

The Crystal Structure of Calcium Dipicolinate Trihydrate (A Bacterial Spore Metabolite)

BY GERALD STRAHS* AND RICHARD E. DICKERSON†

Noyes Chemical Laboratory, University of Illinois, Urbana, Illinois, U. S. A.

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Calcium dipicolinate (the equimolar salt of calcium and 2,6-pyridinedicarboxylic acid) crystallizes as the trihydrate and the sesquihydrate. The trihydrate is monoclinic, space group $P2_1/n$ (no. 14) with cell parameters $a = 9.94$, $b = 17.65$, $c = 5.87$ Å, $\beta = 99.45^\circ$. Its crystal structure has been determined and refined by least squares to a residual of 6.1%. The crystal structure is built up from calcium dipicolinate dimers linked by water molecules to form columns parallel to the c axis. Each calcium has eight nearest neighbor bonds: three to one dipicolinate ion and one to the other of the dimers, one to the linking water molecule and three to other water molecules. The calcium coordination polyhedron is a dodecahedron with twelve triangular faces. Eight of the nine hydrogen atoms have been located. Five hydrogen atoms (of the three water molecules) form intermolecular hydrogen bonds (four to carboxyl oxygen atoms) to two other molecules. One of the hydrogen atoms on the pyridine ring forms an unusual hydrogen bond to the carboxyl group of another dipicolinate ion linking the calcium dipicolinate into infinite ribbons by a 10-membered centrosymmetric ring with sequence



Introduction

The first discovery of dipicolinic acid (2,6-pyridinedicarboxylic acid) in a biological system was reported by Udo (1936) who found dipicolinic acid in the viscous matter of Natto, a Japanese food made of steamed soybeans fermented with *Bacillus natto*. Subsequently dipicolinic acid has been found to be produced by all bacterial spores (Powell, 1953) and by some fungi (Shima, 1955; Oyama, Nakamura & Tanabe, 1960). Calcium and dipicolinic acid are present in the spores in approximately equimolar quantities and recent spectroscopic evidence indicates that the calcium dipicolinate chelate (which will be referred to as CaDPA) is

present in bacterial spores (Bailey, Karp & Sacks, 1965). Murrell & Warth (1965) reported that the amount of calcium in bacterial spores increases with increasing heat resistance and confirmed the approximately equimolar ratio of calcium and dipicolinic acid.

Crystal preparation

Crystals of two hydrates of CaDPA were prepared by L. K. Steinrauf and us by mixing solutions of 0.1M $\text{Ca}(\text{NO}_3)_2$ and 0.1M sodium dipicolinate (Na_2DPA), heating in a boiling water bath and cooling slowly. Sodium dipicolinate, prepared from dipicolinic acid (Aldrich Chemical Company), was used as dipicolinic acid is rather insoluble in cold water.

Large crystals of the trihydrate, the subject of this paper, are difficult to prepare, having been made only twice in this laboratory. The crystallographic parameters are listed in Table 1. The sesquihydrate has been

* Present address: Department of Chemistry, University of California, San Diego, La Jolla, California, U.S.A.

† Present address: Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California, U.S.A.

Table 1. *Cell constants for CaDPA*

Space group	$C2/c$ or Cc (No. 15 or No. 9)	$P2_1/n$ (No. 14)
Formula	CaDPA. $1\frac{1}{2}\text{H}_2\text{O}$	CaDPA. $3\text{H}_2\text{O}$
a	13.54 Å	9.94 Å
b	9.84	17.65
c	13.62	5.87
β	92.6°	99.45°
Extinctions	$hkl: h + k = 2n$ $h0l: l = 2n$	$h0l: h + l = 2n$ $0k0: k = 2n$
ρ measured	1.685 ± 0.013	1.675 ± 0.015
ρ calculated	1.719	1.699
Molecules/cell	8	4
CaDPA = $\text{C}_7\text{H}_3\text{CaNO}_4$	M.W. 205.21	
Mo $K\alpha$ assumed as 0.70997 Å		

prepared many times by keeping the freshly prepared CaDPA solution in a boiling water bath until the large crystals form (about 1 to 2 hours); its crystallographic parameters are also listed in Table 1. As there was uncertainty about the water content of the second crystal form, two chemical analyses were made which proved that it was the sesquihydrate (Table 2). This analysis is compared with that of Powell (1953) who reported a dihydrate. Since we have not found the dihydrate and Powell's elementary analysis can be satisfactorily explained by the sesquihydrate, we believe that it was the sesquihydrate. The two forms appear to be the low temperature form and the high temperature form, respectively (Holden & Singer, 1960; Powell, 1953; Bailey, *et al.*, 1965). Powder photographs and crystal samples have been donated to the U.S. National Bureau of Standards.

Table 2. *Elementary analysis of the sesquihydrate*

	Measured (Illinois)	Calculated (as $1\frac{1}{2}$ H ₂ O)	Measured (Powell, 1953)
%Ca	17.05	17.260	15.8
%C	36.66	36.208	36.3
%N	6.01	6.048	5.7
%H	2.61	2.604	2.7
%O (by difference)	37.67	37.895	39.5

Experimental data

X-ray data were first collected for the three axial projections. Although the calcium atom and several other atoms were located by Patterson and Fourier methods, the molecule could not be discerned. Since we had planned to collect three-dimensional data in the interest of accuracy, work in projection was not continued, and the remaining data were collected. A total of ten intersecting planes were recorded on films with a Buerger precession camera using zirconium-filtered molybdenum $K\alpha$ X-radiation. 1962 independent reflections were recorded, of which 468 were unobserved, representing about two-thirds of the available reflections. The data reduction was done in the usual way, except that no absorption correction was applied because of the crystal's small size (*ca.* 0.3 by 0.1 by 0.05 mm).

Determination of the structure

A three-dimensional Patterson map was calculated and the two Harker sections were examined to determine the position of the calcium atom. The three calcium atom self vectors were fairly obvious, and they defined the calcium position. The calcium position was used in two ways; to calculate an electron density map using experimental data with phases based on the calcium atoms alone, and to calculate a fourfold superposition (by hand). Although the structure was not discerned, the two maps were very similar. A second Fourier map was calculated based on the calcium atom position and

several other atoms common to the previous maps. At this stage, most of the atoms were located, but their identity and their connections to form a molecule were not obvious. Therefore a three-dimensional scale model of the peaks in one unit cell was constructed from styrofoam, clay and baling wire, while a model of the dipicolinate ion was made from wooden applicators, paper and glue (assuming the ion to be planar). The DPA ion was moved through the cell in a searching process, and the correct position was soon determined, a missing carbon atom was located, and the other atoms were identified.

Structure refinement

Several false starts were made in least-squares refinement procedures and therefore we decided to check ourselves by calculating a difference Fourier map with all the atoms assumed to be nitrogen atoms, except for the calcium atom. This difference Fourier map showed that all the assumed carbon atoms had too many electrons, as expected, and all the oxygen atoms had too few electrons. A small positive peak at the calcium site led us to use neutral calcium form factors in later stages. This confirmation of the correctness of the structure gave us the confidence to pursue the refinement through several cycles of least-squares refinement (with unit weights for all reflections and unobserved reflections included as 0.7 times the minimum observable value) until the residual was decreased to 13%. The program used was a version of ACA 317 (Gantzel, Sparks & Trueblood, unpublished) and the atomic scattering factors were taken from *International Tables for X-ray Crystallography* (1962). At this point nine small peaks were found in the difference Fourier map and were included in the refinement as hydrogen atoms, but their parameters failed to refine. The individual atoms were then assigned anisotropic temperature factors when the familiar cloverleaf pattern was seen in difference Fourier maps. The final residual obtained was 9.0%.

Refinement was continued later at the California Institute of Technology using the CRYRM system least-squares program (Duchamp, 1964), in which the quantity minimized is $\sum w(F_0^2 - F_c^2)^2$. The data were reprocessed and the unobserved reflections were identified and eliminated from the refinement procedure, the observed reflections were given weights $1/w$ of $1/F^2$, and three reflections were given zero weights (two were measured only with copper radiation, and the third was in a radiation streak from a more intense reflection). With these changes in calculating the residual, a value of 6.6% was obtained. The hydrogen positions were located anew and refined. One of the hydrogen atoms [hydrogen H(4) on the water oxygen atom O(6)] did not refine. Two three-dimensional difference Fourier maps and a difference Fourier map in the plane of possible positions yielded only three peaks which were too small to be even half of a hydrogen atom and none of these positions would refine. The residual

Table 3. Observed and calculated structure factors (x 10)

A minus sign preceding F(obs) signifies 'less than'. Reflections indicated with an asterisk were omitted from the refinement.

Table with columns for h, k, l, F(obs), and F(calc). The table contains a dense grid of numerical data points for various reflections, with some cells containing asterisks to indicate reflections omitted from refinement.

for this structure with sixteen anisotropic heavier atoms and eight isotropic hydrogen atoms is 6.1%. The structure factors, atomic parameters, and thermal parameters are given in Tables 3, 4 and 5.

Table 4. Atomic parameters

[Coordinates are given within the limits $(x, y, z) = (0-1, 0-\frac{1}{2}, 0-\frac{1}{2})$ as used for refinement.]

Atom	x	y	z
Ca	0.47092	0.07882	0.2307
O(1)	0.8126	0.0183	0.3941
O(2)	0.8651	0.4949	0.3779
O(3)	0.3573	0.1550	0.4599
O(4)	0.6857	0.2697	0.0275
O(5)	0.0064	0.3450	0.4065
O(6)	0.6690	0.1669	0.3667
O(7)	0.1073	0.4596	0.1242
N	0.2239	0.1003	0.0926
C(1)	0.7429	0.4859	0.2837
C(2)	0.6588	0.4321	0.3975
C(3)	0.5215	0.4180	0.3110
C(4)	0.4528	0.3681	0.4334
C(5)	0.0193	0.1647	0.1358
C(6)	0.1558	0.1476	0.2076
C(7)	0.2383	0.1807	0.4246
H(1)	0.071	0.306	0.43
H(2)	0.944	0.337	0.27
H(3)	0.687	0.204	0.32
H(5)	0.187	0.460	0.06
H(6)	0.097	0.424	0.21
H(7)	0.486	0.443	0.17
H(8)	0.355	0.360	0.37
H(9)	0.976	0.197	0.22

Table 5. Thermal parameters

(a) Anisotropic thermal parameters (multiplied by 10^4) used in: $\exp[-(h^2\beta_{11} + k^2\beta_{22} + l^2\beta_{33} + hk\beta_{12} + hl\beta_{13} + kl\beta_{23})]$.

	b_{11}	b_{22}	b_{33}	b_{12}	b_{13}	b_{23}
Ca	34.6	10.6	58.2	0.9	7.2	-4.6
O(1)	46	43	206	8	-15	-124
O(2)	35	17	94	-5	-22	22
O(3)	60	20	144	23	-7	-31
O(4)	94	31	212	-55	-78	84
O(5)	98	20	119	32	-43	-23
O(6)	137	33	189	-68	-73	54
O(7)	62	13	115	9	45	2
N	46	16	102	4	0	-22
C(1)	39	18	112	7	9	21
C(2)	46	17	120	-7	46	37
C(3)	48	30	258	-18	-23	70
C(4)	53	35	340	-21	-46	104
C(5)	56	30	287	15	-29	-83
C(6)	57	15	148	7	12	-30
C(7)	56	16	152	6	0	-24

(b) Isotropic temperature factors for hydrogen atoms

	B
H(1)	3.1
H(2)	0.2
H(3)	3.4
H(5)	3.8
H(6)	-0.7
H(7)	0.1
H(8)	2.7
H(9)	0.0

Table 5 (cont.)

(c) Isotropic temperature factors ($R=12.5\%$) used during refinement.

Ca	1.09
O(1)	2.98
O(2)	1.54
O(3)	2.16
O(4)	3.03
O(5)	2.51
O(6)	3.67
O(7)	1.81
N	1.53
C(1)	1.67
C(2)	1.75
C(3)	2.76
C(4)	3.21
C(5)	2.89
C(6)	1.92
C(7)	1.99

Description of the structure

Dipicolinate ion

The dipicolinate ion was found to be essentially planar and to be coordinated with the calcium ion as a tridentate ligand. Two CaDPA units are joined together forming a dimer as shown in Fig. 1. The bond lengths in the DPA ion show that the ring is essentially an aromatic pyridine ring with two ionized carboxyl groups. The approximate equivalence of the carbon-oxygen bond lengths indicates that the carboxyl groups are stabilized by resonance and that all the carbon-oxygen bonds have about 50% double bond character. Bond lengths and interatomic angles are tabulated in

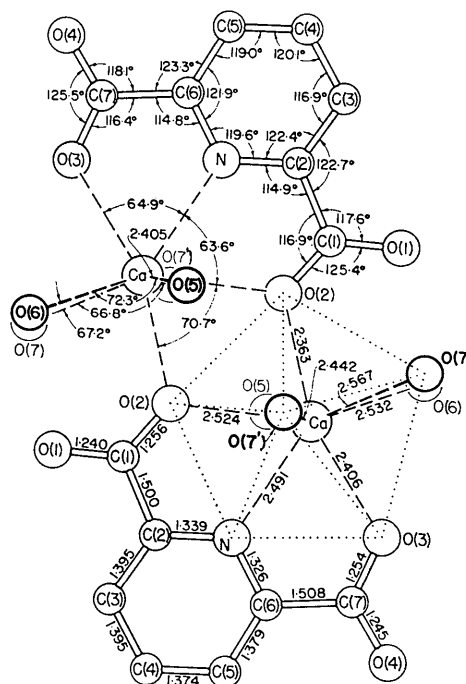


Fig. 1. CaDPA dimer. The numbering of atoms, bond lengths and interatomic angles is indicated.

Tables 6 and 7. The exterior angles at C(2) and C(6) reveal that the carboxyl groups are attracted to the calcium ion.

Table 6. Bond lengths in CaDPA

Bond lengths	Estimated standard deviation
Ca—O(2)	2.525 Å 0.007 Å
Ca—O(2')	2.366 0.007
Ca—O(3)	2.401 0.007
Ca—O(5)	2.403 0.008
Ca—O(6)	2.532 0.010
Ca—O(7)	2.570 0.007
Ca—O(7')	2.444 0.007
Ca—N	2.485 0.008
C(1)—O(1)	1.24 0.01
C(1)—O(2)	1.26 0.01
C(7)—O(3)	1.26 0.01
C(7)—O(4)	1.23 0.01
C(1)—C(2)	1.49 0.01
C(6)—C(7)	1.51 0.01
N—C(2)	1.35 0.01
N—C(6)	1.33 0.01
C(2)—C(3)	1.40 0.02
C(3)—C(4)	1.39 0.02
C(4)—C(5)	1.39 0.02
C(5)—C(6)	1.39 0.02
H(1)—O(5)	0.9 0.1
H(2)—O(5)	0.9 0.1
H(3)—O(6)	0.7 0.1
H(5)—O(7)	0.9 0.1
H(6)—O(7)	0.8 0.1
H(7)—C(3)	1.0 0.1
H(8)—C(4)	1.0 0.1
H(9)—C(5)	0.9 0.1

Table 7. Interatomic angles in CaDPA

Angle	Estimated standard deviation
C(2)—N—C(6)	119.0° 0.8°
C(2)—C(1)—O(2)	117.0 0.8
O(1)—C(1)—O(2)	125.2 0.9
O(1)—C(1)—C(2)	117.8 0.9
C(1)—C(2)—N	115.1 0.8
C(1)—C(2)—C(3)	122.3 0.9
N—C(2)—C(3)	122.5 0.9
C(2)—C(3)—C(4)	117.4 1.1
C(3)—C(4)—C(5)	120.2 1.2
C(4)—C(5)—C(6)	118.3 1.2
C(5)—C(6)—C(7)	122.6 1.0
C(5)—C(6)—N	122.6 1.0
N—C(6)—C(7)	114.8 0.9
C(6)—C(7)—O(3)	115.7 1.0
C(6)—C(7)—O(4)	118.2 0.9
O(3)—C(7)—O(4)	126.0 1.0

The seven carbon atoms and the nitrogen atom are coplanar within 0.02 Å (see Table 8). The calcium atom is significantly out of the DPA plane and the two carboxyl groups are slightly out of the plane; one is bent [O(1)—C(1)—O(2)] and the other is twisted [O(3)—C(7)—O(4)]. The two DPA units in the dimer are almost coplanar since the perpendicular distance between the two planes is only 0.036 Å.

Calcium environment

The calcium ion is coordinated to eight atoms: the nitrogen atom and seven oxygen atoms. Three of the

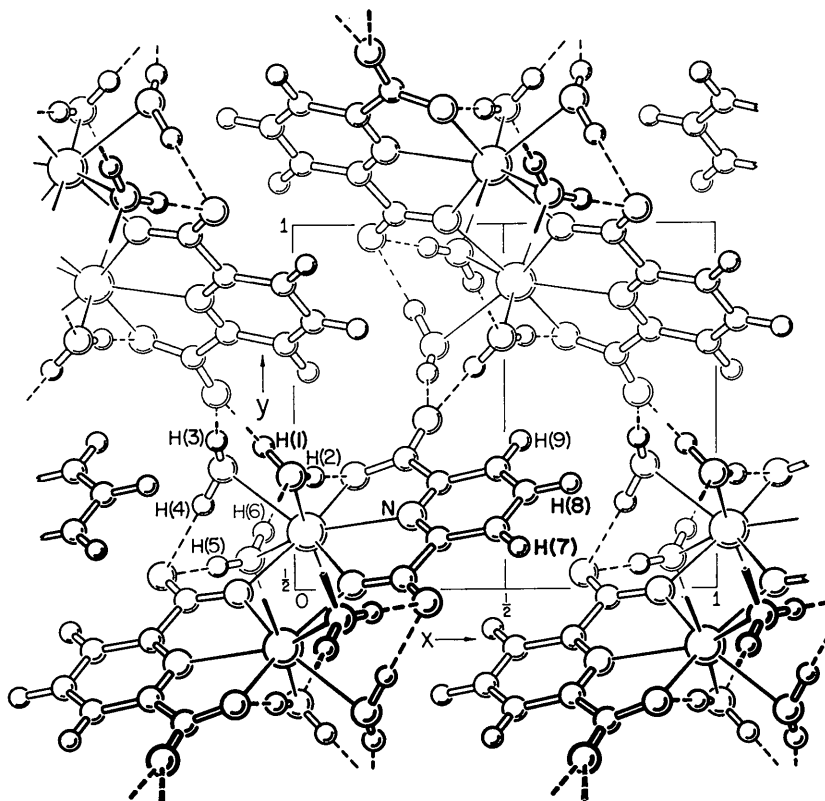


Fig. 2. Projection of the structure down the *c* axis showing the packing and bonding.

Table 8. Planarity of CaDPA

Center of symmetry between O(2) and O(2') -0.018.
 'Best' plane was calculated with the 7 carbon atoms and the nitrogen.

	Distance from 'best' least-squares plane
Ca	-0.142 Å
O(1)	0.032
O(2)	0.021
O(2')	-0.016
O(3)	0.102
O(4)	-0.125
O(5)	-2.414
O(6)	-1.576
O(7)	1.242
O(7')	2.245
N	-0.013
C(1)	0.018
C(2)	-0.008
C(3)	-0.011
C(4)	0.002
C(5)	0.014
C(6)	-0.003
C(7)	0.003

coordinating atoms are part of one DPA ion, and another belongs to the neighboring DPA of the dimer. Four more are water oxygen atoms: O(5), O(6), O(7), and the symmetry-related O(7'). The coordination polyhedron around the calcium ion can be seen in Fig. 1 to resemble a pentagonal bipyramid except that one of the equatorial atoms has been replaced by two atoms slightly above and below the equator. Other ways to describe this figure are by noting that the four water oxygens form a planar trapezoid approximately perpendicular to the planar trapezoid formed by the atoms in the DPA ions, or by stating that the coordination polyhedron has twelve triangular faces and eight vertices.

The coordination polyhedrons are joined together, by the shared edges O(2)-O(2') and O(7)-O(7'), forming a polymer-like chain parallel to the short cell edge (5.87 Å) and the long crystal axis, similar to the chain of pentagonal bipyramids parallel to the 6.87 Å axis of calcium thymidylate (Trueblood, Horn & Luzzati,

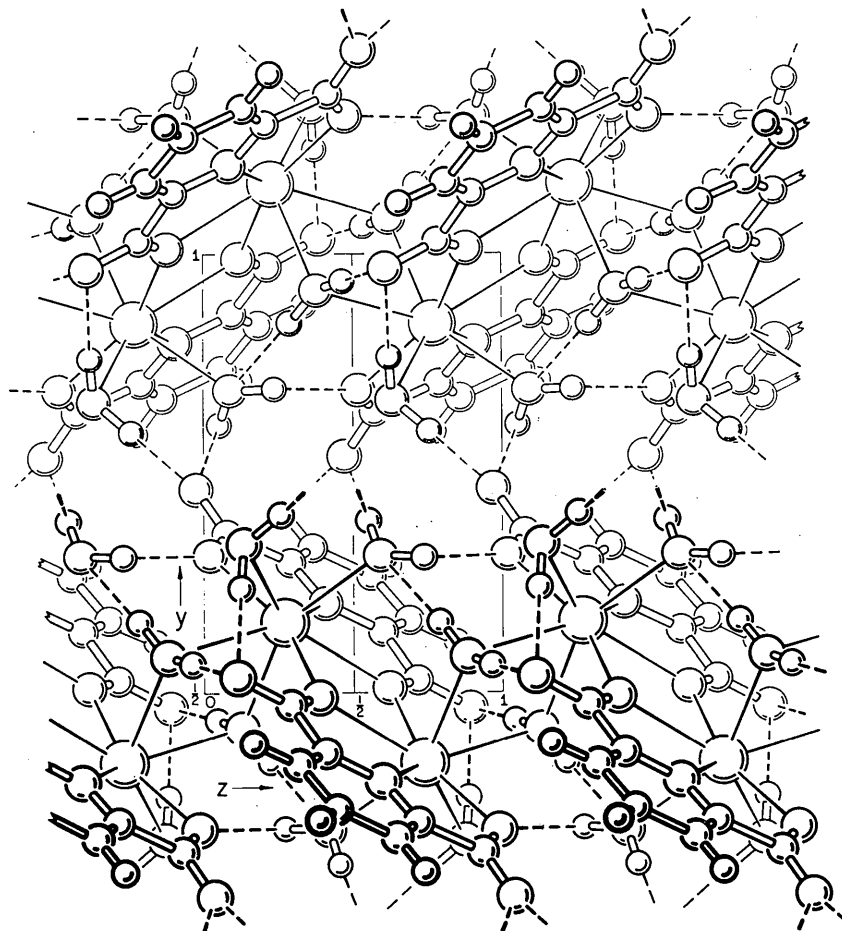


Fig. 3. Projection of the structure down the *a* axis showing the packing and bonding.

Table 9. *Hydrogen bonds*

Hydrogen	Donor atom	Acceptor atom	Donor-acceptor distance (e.s.d. = 0.01 Å)	Hydrogen-acceptor distance (e.s.d. = 0.1 Å)	Pertinent angle
1	O(5)	O(4'')	2.71 Å	1.8 Å	O(4'')-O(5)-O(3)
2	O(5)	O(3)	2.69	1.8	118.7 ± 0.4°
3	O(6)	O(4'')	2.72	2.1	O(4'')-O(6)-O(1')
4 (dubious)	O(6)	O(1')	2.98	2.2	122.7 ± 0.5°
5	O(7)	O(1')	2.65	1.7	O(1')-O(7)-O(5 ^{iv})
6	O(7)	O(5 ^{iv})	2.82	2.1	139.3 ± 0.4°
7	C(3)	O(1 ^v)	3.15	2.2	C(4)-C(3)-O(1 ^v) 108.2 ± 0.6° C(2)-C(3)-O(1 ^v) 134.4 ± 0.6°

1961). This chain is seen in the *hk0* projection (Fig. 2) edge on and in the *0kl* projection (Fig. 3) parallel to the *z* axis. The calcium-calcium distances in this chain are 3.991 Å for the dimer and 4.180 Å.

Hydrogen bonds

The hydrogen atoms were located in two ways: by searching difference Fourier maps, and by considering the hydrogen bonds as deduced from the oxygen-oxygen distances and angles. The three hydrogen atoms on the DPA ring were easily located. Five of the six water hydrogens were found without much difficulty. H(4), as previously mentioned, was not located although a possible location is indicated in the cell illustrations. The pertinent data about the hydrogen bonds are listed in Table 9. Only one of the hydrogen bonds is between water molecules. The other hydrogen bonds are to carboxyl oxygens, except for O(2) (which would be unlikely owing to the presence of two positively charged calcium ions). There is an interesting equivalence among the carboxyl oxygen atoms with each having two bonds besides the carbon-oxygen bond.

One of the ring hydrogen atoms, [H(7), which is covalently bound to C(3)] is part of an unusual C-H...O bond to oxygen atom O(1^v) forming a ten-membered planar ring [O(1)-C(1)-C(2)-C(3)-H(7)...O(1^v)-C(1^v), etc.] with two hydrogen bonds in the ring and a center of symmetry in the middle, somewhat similar to the eight-membered centrosymmetric carboxyl dimer found in the crystal structure of many carboxylic acids. This bonding joins the dimers (Fig. 1) together in a long planar ribbon, an example of the kind of structure which Sutor (1963) describes as occurring in crystal structures of heterocyclic compounds which are activated by another atom.

Thermal motion

The difference in the thermal motion of the various atoms could be seen in the first difference Fourier map when all the atoms were assumed to be nitrogen atoms (except for the calcium atom). The highest peak corresponded to O(2), which has the lowest isotropic temperature factor [Table 5(c)] because of its coordination to two calcium atoms and covalently bound to a carbon atom. The lowest peak corresponded to C(4),

which has the highest temperature factor due to its being furthest from the calcium ion and its restraining influence.

Spore structure

While this work cannot explain the stability of bacterial spores or the unique role that CaDPA fulfils, it does provide some basis for constructive speculation. Perhaps some of the bonding and coordination of the crystal also accounts for the spore stability. As in the crystal, the polymeric calcium oxygen chains may form a rigid polymeric structure as a component of the spore wall or cortex. Such a structure can be combined further to the remainder of the spore through hydrogen bonding with the spore components and the bound water as in this crystal form. The DPA unit occupies only three of the eight coordination positions of the calcium atom and therefore there can be five positions vacant for coordination to another molecule or molecules.

It would be most interesting to have the CaDPA sesquihydrate structure for comparison to see whether a similar intermolecular bonding system exists. The lower water content might favor either the use of more carboxyl oxygens in the calcium coordination polyhedron or a lower coordination number.

The authors want to thank L.K. Steinrauf for the crystal used, and Z.J. Ordal for suggesting the project. Preliminary accounts of this work have appeared previously (Strahs & Dickerson, 1964, 1965; Strahs, 1965). More details can be found in the Ph.D. thesis of G.S., which can be ordered from University Microfilms, Ann Arbor, Michigan. We wish to acknowledge gratefully the financial support of the National Institutes of Health, who made this work possible.

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X-ray Crystal Analysis of the Substrates of Aconitase. VIII. The Structure and Absolute Configuration of Potassium Dihydrogen Isocitrate Isolated from *Bryophyllum Calycinum**

BY DICK VAN DER HELM,† JENNY PICKWORTH GLUSKER, CARROLL K. JOHNSON,‡
 JEAN A. MINKIN, NANCY E. BUROW AND A. L. PATTERSON§

The Institute for Cancer Research, Philadelphia, Pennsylvania 19111, U.S.A.

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Anhydrous potassium dihydrogen isocitrate, prepared from the isomer of isocitric acid which is active in the tricarboxylic acid cycle, crystallizes in the space group $P2_12_12_1$ with four formula units, $K(C_6O_7H_7)$ in the unit cell of dimensions $a = 12.013$, $b = 13.145$ and $c = 5.159$ Å. Two complete sets of three-dimensional data were collected, one set with Cu $K\alpha$ radiation (Mo $K\alpha$ for $hk0$ and $h0l$ reflections) and the other set with Cr $K\alpha$ radiation. The structure was solved by comparing the $|F|^2$ map for the Cu-Mo data with the P_2 map for the Cr data. The final R values are 8.3% for the Cu-Mo data and 2.3% for the (more limited) Cr data. The absolute configuration of the biologically active isomer, (+)-isocitric acid, is (1R : 2S)-1-hydroxy-1,2,3-propanetricarboxylic acid. The ion is fully extended and there is slight disorder in the position of one carboxylic acid group. The central carboxyl group is the one that is ionized in this crystalline form. The ion forms a tridentate chelate with one potassium ion and a bidentate chelate with another.

Introduction

The isomer of isocitric acid present in the leaves of the succulent plant *Bryophyllum calycinum* is a substrate of the enzyme aconitase in the tricarboxylic acid cycle. The relative and absolute configurations of this isomer have already been determined crystallographically from a study of the potassium and rubidium salts of its lactone (Glusker, Patterson, Love & Dornberg, 1963; Patterson, Johnson, van der Helm & Minkin, 1962). When crystalline salts of the acid itself became available the present structure analysis was initiated to determine the shape, the mode of ionization and the packing of the isocitrate ion.

Experimental

Needle-like crystals of potassium and rubidium dihydrogen isocitrate were prepared for us from the leaves of *Bryophyllum calycinum* by Dr H. B. Vickery and Dr D. G. Wilson of the Connecticut Agricultural Experiment Station. These are isomorphous, crystallizing in the space group $P2_12_12_1$ (systematic absences $h00$ if h is odd, $0k0$ if k is odd, $00l$ if l is odd) with four metal ions and four isocitrate ions ($C_6O_7H_7^-$) in the unit cell. There is no water of crystallization. The unit-cell dimensions of the rubidium salt were measured from precession photographs. Those of the potassium salt were measured from high angle reflections on the General Electric XRD-5 diffractometer using both copper and chromium radiation. These values, and the densities measured by flotation, are given in Table 1. The structure determination was done on the potassium salt alone.

Data collection

X-ray intensity measurements were made with crystals which were ground as spheres in order to simplify the

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† Present address: The Department of Chemistry, The University of Oklahoma, Norman, Oklahoma, U.S.A.

‡ Present address: Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, U.S.A.

§ Deceased, 6 November 1966.