± 15, which corresponds with the formula preferred on analysis, $C_{16}H_{22}O_8N_3SNa,2H_2O$ (calculated molecular weight 475).

In the intensity distribution it was observed that $h0l$ reflexions were very weak and $h1l$ reflexions rather weak, for l odd, whereas for higher values of k there was no marked distinction between l-odd and l-even reflexions. The effect suggested that the asymmetric unit consisted of two molecules separated by a translation of $\sim c/2$ but having rather different y parameters.

The structure analysis is based on 1428 X-ray reflexions recorded on multiple film Weissenberg photographs and measured by eye.

Structure analysis

Both the course of the structure analysis and the accuracy achieved were much affected by the limited character of the available data. In spite of the short b cell dimension it proved impossible to solve the structure in projection alone and three-dimensional calculations were undertaken. Fig. 1 shows in outline the stages of the analysis.

Three evaluations of the three-dimensional Patterson distribution were made with terms modified by different sharpening functions. All three illustrate the character of the lattice suggested by the intensity distribution; the whole pattern repeats to a first approximation in the interval $c/2$, the pseudo-origin being more drawn out in the b direction than the true origin.

Of the three distributions the first, I, is very diffuse and was of little use; the modification

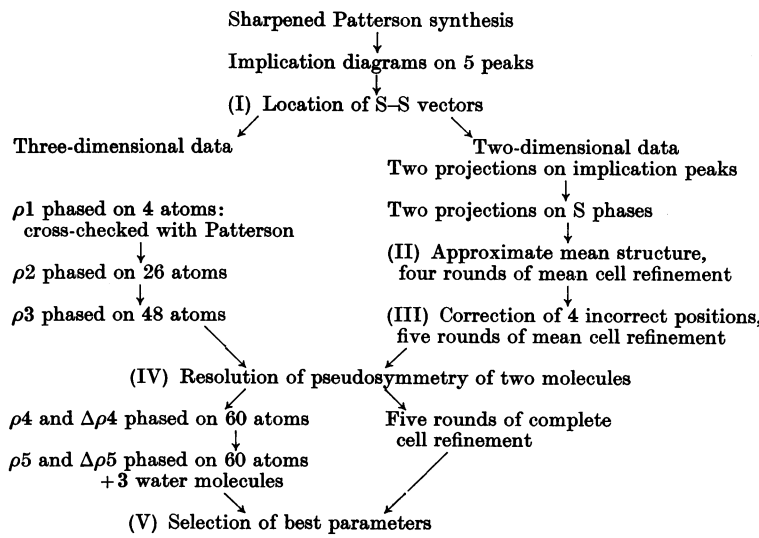


Fig. 1. Flow sheet.

function here reduced the terms approximately to those expected from atoms at rest. The third, III, and sharpest version, corresponding with point atoms at rest, was calculated by Dr S. Abrahamson after the main structure analysis here described was complete. The second, II, the distribution actually used, is nearer to III in character; the modification function chosen here, $100 \sin^4 \theta \exp(-9{\cdot}0 \sin^2 \theta)$, was similar to that used in the analysis of serine (Shoemaker, Barieau, Donohue & Lu, 1953) with some adjustment of the exponential function to reduce diffraction ripples. Here the peaks are well resolved; corresponding peaks in the two halves of the Patterson distribution are alternatively high and sharp in one half, and low but extended in the other, thus making it possible to determine where the co-ordinates of atoms in the two molecules differ appreciably. The section in this function at $y = 0$ is shown in Fig. 2.

Of the prominent peaks in the section, $y = 0$, the six highest (numbered 1–6 in Fig. 2) were selected for trial as possibly representing S–S vectors. Implication diagrams were plotted to show the coincidence of the peak pattern surrounding each of these with peaks surrounding the origin, only peaks above 100 on the arbitrary Patterson scale being included. Seventy-three good coincidences throughout the distribution were observed for 3, 55 for 1 (later identified as an O–O vector), whereas relatively few were found for the other peaks. Further, the peak pattern surrounding 3 (Fig. 3) clearly mapped a six-membered ring. This could be correlated directly with the suggestion made in the same period by E. P. Abraham & G. G. F. Newton (personal communication) that a six-membered ring was present in cephalosporin C. It is perhaps worth observing that peak 3 is actually the highest peak in the later, sharper Patterson distribution III, which would have identified it directly as the S–S vector.

It was clear that many of the other atomic positions in the crystal were defined by the S-light atom vectors isolated in the implication diagram shown in Fig. 3, but their selection as a whole was not immediately obvious in the complex situation thus revealed. Also, whereas the implication diagram defined the x and z co-ordinates, it left the sign of the y co-ordinates of the corresponding peaks indeterminate. Accordingly, initial trial calculations were based on the projection data only.

The first approximate electron-density projection was based on signs given by 53 of the more likely implication peaks selected as possible atomic sites. A difference map suggested the removal of eight of these and the addition of ten others. Further structure-factor calculations showed very poor agreement for l-odd reflexions and these were omitted in the next few rounds of calculation, which led accordingly to a mean structure corresponding with a one-molecule asymmetric unit. Parallel with the series based on the peaks in the implication map, an approximate projection was calculated by using signs calculated on the proposed sulphur atom positions only. In this series only the 55 large terms which were likely to have the same phase as the sulphur contributions were included. These were terms for which the unitary structure factor exceeded 0·1, and the sulphur contribution exceeded 0·5 of its maximum possible value. One large reflexion, $20\bar{1}0$, had a sulphur contribution 0·4 maximum and a separate map was evaluated including this term.

Together, the electron-density projections and implication maps considerably limited the possible distribution of the atoms in the crystal. It became clear that the six-membered ring carried substituents on four of the atoms and that two of these, 7 and 8, could be extended to another possible position forming a β-lactam ring. Beyond them a chain could be traced having the form of the adipic acid side chain known to be present in the antibiotic. A second chain, at position 3, was more obscure, but of length equivalent to acetoxymethyl. On the fourth position, a group of peaks suggested a carboxyl group a short distance from a Na^+ ion. The first orientation derived for this group was soon found to be incorrect by the calculation of a series of further refinements of the projected electron-density and difference maps. But in outline the structure derived at this point has been confirmed by subsequent three-dimensional calculations and the parallel chemical investigation.

The refinement of the electron-density projection at this stage is illustrated by Fig. 4*a–c*. Fig. 4*a* shows a difference map calculated for the structure as first outlined, Fig. 4*b*, a difference map calculated leaving out the uncertain atoms connected with the carboxyl group, Fig. 4*c* the subsequent map derived by using $h0l$ terms with l even only. Fig. 4*a* and *b* give some of the evidence which led to the reorientation of the carboxyl group and movement of the Na^+ ion from a site essentially that occupied by one atom of the carboxyl group to that first thought to be occupied by a water molecule. Fig. 4*c* defines the molecule as a whole to a first approximation, together with the Na^+ ion and possible sites for water molecules. R, the agreement factor, at this stage was 26·6% on observed reflexions only.

Parallel with the calculation of the electron density in projection, calculations of the electron density in three dimensions were made. Here the complete unit-cell data had to be employed throughout. The first stage was independent of the projection evidence. Positions were derived from the implication diagram for four atoms, the

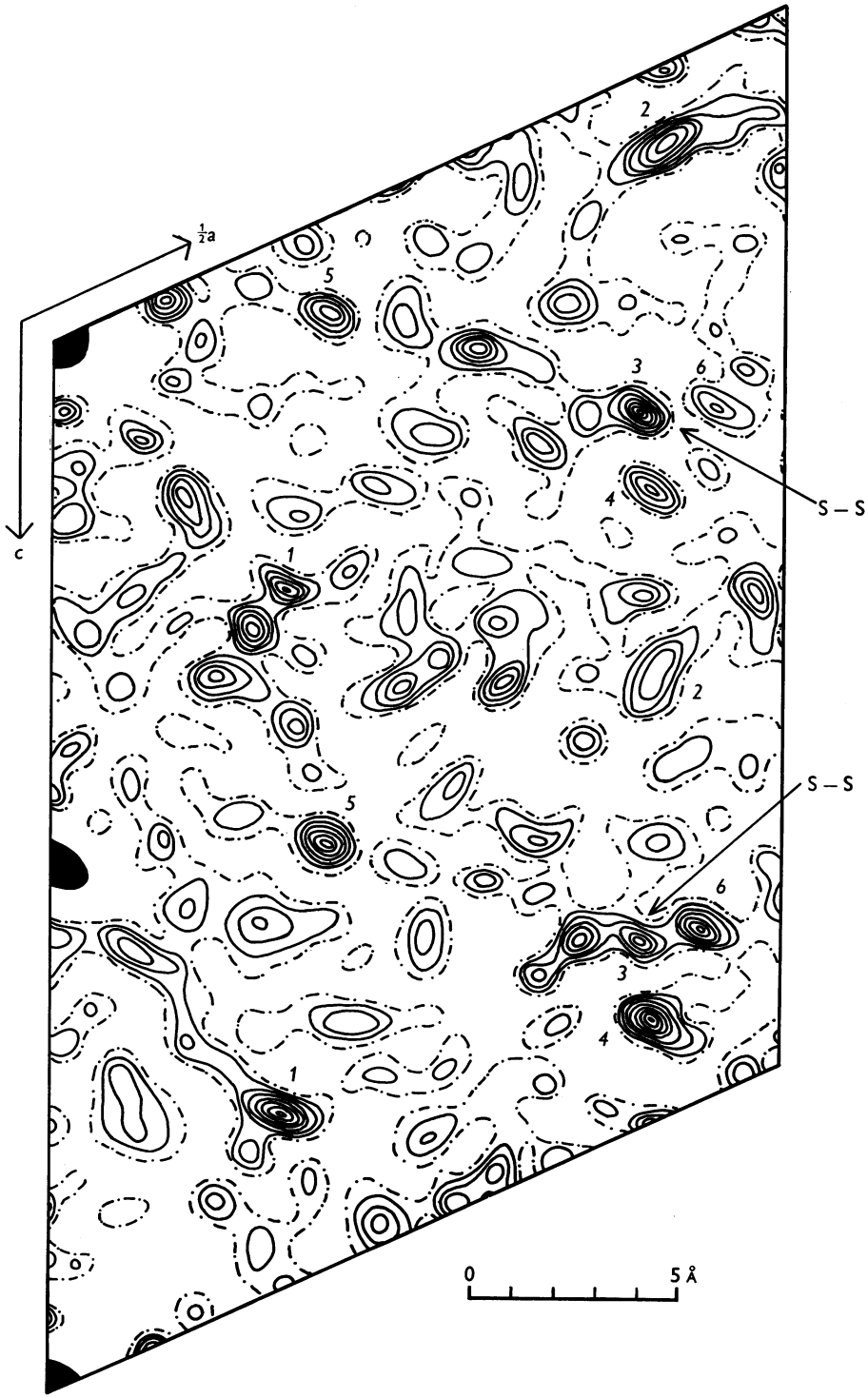


Fig. 2. Section at $y = 0$ in the three-dimensional Patterson distribution II. S–S indicates a peak corresponding to the sulphur–sulphur vector.

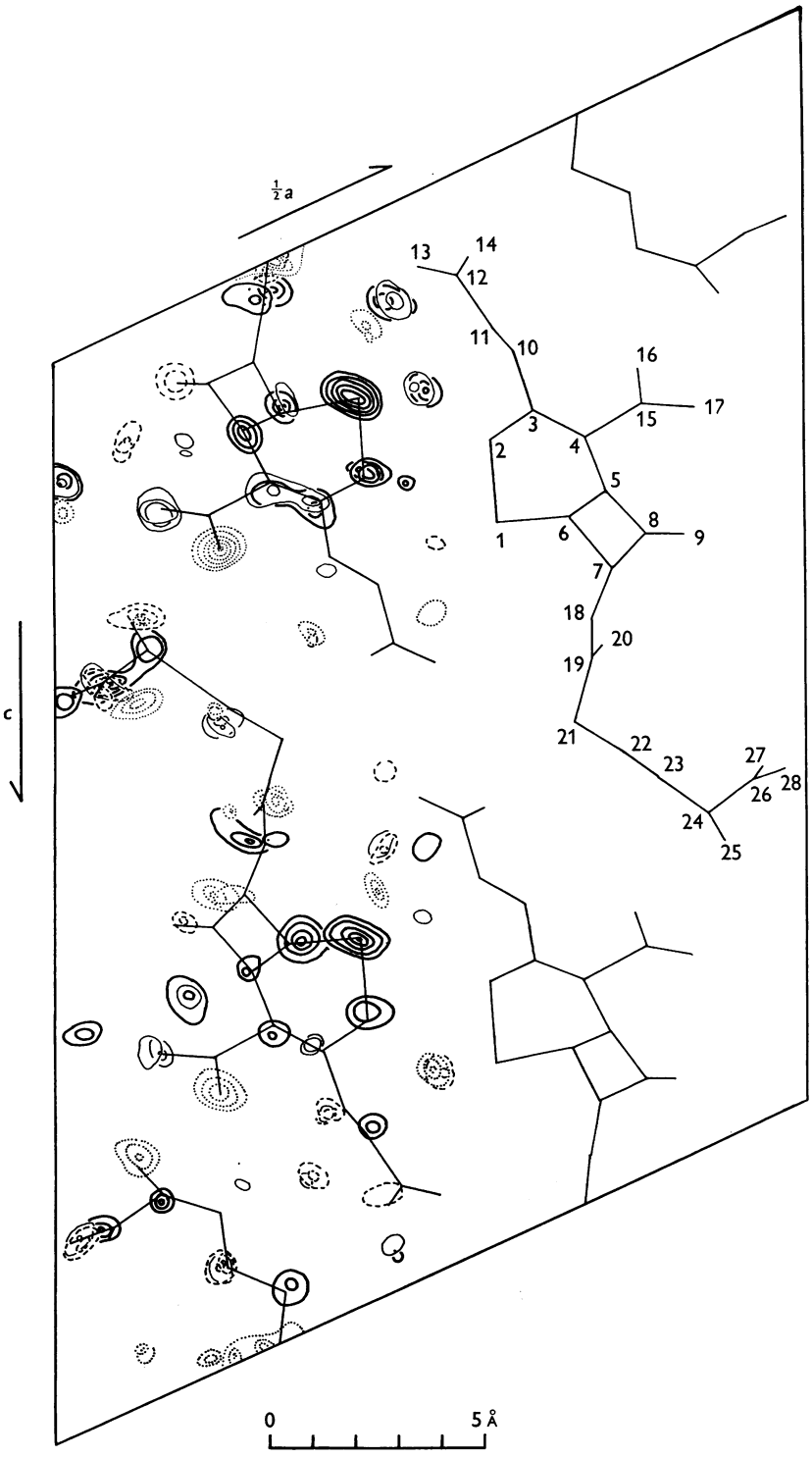


Fig. 3. Implication diagram on peak 3 of Fig. 2. The numbering in this figure corresponds with the numbering of the atoms in Table 1.

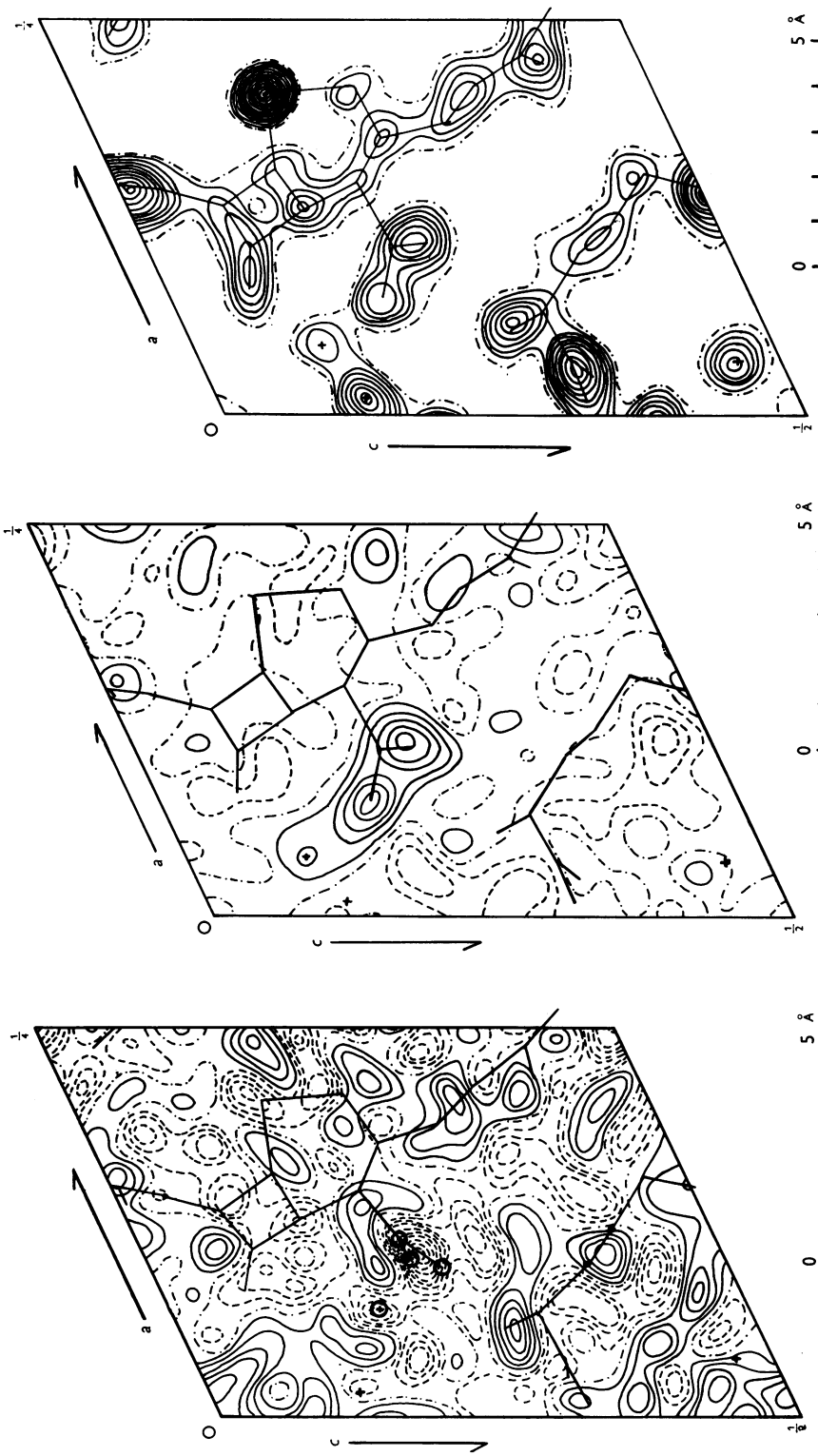


Fig. 4. (*a*) Difference synthesis, (010), in fourth round of the projection refinement. The four atoms thought to be incorrectly placed are ringed. Contour interval, 0·5 e/Å^3. (*b*) Difference synthesis, (010), on eighth round of the projection refinement showing the effect of omitting doubtful atoms. Contour interval, 1 e/Å^3. (*c*) Electron-density projection on (010) obtained through the mean cell refinement. Contour interval, 1 e/Å^2. The broken contour is at 2 e/Å^2, below which the contours are omitted. In these figures the correctly oriented molecule is drawn superimposed on the contours. + indicates position chosen for water molecules and the sodium ion.

sulphur atoms in the two independent molecules, *a* and *b*, and two others thought then to be sodium ions, actually oxygen atoms of carboxyl groups. Phase angles were calculated on these positions, the two oxygen atoms being weighted as sodium atoms.

From the following electron-density distribution 26 atomic positions were selected and used in the next phasing calculations and the series was continued, as shown in Fig. 1, by the progressive selection of 48, 60 and 63 atomic positions. The selection was never automatic. In the first synthesis there were peaks present corresponding to every atom in the molecule, ranging from $1\frac{1}{4}$ to $3\frac{1}{2}$ e/Å^3 in height, but these were accompanied by a set of peaks related to them by a false plane of symmetry through $y = 0$, and by an almost equal number of completely spurious peaks up to 2 e/Å^3 in height. The selection of only 26 atomic sites from among these was perhaps over-cautious; examination of the series shows that in 38 cases the present chosen position was higher than the mirror-image peak and in only 18 cases was the false image substantially higher. The completely spurious peaks did not, in general, have false symmetry peaks associated with them, and a direct cross-check with the Patterson synthesis enabled most of them to be eliminated.

The later rounds of calculation proceeded fairly smoothly with small complications. In the 26 atom series only two of the mirror-image peaks were higher than the true peaks; in the 48 atom series, only one was, C-6 (*a*), which had accidentally been inserted at the wrong mirror-image site. Although then represented by a low peak, it did not show up as well as was expected at the correct site. The main problem in the last two stages proved to be the selection of the water-molecule positions. Until these were inserted differently in the two half-cells the atomic positions belonging to the two crystallographically non-identical molecules tended to drift in opposite directions, making different bond-lengths long and short. This tendency was reduced once different water molecule situations were proposed. At the 60 atom stage all the atoms in both molecules, the sodium ions and one water molecule similarly placed in the two half-cells were inserted. Near the (*b*) molecule there were two peaks in the expected position for the water molecules of $2\frac{1}{2}$ and 2 e/Å^3. Near the (*a*) molecule there were three peaks of 1·8, 1·7 and 1·6 e/Å at *X*, *Y* and *Z* in Fig. 5; these are too near to be simultaneously occupied. In the 63 atom distribution positions *X* and *Y* were weighted as if occupied by $\frac{1}{2}$ molecule of water. A small peak of 1 e/Å^3 still persisted at *Z*.

Similar effects were observed in the later refinement stages of the electron-density projection on (010) which includes all *h*0*l* reflexions, not only

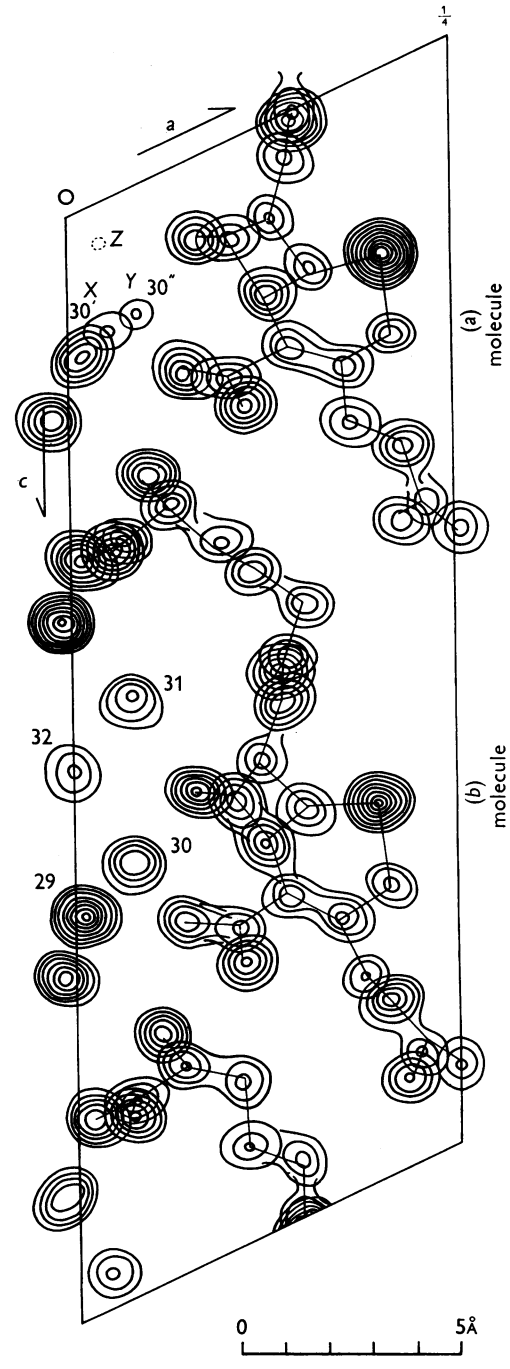


Fig. 5. The arrangement of the molecules in the crystal of sodium cephalosporin C projected on the *b* plane. Sections in the electron-density distribution, $\rho 5$, are shown projected over the atomic positions. *X*, *Y* and *Z* are possible water molecule positions. Contour interval, 1 e/Å^3. The numbers in this figure correspond with the numbers of atoms in Table 1.

those with l even. While the water molecules near the (b) molecule of cephalosporin C appeared to occupy definite positions those near the (a) molecule were represented by a blurred peak covering both positions X and Y of the three-dimensional distribution, together with a small peak at Z. Also, the atoms near the doubtful water molecule belonging to the (a) molecule appeared in negative regions in difference syntheses with positive peaks on either side, as if they too could occupy alternative positions.

It seems clear that there is some disorder in the arrangement of the water molecules between the (a) type molecules. In any one asymmetric unit either the sites Z and X or Y but not all three may be occupied by water molecules and the nearest atoms of cephalosporin C seem to be displaced a little to correspond. This disorder may be responsible for the weakly reflecting character of the crystals which limits the X-ray data. It, in turn, is presumably due to the packing problems posed by the awkward molecular shape.

The progress of the structure analysis as a whole is illustrated by Fig. 6 which shows the general improvement of the reliability index, R, in the different types of structure-factor calculation carried out. On the three-dimensional data, the

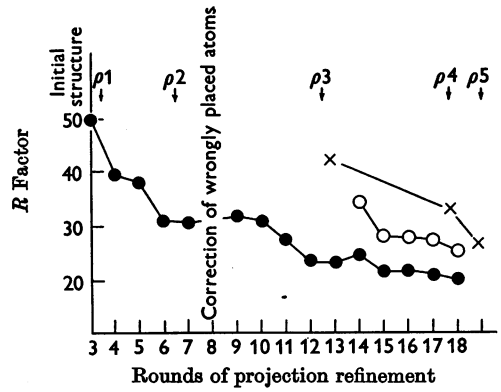


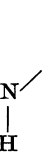
Fig. 6. Reliability index during the refinement of sodium cephalosporin C. ●, *h0l*, *l* even; ○, *h0l*, *l* complete; ×, *hkl*.

Table 1. *Atomic co-ordinates*

	Atom		x/a	y/b	z/c
1	S	(a)	0·2055	0·988	0·1681
		(b)	0·1990	0·926	0·6595
2	C	(a)	0·2100	0·942	0·2442
		(b)	0·2056	0·891	0·2379
3	C	(a)	0·1788	0·821	0·2480
		(b)	0·1744	0·760	0·7477
4	C	(a)	0·1438	0·823	0·2116
		(b)	0·1403	0·780	0·7012
5	N	(a)	0·1290	0·952	0·1552
		(b)	0·1276	0·888	0·6422
6	C	(a)	0·1582	0·116	0·1529
		(b)	0·1525	0·084	0·6323
7	C	(a)	0·1337	0·110	0·0882
		(b)	0·1226	0·026	0·5705
8	C	(a)	0·1063	0·885	0·0884
		(b)	0·1048	0·800	0·5919
9	O	(a)	0·0840	0·717	0·0736
		(b)	0·0805	0·582	0·5719
10	C	(a)	0·1818	0·671	0·3020
		(b)	0·1891	0·651	0·8092
11	O	(a)	0·2182	0·875	0·3475
		(b)	0·2029	0·886	0·8385
12	C	(a)	0·2303	0·813	0·4057
		(b)	0·2281	0·884	0·9048
13	C or O	(a)	0·2547	0·017	0·4482
		(b)	0·2523	0·123	0·9269
14	O or C	(a)	0·2122	0·602	0·4094
		(b)	0·2179	0·666	0·9188
15	C	(a)	0·1055	0·714	0·2127
		(b)	0·1083	0·538	0·7076
16	O^{i-}	(a)	0·1155	0·495	0·2447
		(b)	0·1103	0·428	0·7449
17	O^{i-}	(a)	0·0735	0·817	0·1863
		(b)	0·0719	0·726	0·6812
18	N	(a)	0·1436	0·019	0·0394
		(b)	0·1368	0·927	0·5266
19	C	(a)	0·1484	0·216	0·0030
		(b)	0·1411	0·129	0·4932
20	O	(a)	0·1476	0·498	0·0096
		(b)	0·1338	0·340	0·4999
21	C	(a)	0·1469	0·134	0·9497
		(b)	0·1530	0·967	0·4486
22	C	(a)	0·1127	0·087	0·9100
		(b)	0·1188	0·875	0·3945
23	C	(a)	1·1094	0·992	0·8500
		(b)	0·0959	0·145	0·3591
24	C	(a)	0·0731	0·995	0·8130
		(b)	0·0632	0·071	0·3016
25	N	(a)	0·0578	0·226	0·7770
		(b)	0·0486	0·304	0·2678
26	C	(a)	0·0403	0·883	0·8212
		(b)	0·0341	0·962	0·3151
27	O^{i-}	(a)	0·0371	0·643	0·8303
		(b)	0·0255	0·721	0·3193
28	O^{i-}	(a)	0·0121	0·035	0·8214
		(b)	0·0094	0·150	0·3171
29	Na^{+}	(a)	0·0081	0·418	0·1253
		(b)	0·0078	0·533	0·6376
½30	O	(a')	0·0247	0·938	0·1133
½		(a'')	0·0425	0·196	0·1137
		(b)	0·0375	0·188	0·6125
31	O	(a)	0·0218	0·910	0·9634
		(b)	0·0377	0·660	0·4589
½32	O	(a)	0·0183	0·331	0·0500
½32	O	(b)	0·0000	0·044	0·5000

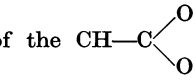
latest value of R is 26·4 % and on the $h0l$ term, with l even, 19·7 %. Some further improvement on these values should be possible, even with the present limited data, and further cycles of refinement are at present being carried out. In the meantime, it seems worth recording the general characteristics of the molecular and crystal structure found. These are illustrated by Fig. 5, which shows the electron-density peaks corresponding with the two cephalosporin C molecules as they appear in the 63-atom electron-density distribution. The atomic co-ordinates derived from this distribution are listed in Table 1. They define bond lengths which deviate by a maximum of 0·17 Å (in one case) and by an average of 0·07 Å from acceptable values for formula I. Such deviations are only to be expected at the present stage of structure refinement; it seems best to defer a full record of the bond lengths and bond angles in the molecule until refinement has been carried as far as possible.

Crystal and molecular structure of sodium cephalosporin C

In outline the crystal and molecular structure found for the sodium salt of cephalosporin C is very different from those found for sodium benzylpenicillin. Cephalosporin C, in Fig. 5, is shown in the correct absolute configuration, unlike benzylpenicillin in the first publication of the structure (Crowfoot, Bunn, Rogers-Low & Turner-Jones, 1949). Only the small region of the two molecules, including the β-lactam ring and the directly attached atoms, is closely similar. The amide chain in the cephalosporin C crystal is turned about the bond C-7–N-10, compared with that of benzylpenicillin, to a position with the amide carbonyl group nearly normal to the β-lactam ring. And the whole of the substituted adipic acid side chain is swung about the C-19–C-21 bond into a direction exactly opposite to that of the benzyl group in benzylpenicillin relative to the sulphur-containing ring. These changes in conformation may, of course, represent only temporary molecular situations which are stabilized in crystallization. They make possible a type of packing in sodium cephalosporin C reminiscent of many peptide crystals where a short-cell dimension of 4·8–4·9 Å corresponds with the hydrogen-bonded distance between parallel peptide chains. Here the hydrogen bond is between the amide  groups of successive molecules in the direction of the short b axis.

The six-membered sulphur-containing ring is rather flat. All of the atoms C-2, C-3, C-4, N-5, C-10 and C-15 lie in a plane within the limits of experimental error, with the sulphur atom about 0·6 Å above the plane and C-6 0·6 Å below it. This is in agreement with the formulation of C-3–C-4 as a double bond, which is also supported by its observed bond length of 1·31 Å. The changes in this part of the molecule make it possible for the attached carboxyl group here to lie much more nearly in the plane of the ring than it does in benzylpenicillin, where the carboxyl group is turned at about right angles to the five-membered ring.

The side chain at C-3 has the general form of an acetoxy group but is not very well defined. The four terminating atoms of the chain have an approximately planar configuration, but the last two are indistinguishable in electron density. This suggests that the two positions are occupied at random by oxygen and methyl groups corresponding to alternative positions reached by rotation about  . There appear to be no strong intermolecular forces in this region which might favour one packing arrangement rather than another.

The terminating atoms of the adipic acid side chain are, on the other hand, well defined. The two carboxyl oxygen atoms, by contacting succeeding Na^+ ions along the b axis, link the molecules together through ionic bonds. The amino group, by contrast with many amino acid structures, lies about 1·27 Å from the plane of the  system in a position at which it can form a hydrogen bond with the ring carboxyl group of a neighbouring molecule, O-16. The co-ordination sphere of the sodium ion is completed by contact with the second oxygen atoms, O-17 of this group and by two water molecules.

The differences that appear in the electron-density maps suggest that the two molecules, (*a*) and (*b*), lie at slightly different angles to the crystallographic b plane. This difference is probably to be correlated with the geometrical relation of the hydrogen bond between the amino group in one molecule and carboxyl group in another and the requirements of the sodium co-ordination sphere. Some modification of the detailed appearance of the atomic positions indicated in Fig. 5 must be expected on further refinement. But it seems unlikely that this could be sufficient to change our interpretation of the chemical structure of the molecule.

EXPERIMENTAL

X-ray photography

The b unit-cell dimension of cephalosporin C was measured on an oscillation photograph while d_{100}, d_{001} and β were determined on a Weissenberg photograph showing the $h0l$ reflexions together with lines from a copper specimen. The main series of reflexions were recorded on multiple film Weissenberg photographs taken with the b axis as rotation axis and copper K_α radiation. Three different crystals were used, of size $0{\cdot}6 \times 0{\cdot}25 \times 0{\cdot}1$ mm.^{3} for the zero and first layers, $1{\cdot}5 \times 0{\cdot}30 \times 0{\cdot}12$ mm.^{3} for the second and third layers, $1{\cdot}8 \times 0{\cdot}30 \times 0{\cdot}15$ mm.^{3} for the fourth layer. The intensities were first estimated visually by Miss G. Hurdman with some help from Miss B. Prestwood and entirely cross-checked by E.N.M. They were corrected for Lorentz, polarization and inclination factors by using a programme designed by C. K. Prout. Correlation between the layer lines was mainly effected by using a zero-layer Weissenberg photograph of a crystal rotating about the c axis. The intensities of reflexions in this film were measured both visually and by means of a double-beam microdensitometer. Correlation factors obtained from this film for the $h0l$, $h1l$, $h2l$ and $h3l$ reflexions were in good agreement with approximate values obtained from Wilson plots on each layer. $h4l$ reflexions did not appear on the c axis photograph; these were given similar scale and temperature factors to the other photographs. The whole of the data, placed on a common scale, were then set on an approximately absolute scale by means of a three-dimensional Wilson plot; B from this plot was 3·5.

Calculations

The main part of the calculations were carried out on the Ferranti Mercury automatic electronic computer at Oxford with a variety of programmes.

For the structure factor calculations the f curves calculated for S by Viervoll & Ögrim (1949) were employed, for Na^+ by Freeman (1959) and for O, N and C by Berghuis *et al.* (1955). The signs and phase angles calculated were not automatically carried over to the next electron-density calculation. In the early stages of projection analysis, a selection of terms where the calculated S contribution was above 0·5 was made. In the first three-dimensional series, ρ_1, hkl terms were only introduced where F_0 was above 0·04 and the calculated contribution of the four atoms above 0·25 of their possible maximum.

In addition, the $h0l$ terms were given the signs determined from the partly refined projection. At $\rho 4$–60 atoms, an attempt was made to derive the atomic positions precisely by a nineteen point routine analysis of the peaks in both $\rho 4$ and $\Delta\rho 4$ followed by an n shift where n was 1·75. Examination of the results of this calculation suggested that the peak positions at this stage were too poorly resolved for really satisfactory operation of the routine, particularly over the short bond C-3–C-4.

Interatomic distances were calculated, with programmes devised by Dr R. A. Sparks, at various stages of the analysis.

The full record of the intensity-data and structure-factor calculations preceding the evaluation of $\rho 5$ is given by Maslen (1960).

SUMMARY

1. Crystals of the sodium salt of cephalosporin C were grown slowly from water and ethanol and found to have a more complex monoclinic lattice than that earlier recorded, with

$$a = 38{\cdot}88 \pm 0{\cdot}15\text{Å}, \quad b = 4{\cdot}99 \pm 0{\cdot}04\text{Å},$$
$$c = 25{\cdot}65 \pm 0{\cdot}10\text{Å}, \quad \beta = 115° 25 \pm 15',$$

space group C2. The asymmetric unit contains two molecules of cephalosporin C, $C_{16}H_{22}O_8N_3SNa + 4$–5 molecules of water of crystallization.

2. The crystals were poorly reflecting. The intensities of all X-ray reflexions were measured visually out to the spacing limit observed about 1·04 Å.

3. From sharpened three-dimensional Patterson distributions, the positions of the sulphur atoms and of a number of other possible atoms in the crystal unit were found. These other atoms included five connected in a six-membered ring to the sulphur atom.

4. Further calculations, including electron-density distribution in two and three dimensions, enabled positions to be assigned to all the atoms in the unit cell. These positions cannot be given with great accuracy owing to the limited data available; they correspond closely with the requirements of formula (I) (p. 393).

5. Both in conformation and in molecular packing in the crystalline state sodium cephalosporin C is markedly different from sodium benzylpenicillin.

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