Quantitative Crystallinity Determinations for β-Lactam Antibiotics by Solution Calorimetry: Correlations with Stability

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Abstract □ The solution calorimetry method is based on the observation that amorphous forms are normally significantly higher in energy than are crystalline forms. The utility and validity of the calorimetric method were investigated for cephalothin sodium, cefazolin sodium, cefamandole nafate, and cefamandole sodium. Amorphous, partially crystalline, and crystalline forms were prepared and characterized by X-ray diffraction (powder), by solution calorimetry, and, for cephalothin sodium, by the thermal decomposition rate at 50°. Qualitatively, there was a good correlation between calorimetric crystallinity and the (less precise) crystallinity derived from X-ray data. The energy and structure of the amorphous state depend on the history of the sample; even samples of the same crystalline polymorph, containing no amorphous phase, may differ in energy. Thus, the *absolute* value of the crystallinity (X-ray or calorimetric) depends on the choice of amorphous and crystalline standards. The heat of solution is a precise (±1%) and unambiguous measure of the *relative* crystallinity; and provided amorphous and crystalline standards are appropriately chosen, the calorimetric crystallinity correlates well with chemical stability.

Keyphrases □ Crystallinity—determination by solution calorimetry, β-lactam antibiotics, correlated with chemical stability □ Antibiotics, β-lactam—crystallinity determined by solution calorimetry, correlated with chemical stability □ Calorimetry, solution—determinations of crystallinity of β-lactam antibiotics, correlated with chemical stability □ Stability, chemical—β-lactam antibiotics, correlated with crystallinity determined by solution calorimetry

The importance of polymorphism to an understanding of pharmaceutical systems is well recognized (1–6). Partial crystallinity is a special class of polymorphism and may have significant effects on dissolution rates (5, 6) and chemical stability (7). The magnitude of the observed difference in the dissolution rate (5, 6) or stability (7) between amorphous and crystalline phases suggests that even a small amount of amorphous phase in an otherwise crystalline sample is sufficient to alter measurably physical and chemical properties.

Methodology for determining precise degrees of crystallinity is needed to characterize fully the solid phase. Such methodology, specifically applied to β-lactam antibiotics, is the subject of this report. In principle, any extensive property that varies smoothly with the fraction of the crystalline phase in crystalline/amorphous mixtures may be used to measure crystallinity.

BACKGROUND

X-ray diffraction is the traditional method for crystallinity measurements (8–13). Although essential for qualitative identification of the crystalline phase present, X-ray powder patterns are not well suited for quantitative crystallinity measurements on β-lactam antibiotics. Precision is lacking, orientation effects[1] may result in systematic errors, and, because of pattern complexity, the separation of amorphous scattering from the total diffraction pattern is somewhat ambiguous. These problems are not unique to β-lactam antibiotics. Crystallinity measurements on polymers are subject to the same artifacts and ambiguities (10–13).

Quantitative degrees of crystallinity may be evaluated from heat of solution measurements (17). This method is based on the observation (14, 17–21) that, for many solids, the energy of the amorphous form is significantly higher than the energy of the crystalline form. The heat of solution, or calorimetric, percent crystallinity, P_c, is defined by:

$$P_c = 100\left[\frac{\Delta\bar{H}^\circ_s - \Delta\bar{H}^\circ_a}{\Delta\bar{H}^\circ_c - \Delta\bar{H}^\circ_a}\right] \qquad \text{(Eq. 1)}$$

where $\Delta\bar{H}^\circ_s$, $\Delta\bar{H}^\circ_c$, and $\Delta\bar{H}^\circ_a$ are the heats of solution to infinite dilution (in any fixed solvent) of the sample, the 100% crystalline standard, and the 100% amorphous standard, respectively. Thus, as with the usual relative X-ray and density methods (8–12), the partially crystalline sample is assumed to be a mixture of the two standard states (two-state model). Since the energy of a mixture of two solid phases is rigorously given by the sum of the energies of each phase, Eq. 1 is exact for mixtures of amorphous and crystalline standards. Provided the energy difference between crystalline and amorphous states is large, calorimetric crystallinities are potentially more precise, less subject to artifacts, and less ambiguous than crystallinity data derived from X-ray diffraction.

The objective of this research was to investigate the heat of solution as a measure of the crystallinity of β-lactam antibiotics, with specific consideration given to: (*a*) interference from contaminants (*e.g.*, water), (*b*) the magnitude of the energy difference between crystalline and amorphous forms, (*c*) the uniqueness of the amorphous and crystalline standards, (*d*) correlation of calorimetric and X-ray diffraction data, and (*e*) correlation of chemical stability with calorimetric crystallinity for partially crystalline samples.

The compounds and polymorphs studied are summarized in Table I. A sample was classified as amorphous when the X-ray diffraction pattern (powder) showed no distinct peaks and the sample was nonbirefringent (microscopic examination under polarized light).

EXPERIMENTAL

Sample Preparation—*Cefazolin Sodium*—The crystalline α-form (pentahydrate) was prepared by recrystallization from aqueous ethanol (22). The monohydrate was prepared by suspending the α-form in anhydrous ethanol. The identity of each crystal form was verified by X-ray diffraction.

The precipitated forms (Samples 4 and 5, Table I) were prepared by rapidly pouring 20 ml of a 50% solution of cefazolin sodium in water–acetonitrile (1:1) into 300 ml of anhydrous ethanol. A gel first formed, which then was transformed into nonbirefringent beads about 80 μm in diameter. The system was allowed to age for several hours and then was filtered. The solid was air dried overnight and then was vacuum dried. The solid product exhibited birefringence. The X-ray diffraction patterns were qualitatively similar to the monohydrate pattern. However, the samples were very poorly crystallized, and a positive identification of the crystal form could not be made.

Sample 7 (Table I) was spray dried[2] from a 25% aqueous solution with an inlet temperature of 150° and an outlet temperature of 100°, yielding glassy amorphous beads 10–20 μm in diameter. The amorphous freeze-dried sample (Sample 8, Table I) was prepared by freeze drying an aqueous solution at low temperature as previously described (7).

[1] Orientation effects may be eliminated by reducing the sample to a fine powder by grinding. However, since grinding reduces the crystallinity of many materials (6, 14, 15), it cannot be employed in crystallinity determinations. Sample rotation may be used to minimize orientation effects if the diffractometer is modified suitably (16). These specialized modifications were not available for the research described in this report.

[2] Komline-Sanderson, 91-cm (36-in.) diameter, or equivalent laboratory model spray drier.

Table I—Effect of Physical Form on Energy of Solids: Heats of Solution at Infinite Dilution in Water at 25°

Sample	Compound	Physical Form[a]	Water Content, % (w/w)	Heat of Solution[b], $\Delta H°_s$, kcal/mole
1	Cefazolin sodium	Crystalline, pentahydrate (α-form)	15.9	+7.8
2	Cefazolin sodium	Crystalline, monohydrate	4.0	+4.4
3	Cefazolin sodium	Weak crystalline, vacuum-dehydrated monohydrate	0.2	−1.4
4	Cefazolin sodium	Weak crystalline, precipitated[c]	0.3	−2.0
5	Cefazolin sodium	Weak crystalline, precipitated[c]	0.3	−3.5
5a	Cefazolin sodium	Weak crystalline, precipitated[c]	4.0	+0.8
6	Cefazolin sodium	Weak crystalline, vacuum-dehydrated α-form	0.0	−3.2
7	Cefazolin sodium	Amorphous, spray dried	0.3	−4.5
8	Cefazolin sodium	Amorphous, freeze dried	0.1	−5.4
9	Cefamandole nafate[d]	Crystalline, γ-form	0.0	1.92 ± 0.05
10	Cefamandole nafate[d]	Crystalline, γ-form[e]	0.1	1.48 ± 0.05
11	Cefamandole nafate[d]	Weak crystalline, vacuum-desolvated α-form	0.1	−1.0
12	Cefamandole nafate[d]	Amorphous, spray dried	0.3	−3.4 ± 0.1
13	Cefamandole nafate[d]	Amorphous, freeze dried	0.1	−4.4 ± 0.1
14	Cefamandole sodium[d]	Crystalline, monohydrate	4.0	0.26 ± 0.05
15	Cefamandole sodium[d]	Crystalline, vacuum-dehydrated monohydrate	0.2	−1.84 ± 0.05
16	Cefamandole sodium[d]	Amorphous, spray dried	0.1	−3.1
17	Cefamandole sodium[d]	Amorphous, freeze dried	0.4	−5.3
18	Cephalothin sodium	Crystalline	0.1	1.9 ± 0.1
19	Cephalothin sodium	Crystalline	0.0	1.67 ± 0.02
20	Cephalothin sodium	Crystalline	0.0	1.47 ± 0.05
21	Cephalothin sodium	Crystalline, mortar ground Sample 20	0.1	1.20 ± 0.08
22	Cephalothin sodium	Amorphous, freeze dried	0.1	−4.1 ± 0.1
23	Penicillin G potassium	Crystalline	0.1	−0.32 ± 0.05
24	Penicillin G potassium	Amorphous, freeze dried	0.5	−5.4

[a] Crystalline = 100% birefringent particles, sharp X-ray pattern with low diffuse scattering; weak crystalline ≈ 100% birefringent particles, weak and diffuse X-ray pattern; amorphous = nonbirefringent, no distinct peaks in the X-ray pattern. [b] Uncertainty, ~ ±0.2 kcal/mole for the sample studied except when specifically indicated otherwise. The uncertainty corresponds roughly to the 90% confidence level. [c] Weakly crystalline even with 5% water. Appears to be poorly crystallized monohydrate. [d] The chemical structure of this compound may be found in Table I of Ref. 7. [e] Probably has an amorphous layer coating the crystals (see *Experimental*).

Cefamandole Nafate—Samples 9 and 10 (Table I) were crystallized as one lot from a methanol solution by the addition of 2-propanol, yielding nonsolvated crystalline material. One portion of the filter cake was first air dried overnight and then vacuum dried (25°/24 hr; 40°/24 hr) to yield Sample 9. The remaining portion was placed directly into a 50° oven and vacuum dried overnight to yield Sample 10. This procedure presumably caused an amorphous coating on the crystal surface because of partial dissolution at high temperature and subsequent evaporation of solvents (methanol, 2-propanol, and atmospheric moisture contamination).

Crystalline α-form cefamandole nafate (Sample 11) was prepared by adding an acetone solution of sodium 2-ethylhexanoate to an acetone solution of the acid form of cefamandole nafate. The resulting crystals (probably an acetone–water mixed solvate) were vacuum dried to remove all traces of solvent.

Amorphous freeze-dried cefamandole nafate was prepared as previously described (7). Sample 12 was spray dried from a 25% aqueous solution with inlet and outlet temperatures of 107 and 75°, respectively.

Cefamandole Sodium—The monohydrate (Sample 14) was prepared by passing nitrogen at 50% relative humidity through a fluidized bed of crystalline methanol solvate at 25°. The conversion of methanolate to monohydrate was complete within 24 hr. The methanolate crystals were obtained by addition of sodium acetate to a methanol solution of purified cefamandole acid. The freeze-dried amorphous sample was prepared as previously described (7). The spray-dried amorphous sample was prepared by spray drying a 20% aqueous solution with inlet and outlet temperatures of 90 and 70°, respectively.

Cephalothin Sodium—Sample 20 (Table I) was commercial[3] cephalothin sodium used as received except for vacuum drying. Samples 18 and 19 were commercial samples that were recrystallized four times by salting out cephalothin sodium from a 20% aqueous solution with sodium chloride and sodium lactate, respectively. Sample 21 was prepared by vigorous mortar grinding of Sample 20.

Amorphous freeze-dried cephalothin sodium (Sample 22) was prepared by freeze drying a 20% aqueous solution at low temperatures (7). The partially crystalline freeze-dried samples (Samples 26–28, Table II) were prepared using essentially the same procedure, except that the frozen solutions were allowed to anneal at −5° for 3–18 hr. A sample of the freeze-dried material in each vial was examined microscopically under polarized light, and the vials were separated into three classes. The contents of all vials of a given class were combined to yield three samples: Sample 26, essentially all particles showing birefringence; Sample 27, mostly birefringent material[4]; and Sample 28, roughly one-half birefringent material.

Sample 25 (Table II) was spray dried from a 20% aqueous solution, using an inlet air temperature of 150° and an outlet temperature of 70°. The sample was initially poorly crystallized (heat of solution, −0.06 kcal/mole) and was allowed to anneal at ambient temperature for 2 months to yield essentially crystalline material (heat of solution, +1.39 kcal/mole; essentially 100% birefringent beads ~30 μm in diameter). Although some discoloration of the powder occurred during the annealing process, the extent of decomposition was too small to detect by iodometric assay or TLC (7). Sample 29 was spray dried from a solution composed of 18% cephalothin sodium, 2% diethylcarbonate, and 80% water to yield a mixture of birefringent and glassy nonbirefringent beads 10–20 μm in diameter. The air temperatures were as indicated for Sample 25.

Penicillin G Potassium and Cephaloridine—Penicillin G potassium samples were prepared as previously described (7). Cephaloridine was δ-form (3) crystalline material of commercial origin[2].

Assay Methods and Sample Purity—Cefazolin sodium, cefamandole nafate, and cefamandole sodium were assayed by polarographic techniques (24, 25); an automated iodometric procedure (26) was used for the other β-lactams. Assay for the free 3′-side chain of cefamandole, 1-methyl-5-mercapto-1,2,3,4-tetrazole, a decomposition product, was performed by polarography (24, 25). TLC (7) was used as a qualitative check on purity.

The crystalline and partially crystalline samples were 98+% pure as determined by assay and were one-spot materials on TLC. The amorphous samples were 96+% pure (assay results), and some showed trace quantities (~1%) of decomposition products by TLC.

[3] Eli Lilly and Co., Indianapolis, Ind.

[4] The fraction of birefringent material was estimated by microscopic examination under polarized light as the stage was rotated. The resulting visual estimation was highly approximate.

Table II—Comparison of Degrees of Crystallinity for Cephalothin Sodium Evaluated from X-ray Diffraction, Calorimetry, and Chemical Stability

Sample	Sample Origin	$\Delta H°_s$, kcal/mole (≤0.1% H_2O)	Percent Crystalline				
			X-Ray[a]			50° Stability	
			External Method	Internal Method	Calorimetry[b]	Dry	31% R.H.
18	Laboratory crystallized	1.9	100	100	100	100	100
19	Laboratory crystallized	1.67	77	70	96	—	—
20	Commercial lots	1.47	72	67	93	101(3)	100 (0.5)
25	Spray dried[c]	1.39	69	55	92	102 (1)	97 (6)
26	Freeze dried[d]	1.15	62	51	88	101 (2)	100 (0)
27	Freeze dried[e]	0.32	57	48	74	86 (11)	—
28	Freeze dried[e]	−0.84	47	40	54	77 (2)	85 (1)
29	Spray dried[f]	−1.30	37	34	47	54 (1)	44 (10)

[a] With fixed data for amorphous and crystalline standards, the reproducibility (standard deviation) of crystallinity for a given sample is about ±3% for both methods when all data are taken on the same day, as with the data in Table II. From the reproducibility of the data for a given sample and the reproducibility of the data for crystalline and amorphous standards, and standard error in an X-ray crystallinity was estimated to be about ±5%. The internal X-ray method may be subject to an additional (systematic) error because of orientation effects. [b] The reproducibility of the calorimetric crystallinity was about ±0.6% (*SD*). Considering also uncertainty in the calorimetric data for the amorphous and crystalline standards, the standard error in a calorimetric crystallinity was estimated to be about ±1%. [c] Heat of solution did not change upon aging at 50° for 2 months. [d] During 24 months at 25°, the heat of solution increased from 1.15 to 1.46 kcal/mole. [e] Partial crystallization occurred during the stability determination (microscopic examination). [f] No crystallization was detected during the stability determination (microscopic examination).

Water contents were determined by Karl Fischer titrations. Residual solvents, other than water, were determined to be less than ~0.1% by GLC, NMR spectroscopy, or the difference between thermal gravimetric analysis mass loss and the water content.

Calorimetry—Heats of solution were determined with a commercial isothermal calorimeter system[5] similar in design to the calorimeter developed by Arnett *et al.* (27). The sample container used in this research (Fig. 1) was essentially a stainless steel tube sealed at both ends with removable plastic plugs. The plugs were fitted with rubber O-rings coated with silicone stopcock grease to ensure a good seal between the contents of the tube (*e.g.*, the sample) and the calorimeter solvent (*e.g.*, water).

The sample container was placed in the calorimeter cell through an opening in the cell head. The sample was exposed to the solvent by pulling up on disk A (Fig. 1) to remove the top plug and then pushing down on disk B to remove the bottom plug. The heat of opening was zero within the sensitivity of the calorimeter (0.04 cal). For most samples, complete dissolution was achieved within 30–60 sec.

Standard calorimetric procedures were followed (27, 28). The final solution concentrations were normally less than 0.02 *M*. Heats of solution for sodium and potassium salts were corrected to infinite dilution, assuming that, as a first approximation, the heats of dilution were the same as for potassium chloride (29) at the equivalent molar concentration. The accuracy of this approximation, within ±0.03 kcal/mole, was verified for cephalothin sodium at concentrations up to 0.1 *M*.

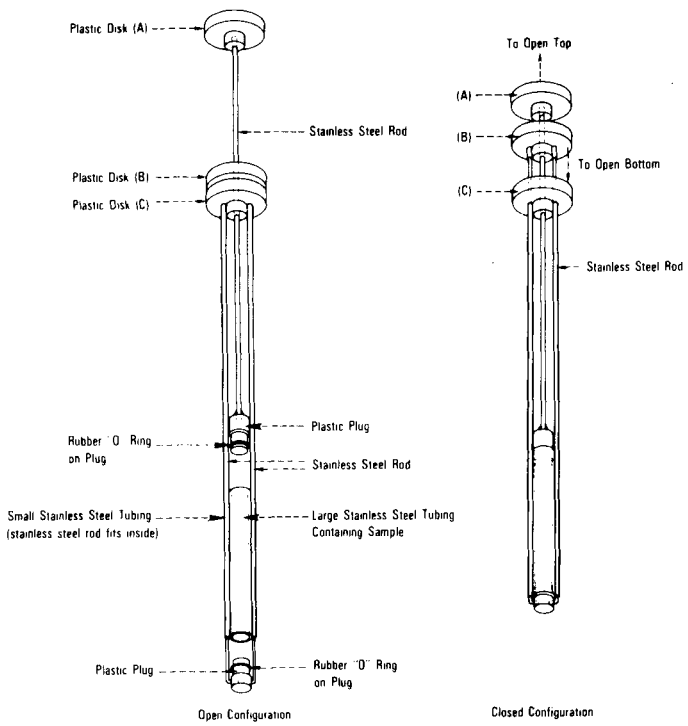

Figure 1—*Diagram of calorimeter sample container.*

[5] Guild Electronics, 400-115, SKC Inc., Pittsburgh, PA 15200.

The calorimetric accuracy was periodically checked by measuring the heat of solution of potassium chloride[6] in water and by measuring the heat of dissolving 2-amino-2-(hydroxymethyl)-1,3-propanediol[7] in an excess of 0.1 *N* HCl. Results were within 1% of the accepted literature values (30, 31).

All sample transfers of anhydrous material were carried out at near zero relative humidity[8].

X-Ray, Stability, and Water Absorption Measurements—X-ray scattering data were obtained using commercial X-ray diffractometers[9] with CuKα radiation and a nickel filter.

Stability data were generated as described previously for cephalothin sodium (7). The extent of decomposition at 50° was determined at a single predetermined aging time (3 weeks to 6 months, depending on crystallinity and water content). The decomposition rate was calculated assuming first-order decomposition.

For Samples 22 (Table I) and 27–29 (Table II), water absorption was determined at 25° and 31% relative humidity by placing 2–5 g of dried sample in a desiccator containing solid calcium chloride hexahydrate in equilibrium with its saturated aqueous solution. The desiccator was evacuated, and the water absorbed at equilibrium was determined gravimetrically. The uncertainty in water content was estimated to be less than ±0.1% H_2O. Water absorption for samples of high crystallinity and low water absorption was determined by a more sensitive procedure. Nitrogen at 31% relative humidity was passed over a sample suspended from one arm of an electromagnetic microbalance. The increase in mass was monitored, and the water sorbed was calculated from the equilibrium mass. The uncertainty in water content measured by this method was about ±0.01% H_2O.

RESULTS AND DISCUSSION

Effects of Solvation (Table I and Figs. 3 and 5)—Dehydration of a cephalosporin in either amorphous or solvated crystalline form results in a strong exothermic shift in the heat of solution. With the exception

[6] Mallinckrodt reagent grade, recrystallized from water.

[7] Baker Ultrex grade.

[8] Glove bag, Instruments for Research and Industry, Cheltenham, Pa. The glove bag was continuously purged with air equilibrated with anhydrous calcium sulfate.

[9] Norelco, Mt. Vernon, N.Y. The data reported in Fig. 2 were obtained from a recently acquired instrument, model XRG-3000 (scintillation counter detector). All other data were obtained from another (older) instrument, type 12045B/3 (Geiger counter detector). The settings on model XRG-3000 were chosen to give peak intensities (for a given crystalline sample) comparable to the corresponding intensities measured with instrument 12045B/3. With each instrument, all data were taken with the same instrument settings using the same procedures. The detector of the XRG-3000 was less sensitive to diffraction of the continuous spectrum; therefore, the apparent background scattering was slightly lower with the XRG-3000 model, particularly at low angles. Thus, the data in Fig. 2 may not be compared quantitatively with the scattering data reported in Figs. 3 and 4.

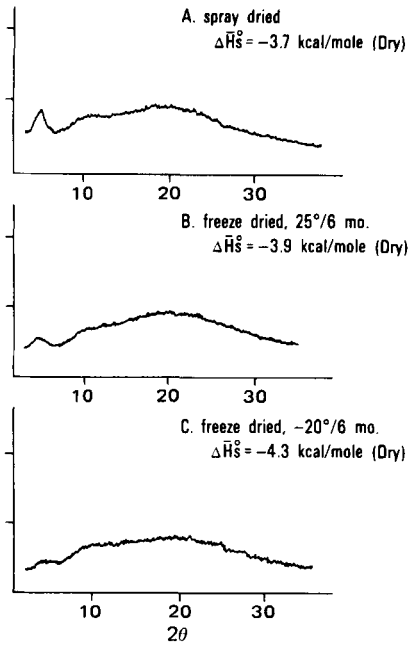

Figure 2—*X-ray diffraction and heats of solution for amorphous forms of cefamandole nafate. Experimental heats of solution (Tables I and III) were corrected to zero water content using 1 kcal/mole/1% H_2O (w/w) as the correction factor.*

of cefamandole sodium monohydrate, desolvated crystals exhibited a weak and diffuse X-ray pattern (Fig. 3), indicating some loss of crystallinity upon desolvation. However, at least for cefazolin sodium pentahydrate, the structural changes occurring on dehydration were completely reversible. Rehydration restored the strong X-ray pattern, and the heat of solution returned to the value characteristic of the pentahydrate (+7.8 kcal/mole).

The exothermic shift of the heat of solution on dehydration is probably due to two effects: (*a*) the crystallinity decreases on dehydration; and (*b*) since a wet solid is already partially hydrated, the (exothermic) hydration energy upon solution is less in magnitude, and the heat of solution is more

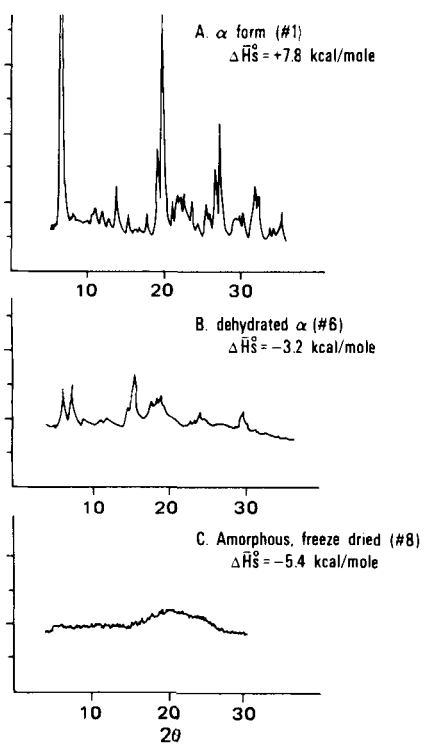

Figure 3—*X-ray diffraction and heats of solution for crystalline, weakly crystalline, and amorphous forms of cefazolin sodium.*

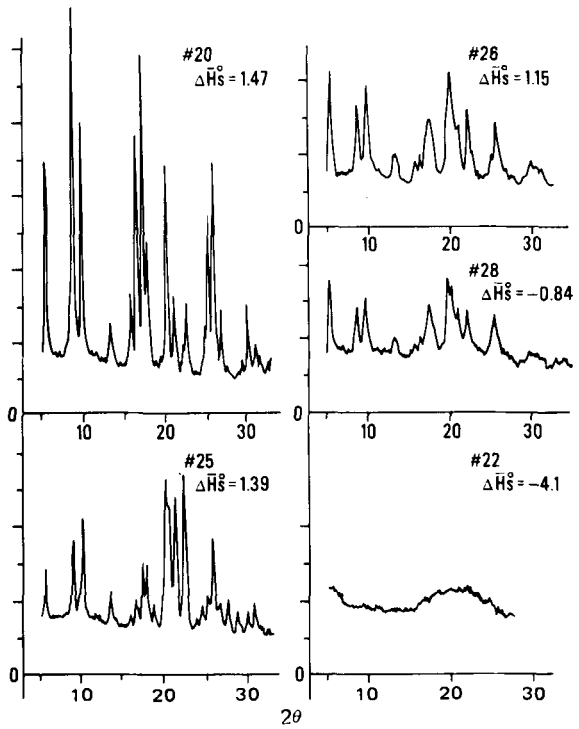

Figure 4—*X-ray diffraction and heats of solution for cephalothin sodium samples of differing degrees of crystallinity.*

endothermic. The magnitude of the exothermic shift for amorphous cephalothin sodium (Fig. 5) was comparable to that observed for crystalline solvates (Fig. 5 and Table I), suggesting that the second effect was the greatest.

Protection of calorimetry samples from atmospheric moisture is essential. Amorphous and desolvated crystalline cephalosporins are extremely hygroscopic, absorbing 3–5% (w/w) water at 30% relative humidity. In view of the sensitivity of the heat of solution to water content, water contamination could result in serious errors.

Excess Energy of Amorphous Forms—As expected, amorphous forms of a compound were energy rich and had more exothermic heats of solution than corresponding crystalline forms (Table I). In general, the energy difference was large, ~6 kcal/mole. However, the magnitude of the energy difference between crystalline and amorphous forms for a given compound (excess energy) was not unique.

The differences in heats of solution between spray-dried and freeze-dried amorphous samples of the same compound (Table I) were real and could not be attributed to residual impurities. Moreover, at least for cefamandole nafate and cefamandole sodium, freeze-dried amorphous samples appeared to anneal slightly upon aging (Table III).

Although the heat of solution clearly becomes more endothermic upon aging (Table III), interpretation of the results in terms of a change in structure is obscured by the increase in decomposition that occurs on aging. As a measure of decomposition upon aging, the mole percent of the free 3′-side chain (1-methyl-5-mercapto-1,2,3,4-tetrazole) is given for each sample (Table III). One might argue that the observed endo-

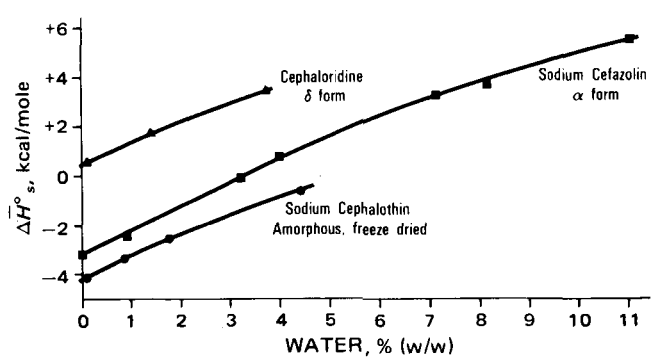

Figure 5—*Effect of water content on the heat of solution of cephalosporins in water at 25°.*

Table III—Changes in Heats of Solution on Aging for Amorphous Freeze-Dried Cefamandole Sodium and Cefamandole Nafate

Aging Condition	Cefamandole Nafate (0.1% H_2O) $\Delta H°_s$, kcal/mole[a]	Cefamandole Nafate (0.1% H_2O) Free 3′-Side Chain, mole %	Cefamandole Sodium (0.4% H_2O) $\Delta H°_s$, kcal/mole[a]	Cefamandole Sodium (0.4% H_2O) Free 3′-Side Chain, mole %
Initial	−4.4	1.2	−5.3	1.7
6 months/−20°	−4.2	1.4	−5.2	2.0
3 months/−5°	−3.9	1.3	−5.2	1.1
1 month/25°	−4.0	2.1	−5.3	2.4
3 months/25°	—	—	−4.9	2.9
6 months/25°	−3.8	2.7	−4.8	3.5
3 weeks/40°	−3.8	3.6	−4.9	3.9
24 hr/60°	−3.6	4.0[b]	−4.6	5.9[c]

[a] $\Delta H°_s$ is heat of solution to infinite dilution in water at 25°. Estimated uncertainty = ±0.1 kcal/mole. [b] Extent of decomposition by polarographic assay for cefamandole nafate, 3%. [c] Extent of decomposition by polarographic assay for cefamandole sodium, 7%.

thermic shift in the heat of solution upon aging reflects contamination of the sample by decomposition products. If the endothermic shift is attributed to decomposition, the heat of solution of the decomposition products would have to be ~ +210 cal/g, which seems much too high. (The heat of solution of crystalline 1-methyl-5-mercapto-1,2,3,4-tetrazole was only +46 cal/g.) Therefore, some type of annealing phenomenon apparently occurred.

Slight differences in the X-ray diffraction patterns of amorphous samples were also evident, as demonstrated in Fig. 2 for cefamandole nafate. Differences were particularly evident at $2\theta = 5°$. The annealed freeze-dried sample (Fig. 2B), aged at 25° for 6 months, was intermediate between the spray-dried sample (Fig. 2A) and the freeze-dried sample stored at −20° for 6 months (Fig. 2C). The latter sample (Fig. 2C) was typical of freshly prepared freeze-dried material.

Although subtle, the differences shown in Fig. 2 are reproducible. Two additional lots of both spray-dried and freeze-dried cefamandole nafate were examined by X-ray diffraction and calorimetry with essentially the same results. To facilitate a quantitative comparison independent of systematic intensity errors, an order parameter, X, is defined by:

$$X = \frac{h_p}{h_D} \qquad \text{(Eq. 2)}$$

where h_p is the height of the broad peak at $2\theta = 5°$ and h_D represents the corrected diffuse (background) scattering at 5°. The parameters h_p and h_D are calculated as follows. A straight line is drawn connecting the point on the diffraction curve at $2\theta = 3.5°$ with the minimum at $2\theta = 7°$; h_p is the vertical distance between this line and the peak at 5°. The distance between the straight line and the zero intensity axis represents the sum of h_D and various extraneous background effects such as incoherent scattering, air scattering, and diffraction of the continuous spectrum. Then a straight line is constructed as described for a 100% crystalline standard (Sample 9). The vertical distance between zero intensity and this line at 5° is taken as the intensity of the extraneous background (incoherent scattering, air scattering, and diffraction of the continuous spectrum).

The mean order parameters (for the three samples studied) and their corresponding error limits (90% confidence) were: spray dried, $X = 1.34 \pm 0.09$; aged freeze dried (6 months/25°), $X = 0.92 \pm 0.15$; and fresh freeze dried (or 6 months/−20°), $X = 0.62 \pm 0.15$. While the difference between aged and fresh freeze-dried material was significant at the 90% confidence level, more data are needed to verify the difference. However, the difference between spray-dried and fresh freeze-dried materials was clearly significant. Thus, spray-dried amorphous material appears to be an annealed form of amorphous material. While there is little experimental precedent for this observation, differences in structure between amorphous samples of the same compound have ample theoretical support (15, 32–34).

Samples 18–20 (Table I) were crystalline cephalothin sodium samples (same polymorph) with slightly different heats of solution. The X-ray patterns were qualitatively alike (see Sample 20, Fig. 4) but differed slightly in the magnitude of background scattering. All samples were crystallized from aqueous solution by salting out with either sodium lactate (Samples 19 and 20) or sodium chloride (Sample 18). Sample 20 was a representative commercial sample[3], while Samples 18 and 19 were laboratory samples prepared by four recrystallizations of the commercial sample. No difference in sample purity was detected (see *Experimental*), and none of the samples appeared to be a mixture of amorphous and crystalline phases[10].

For lack of a plausible alternative interpretation, the energy differences among Samples 18–20 were attributed to differences in crystal perfection, *e.g.*, point defects or dislocations. While similar observations were noted for potassium chloride (30), the energy differences observed for cephalothin sodium were much greater.

The ambiguity in the definition of crystalline and amorphous causes uncertainty in the choice of standards for use in crystallinity measurements. While this uncertainty does not affect a precise comparison between samples, the numerical value of the crystallinity is obviously not unique. In this study, the lowest energy crystalline preparation (most endothermic heat of solution) was selected as the crystalline standard and the highest energy amorphous preparation (most exothermic heat of solution) was the amorphous standard. In some applications, it may be desirable to use a crystalline standard similar in perfection to the crystalline phase in the partially crystalline samples investigated.

Correlation of Calorimetric and X-Ray Data—Qualitatively, there was a good correlation between X-ray crystallinity and calorimetric data for partially crystalline samples.

1. Heats of solution of cephalothin sodium became more exothermic as the X-ray patterns became weaker and more diffuse[11] (Fig. 4).
2. Cefazolin sodium Samples 4 and 5 (Table I), which appeared to be poorly crystallized monohydrate, had weak X-ray patterns even at the monohydrate composition and also had heats of solution more exothermic than well-crystallized material (Samples 2 and 3).
3. The sample of the crystalline γ-form of cefamandole nafate, believed to have an amorphous layer coating the crystals (Sample 10, Table I), had a slightly more exothermic heat of solution than the "standard" 100% crystalline Sample 9.
4. The more exothermic heat of solution for mortar-ground cephalothin sodium (Sample 21, Table I) was consistent with the observed loss of crystallinity on grinding (6, 10, 14).

A quantitative comparison of X-ray and calorimetric crystallinities is given in Table II for a series of cephalothin sodium samples. Percent crystallinity by calorimetry was evaluated from Eq. 1, using Samples 18 and 22 as the crystalline and amorphous standards, respectively. With the same standards, X-ray crystallinities were evaluated[12] by an external

[10] Since microscopic examination indicated that all particles were birefringent, the only plausible postulate involving an amorphous phase is that the crystals are coated with a thin layer of amorphous phase, presumably formed during vacuum drying. However, cephalothin sodium readily crystallizes when an aqueous solution is vacuum dried. Moreover, even under relatively mild conditions, amorphous cephalothin sodium degrades to yield small amounts of highly colored product, which gives the amorphous powder an amber color. Therefore, the presence of a surface amorphous phase in Sample 20 of the proportions indicated by the calorimetric data (~7%) would cause the solid to develop an amber color upon aging in much the same manner as an amorphous solid. However, Sample 20 did not develop significant color upon aging.

[11] The peak broadening observed in Samples 26 and 28 may be a result of small crystal size and/or strain. Differences between Samples 20 and 25 were probably due mainly to orientation effects. The crystals in Sample 20 were large (~100 μm longest dimension) bladed and prismatic particles, likely to exhibit preferred orientation when compacted into the sample holder. Preferred orientation should be minimal for the other samples in Fig. 4. Scanning electron microscope studies showed that each birefringent bead in Sample 25 had a very irregular rock-like surface with no single bladed or prismatic crystals in evidence. Sample 26 was composed of bundles of small (~20 μm longest dimension) bladed and needle-like crystals which appeared to be fused together and, in many cases, bent. Specific surface areas for Samples 20, 26, and 22 were 0.59, 7.5, and ≤0.2 m^2/g [Brunauer, Emmett, and Teller (BET) N_2 adsorption], respectively.

[12] Crystalline and background scattering were separated by a smooth background curve drawn through the minima between Bragg reflections (11), maintaining a contour similar to that shown by the amorphous standard (Sample 22). The intensity between this curve and the zero intensity axis (the background) is due to the coherent amorphous scattering of interest and various extraneous background effects (incoherent scattering, air scattering, and diffraction of the continuous spectrum). It was assumed that the background scattering for the crystalline standard (Sample 18) was due only to the extraneous background. Thus, the desired amorphous scattering for a given sample was obtained by subtracting the crystalline standard background from the sample background.

The external method defines the crystallinity P_x as $P_x = 100\,(1 - I_a/I°_a)$, where I_a and $I°_a$ are the amorphous scattering intensities at $2\theta = 21°$ for the sample of interest and the amorphous standard, respectively. For the internal method, crystalline and background intensities are integrated (with a planimeter) between 2θ values of 5 and 28.8°. The integrated crystalline and amorphous intensities, A_c and A_a, respectively, are assumed to be proportional to the weight fraction of the respective phase, f_i ($i = c, a$); $A_c = k_c f_c$ and $A_a = k_a f_a$, where k_c and k_a are constants evaluated by calibration with the crystalline and amorphous standards, respectively. The percent crystallinity is then given by $P_x = 10^2 f_c = [100\,K(A_c/A_a)]/[1 + K(A_c/A_a)]$, where $K = k_a/k_p = 0.83$. The external method described here is probably more accurate since it is free of systematic errors due to orientation effects. The only advantage of the internal method is that some systematic intensity errors tend to cancel since both intensities in the ratio refer to the same diffraction pattern.

method based on the reduction in intensity of the background scattering at $2\theta = 21°$ (the amorphous halo peak) resulting from an increase in crystallinity (12, 17) and by an internal method (17) based on the ratio of integrated amorphous and crystalline intensities.

If a partially crystalline sample were simply a mixture of the amorphous and crystalline standards (two-state model), all valid measures of crystallinity would give identical results. The lack of quantitative agreement between X-ray and calorimetric crystallinities (Table II) is due mostly to the failure of the two-state model. Since the two-state model recognizes neither variations in defect structure of crystals nor variations in the structure of amorphous materials, quantitative agreement between different measures of crystallinity cannot be expected. A similar lack of agreement between different measures of crystallinity was noted for polymers (10–13). However, the order of decreasing crystallinities (Table II) was the same for both X-ray and calorimetric data.

Crystallinity and Chemical Stability—Since amorphous cephalothin sodium is much less stable than the crystalline form, one would expect a correlation between calorimetric crystallinity and chemical stability. To facilitate comparison of calorimetric crystallinity and stability, the percent crystallinity *via* stability, P_s, is defined by:

$$P_s = 100(1 - k_s/k_a) \qquad \text{(Eq. 3)}$$

where k_s and k_a are the apparent first-order decomposition rates for the sample and amorphous standard, respectively. The crystalline standard is stable under the test conditions (7), so its decomposition rate is essentially zero and does not appear in Eq. 3. Stability at 50° was determined for both dry samples ($\leq 0.1\%$ H_2O) and samples exposed to a relative humidity of 31% (at 25°). The P_s values are compared with calorimetric crystallinities in Table II. The number in parentheses after the stability crystallinity figure is the difference in crystallinity measured between duplicate samples. If the sample crystallinity remained constant during the stability test and if the two-state model was rigorous, quantitative agreement of calorimetric and stability crystallinities would be observed. However, the two-state model is not rigorous, and annealing during the stability test probably slightly increased the crystallinity measured *via* stability for Samples 26–28 (footnotes *c–f*, Table II). Calorimetric crystallinity in excess of 93% ($\Delta\overline{H}°_s = 1.47$ kcal/mole) does not appear to be obtainable by annealing (footnotes *c–f*, Table II).

With Sample 18 as the crystalline standard, agreement between stability and calorimetric crystallinities (Table II) was only approximate. The chemical stability data for Samples 20, 25, and 26 were not consistent with a sample containing more than 1–2% of amorphous phase. The 7–12% loss in calorimetric crystallinity (28–38% by the external X-ray method) for Samples 20, 25, and 26 cannot be attributed to the presence of an amorphous phase. This apparent anomaly may be resolved if one accepts the proposal of significant differences in crystal perfection between crystalline samples and further postulates that differences in crystal perfection have little effect on chemical stability.

Both Samples 18 and 19 were highly purified, carefully crystallized samples and may not be representative of the crystal perfection obtainable by routine crystallization, freeze drying, or spray drying. Thus, if the objective of crystallinity measurements on cephalothin sodium is to predict stability of samples prepared by routine processes, Sample 20 would be a more reasonable choice for the 100% crystalline standard.

Crystallinity and Water Absorption—Amorphous cephalothin sodium absorbs far more water than the crystalline form, and the amount of water absorbed at 25° and a relative humidity of 31% can serve as a reasonably good measure of the degree of crystallinity[13] (Fig. 6). However, the correlation between crystallinity and water absorption was poor at calorimetric crystallinities above 88%. For samples of 88, 92, 93, 96, and 100% crystallinity, the corresponding water absorption data were 0.23, 0.28, 0.18, 0.24, and 0.04% (w/w). The lack of a good correlation was not due to experimental error in either calorimetric crystallinities (footnote *b*, Table II) or water contents (see *Experimental*). Perhaps factors other than crystallinity (*i.e.*, surface area or trace surface impurities) affect water absorption.

For highly crystalline samples, one might expect that water absorption would be directly proportional to surface area. However, from the limited data available, this reasonable speculation does not appear valid. Samples 20 and 26 had specific surface areas of 0.59 and 7.5 m^2/g (BET nitrogen adsorption), respectively. However, the water absorption was nearly the same for both samples: 0.18% (Sample 20) and 0.23% (Sample 26).

[13] It appears that water absorption accelerates crystallization of the amorphous phase. Thus, to avoid changes in crystallinity during the measurement process, high humidity should be avoided and the absorption experiment should be done quickly (within 24 hr).

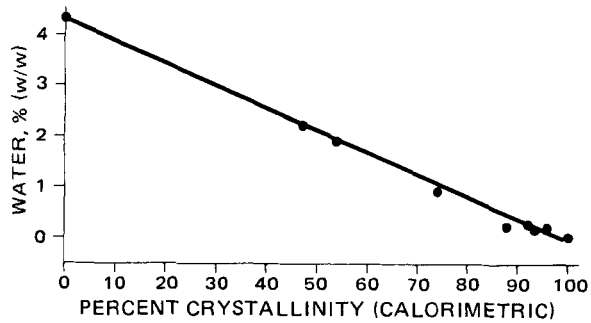

Figure 6—*Water absorption at 31% relative humidity at 25°; correlation with calorimetric crystallinity data from Table II.*

CONCLUSIONS

The heat of solution is a precise unambiguous measure of the relative crystallinity of a sample containing only one crystal form. The energy of the amorphous state depends, to some extent, on the method of preparation, and even crystalline samples of the same polymorphic form containing no amorphous phase may differ in energy. Thus, the absolute value of the percent crystallinity depends on the choice of amorphous and crystalline standards. Provided the standards are appropriately chosen, the calorimetric crystallinity can provide a useful indicator for the chemical stability of a β-lactam antibiotic.

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Interaction of Doxorubicin with Phospholipid Monolayers

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Abstract □ The energies of interaction of doxorubicin hydrochloride and sodium 1,2,4-trihydroxy-9,10-dioxo-3-anthracenesulfonate with dipalmitoylphosphatidylethanolamine and dipalmitoyllecithin monolayers spread at the air–water interface were estimated from the increase in surface pressure with increasing concentrations of the subphase-injected compound. Their orders of magnitude were consistent with those of the energies of interaction of doxorubicin and acridines with double-stranded DNA, which suggests that the same type of van der Waals forces are operative.

Keyphrases □ Doxorubicin—energy of interaction with phospholipid monolayers at air–water interface □ Phospholipid monolayers—energy of interaction with doxorubicin and substituted anthracenesulfonate at air–water interface □ Monolayers, phospholipid—energy of interaction with doxorubicin and substituted anthracenesulfonate at air–water interface □ Antineoplastic agents—doxorubicin, energy of interaction with phospholipid monolayers at air–water interface

Doxorubicin is an anthracycline glycoside antibiotic formed by the tetracyclic quinoid aglycone doxorubicinone and the amino sugar daunosamine (1–3). Its cationic form shows antitumor activity (4). The anthracyclines are representative of a class of drugs whose pharmacological activity depends on their binding with nucleic acids and the subsequent inhibition of nucleic acid synthesis (5).

The energies of interaction of alkanols with dipalmitoyllecithin and dipalmitoylphosphatidylethanolamine monolayers spread at the air–water interface were recently correlated with their permeabilities across biomembranes and with the partition coefficients between (*a*) red cell membranes and water and (*b*) phospholipid liposomes and water (6). The present study examines the surface activity of doxorubicin and its energy of interaction with phospholipid monolayers spread at the air–water interface.

EXPERIMENTAL

Reagents—Doxorubicin (I) hydrochloride[1] and sodium 1,2,4-trihydroxy-9,10-dioxo-3-anthracenesulfonate[2] (II) were used without further purification. Dipalmitoyllecithin[3] (III), dipalmitoylphosphatidylethanolamine[4] (IV), the hexane[5] used for the preparation of the spreading solutions, and the distilled water used as subphase and for the preparation of the aqueous solutions fulfilled the requirements previously specified (7, 8).

Instruments and Methods—A 9-cm diameter polytef dish, provided with two identical microburets[6] and a polytef-coated stirring bar, was used as a trough. Surface tension was measured with a Wilhelmy platinum plate attached to an electrobalance[7] whose output was fed into a dual-pen recorder[8]. The methods for the measurement of the surface tension of aqueous solutions and of the change of the surface pressure of the phospholipid monolayer as a function of time after the injection of the drug in the subphase already were described (6–8). The criterion of equilibrium was the constancy, ± 0.1 dyne/cm, of the surface pressure increment, $\Delta\pi$, over 30 min. In all injection experiments, the initial surface pressure, π, of the phospholipid monolayer was 5 ± 0.1 dynes/cm and the temperature was 20 ± 1°.

RESULTS

The surface tensions of aqueous solutions (10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} *M*) I hydrochloride and II were equal to the surface tension of the pure distilled water used to prepare the solutions (72.80 dynes/cm) within the limits of experimental error (±0.1 dyne/cm). The pH values of the I hydrochloride solutions were 5.3, 5.5, 5.4, and 5.6, respectively, and those of the II solutions were 4.1, 4.3, 4.8, and 5.0, respectively.

Typical plots of the increment of the surface pressure, $\Delta\pi$ (dynes per centimeter), as a function of time, *t* (minutes), after the injection of I hydrochloride and II beneath III and IV monolayers are given in Fig. 1 for the same final concentration (4.28×10^{16} molecules/cm^3) of the drug injected in the subphase. The kinetics of the processes are similar, but the highest value of the equilibrium surface pressure was found for the injection of I beneath the IV monolayer (Table I). The energies of interaction were estimated from the slopes of the reciprocals of the equilibrium surface pressures, $\Delta\pi_{eq}$ (dynes per centimeter), after the injection against the reciprocals of increasing final concentrations, *n* (molecules per cubic centimeter), of the subphase-injected drug (7, 8). Such energies are given in Table I.

DISCUSSION

In accordance with the Gibbs adsorption equation, the fact that the surface tension of water is not affected by the presence of 10^{-4}–10^{-7} *M* concentrations of I hydrochloride or II indicates that the concentrations of these solutes at the interfacial region are identical with the concentrations of the bulk aqueous solutions in both cases; *i.e.*, no spontaneous adsorption of those molecules takes place at the air–aqueous solution interface between these concentrations.

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