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A Crystalline Form of Benzylpenicillinic Acid

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Numerous investigators have attempted to obtain without success one or more of the free acids of the various penicillins in well-defined crystalline form.[1] In the course of some recent investigations of the properties of benzylpenicillin we found conditions under which its free acid could be obtained in well-defined crystalline form. Investigation of this crystalline product has revealed that it contains one mole of diisopropyl ether of crystallization per mole of benzylpenicillin. The properties of this crystalline form of benzylpenicillinic acid are of interest chiefly in connection with the separation of benzylpenicillin from its homologs and as a standard of purity of demonstrable merit.

The results of ultimate analysis (see experimental section), potentiometric titration, optical rotation and microbiological assay all indicated that the crystalline product we had obtained contained only about 77% of the expected active mass. This suggested the presence of about 102 units of inactive mass for every 334 units (the molecular weight of free benzylpenicillinic acid) of active mass. The molecular weight of diisopropyl ether is 102 suggesting its presence in our crystalline product. This supposition was confirmed by an investigation of the infrared absorption spectrum of our product (in tetrachlorethane solution) which revealed the presence of an intense band at 9.05 μ. The aliphatic ethers in general are known to have strong absorption bands in the region of 9.0 to 9.5 μ characteristic of the C–O–C linkage and, in fact, for diisopropyl ether (in tetrachlorethane solution) we observed a strong band at 9.00 μ.

Evidence of the purity of our crystalline benzylpenicillinic acid was obtained by means of the N-ethylpiperidine salt assay.[2,3,4] Values of 99, 101 and 99% of theory (based on a molecular weight of 436) were obtained by this assay when the extraction step was eliminated from the assay procedure, it being unnecessary in view of the ready solubility of the crystalline acid in amyl acetate. When the sample was treated with water and extracted with amyl acetate as in the case of the sodium salt assay consistently low results (97%) were obtained. The elimination of the extraction step in this assay proved of value therefore in pointing out the reason for the low results obtained with the best samples of the crystalline sodium salt which were also found to give 97% recoveries. The low recoveries with the sodium salts were therefore ascribable to about 3% loss due to inactivation in the aqueous solution after acidification.[5] The crystalline benzylpenicillinic acid diisopropyletherate is, therefore, a substance of demonstrable purity and since we have found it to be very stable as a solid even when exposed to ordinary laboratory air for long periods we feel that it is superior to any other form of benzylpenicillin as a primary standard of purity.

The crystalline acid decomposes on melting, beginning to soften at about 80°, and at 87° the partial melt gases violently (probably due to the elimination of the diisopropyl ether since the 9 μ infrared band is absent after such treatment) leaving a porous glass. The microbiological activity of the crystalline acid after dissolving in a *p*H 7 phosphate buffer was found to be 1420 units/mg. against *Staphylococcus aureus* (cup assay). Corrected to sodium salt this corresponds to 1740 units/mg. This is within 4% of the accepted value for the pure crystalline sodium salt of benzylpenicillin which is 1667 units/mg.[1] The optical rotation in a 0.1 *M* *p*H 7 buffer is $[\alpha]^{23}D$ +241° ($C = 1.0$). By correcting for the molecular weight difference between the crystalline acid (436) and sodium benzylpenicillinate (356) the apparent optical rotation as the sodium salt is (436/356) × 241 = +296°, which is in satisfactory agreement with the generally accepted value of +290° for crystalline sodium benzylpenicillinate in *p*H 7 buffer.[1] In undertaking a potentiometric titration of the crystalline acid special conditions had to be resorted to due to its insolubility and instability in water. To circumvent these difficulties the weighed sample (about 60 mg.) was placed in the dry titration vessel, a few drops of acetone added to the solid to dissolve it followed immediately by the addition from a microburet of 90% of the theoretical amount of standardized 1 *N* lithium hydroxide and 3 cc. of water. After introduction of the calomel and hydrogen electrodes and equilibration with a hydrogen atmosphere the titration was completed to the neutralization point. In this manner an average equivalent weight of 442 was obtained (theory 446). By the subsequent addition of about 1.5 additional equivalents of alkali, a three hour hydrolysis period at 23° and back-titration with standardized hydrochloric acid to determine the remaining unbound alkali it was found that an additional amount of alkali was now bound (due to the hydrolysis of the lactam ring whereby penicillin changes to penicilloic acid[1]) which corre-

(1) Chemistry of Penicillin, National Academy of Science Monograph, Princeton University Press, forthcoming publication.

(2) J. C. Sheehan, W. J. Mader and D. J. Cram, THIS JOURNAL, **68**, 2407 (1946).

(3) Federal Register, Thursday, November 28, 1946. Title 21, Chapter I, Part 141, Section 141.3 (f).

(4) W. J. Mader and R. R. Buck, *Anal. Chem.*, **20**, 284 (1948).

(5) Private communication from Mr. R. N. Boos of the Merck Analytical Laboratory to whom we are indebted for the assay figures quoted here as well as for the C, H, N analyses.

sponded to 96% of one theoretical equivalent. The ultraviolet absorption spectrum of the crystalline acid when dissolved in *p*H 7 buffer closely resembles that of the crystalline sodium salt.[1] The observed $E_{1\%}^{1\,cm.}$ of the central benzyl band at 2580 Å. was 6.06 which on conversion to E_M using 436 as the molecular weight gives a value of 265 in satisfactory agreement with the accepted value for the crystalline sodium salt of 257.[1] Although the crystalline acid has but slight solubility in cold water it slowly (overnight) goes into solution and an examination of the ultraviolet absorption of this solution clearly shows the presence of the 2350 Å. band characteristic of benzylpenillic acid. This is the well-known isomerization of sodium benzylpenicillinate in acid solution, *p*H about 3, to benzylpenicillic acid.[1,6] Table I catalogs the wave length positions of the principal strong infrared absorption bands for the solid crystalline acid as well as for the acid in solution. These were determined with a Model 2A Perkin–Elmer Infra-red Spectrometer which had been carefully calibrated as to wave length.[7] As will be seen our wave length values for the invariant bands are in good agreement with those reported by other laboratories such as Shell and the University of Michigan[1] for the solid amorphous benzylpenicillinic acid. For the lactam carbonyl these workers reported 5.61 μ, for the carboxylic carbonyl 5.76 μ, for the amide carbonyl 6.06 and 6.68 μ, and for the phenacyl grouping 14.33 μ. The resolution of our instrument is better than 3 wave numbers in the 10 μ region.

Table II contains data representing the X-ray diffraction spectrum of the crystalline acid. The intensity ratios are based upon the magnitude of the most intense diffraction band observed, designated as line 9 in the table.

TABLE I

INFRARED ABSORPTION BANDS (μ)

In isopropanol	Solid in petrolatum	In tetrachlorethane
5.58 S	3.03 W[a]	5.60 S
5.75 S	5.60 S	5.75 S
5.96 S	5.82 S	5.94 S
6.52 S	5.93 S	6.65 S
	6.61 S	.....
	6.70 M	.....
	7.58 M	.....
	7.92 M	
	8.00 M	
	8.37 M	
	8.67 M	8.65 M
	8.85 M	8.85 M
	9.13 M ← ether band →	9.07 M
	9.25 M	
	9.70 M	
	9.85 M	
	9.98 M	
	10.25 M	
	10.52 M	
	10.64 M	
	10.92 M	
	11.08 M	
	11.33 M	
	11.54 M	
	11.79 M	
	12.54 S	
	13.02 S	
	13.80 S	
	14.22 M	
	14.41 M	

[a] S, M, W indicates strong, moderate and weak intensities, respectively.

TABLE II

X-RAY DIFFRACTION[a]

X-Ray Source CuKα

Penicillinic acid isopropyl etherate

Line	θ	d	Relative I/I_9, %
1	3.75	12.57	18.4
2	4.23	10.42	21.3
3	4.70	9.39	54.4
4	5.05	8.74	14.7
5	5.74	7.70	20.6
6	6.00	7.36	34.6
7	6.30	7.02	41.9
8	7.22	8.65	37.5
9	8.07 8.17	5.48 5.41	(100.0)
10	8.83	5.02	59.6
11	9.14	4.85	20.6
12	9.42	4.70	52.2
13	9.62	4.60	62.5
14	10.11	4.38	31.6
15	10.96	4.05	16.2
16	11.62	3.82	65.4
17	12.28	3.62	42.7
18	12.50	3.56	34.6
19	13.28	3.35	25.0
20	14.15	3.15	38.3
21	14.25	3.13	39.7
22	14.94	2.98	15.5
23	15.36	2.90	28.7
24	16.04	2.79	14.0
25	16.69	2.68	5.2
26	17.30	2.59	10.3
27	18.00	2.49	25.0
28	18.96	2.37	7.4
29	19.57	2.30	16.9
30	20.00	2.25	19.1

[a] We are indebted to Dr. J. B. Conn of the Merck Research Laboratories for these measurements.

A brief study has been made of the stability of the crystalline acid both as solid and in solution. As a solid our experience with the crystalline acid has failed to give any indication of instability over a period of several months exposed to ordinary laboratory conditions. The solid is entirely free of hygroscopicity. In non-aqueous solutions we have found that benzylpenicillinic acid diisopro-

(6) Committee on Medical Research, *Science*, **102**, 628 (1945).

(7) R. A. Oetjen, Chao-Lan Kao and H. M. Randall, *Rev. Sci. Instruments*, **13**, 515 (1942).

pyl etherate is rather unstable. We have followed the decomposition in isopropanol, tetrachloroethane, and chloroform solutions by means of the disappearance of the 5.60 μ (lactam) infrared band and have found that it is completely lost in about fifteen hours at 23°. Examination of the whole infrared absorption spectrum after such decomposition revealed the formation of significant quantities of benzylpenillic acid and led to the actual isolation of that acid in 27% yield. The remaining 73% of the decomposed benzylpenicillinic acid was obtained from the chloroform mother liquors as a gum from which no well-defined product has been obtained. The infrared absorption spectrum of this gummy material, however, shows very strong absorption bands at 6.0 and 6.6 μ suggesting thereby the presence of a product having a mono-substituted amide grouping in its structure. How the penicillin molecule could rearrange under these experimental conditions to give a product having an amide grouping is difficult to understand unless some process of molecular disproportionation takes place. This instability of the free acid of benzylpenicillin in non-aqueous media is not unique. We have found that N-ethylpiperidinium benzylpenicillinate, which is also very soluble in chloroform, is also quite unstable in such solutions.

It has been a matter of quite general experience that the various salts, esters and even some of the derivatives of benzylpenicillin show a much greater tendency to give well-defined crystalline products than the corresponding members of the other penicillin types and this has been the basis of several assay procedures for benzylpenicillin.[2,3,4] In accord with such experiences we have not found it possible to obtain crystalline free acids using diisopropyl ether with the other well-known penicillin types such as Δ^2-pentenylpenicillin, *p*-hydroxybenzylpenicillin and *n*-heptylpenicillin. This observation makes it possible, therefore, to utilize the crystalline benzylpenicillinic acid diisopropyl etherate as a means of separating benzylpenicillin from mixtures containing the other penicillin types.

Since diisopropyl ether might possibly form an oxonium salt with benzylpenicillin and in this manner give the crystalline form observed it was thought of interest to determine whether or not other ethers would also form crystalline products. In this connection we tried diethyl cellosolve, 2-methylfuran, dihydropyran, ethyl tertiary butyl ether, dioxane and a γ-pyrone (maltol). None of these could be induced to give crystalline products which inclines us to believe that the diisopropyl ether merely permits the formation of a well-defined crystalline lattice and so is present as a simple solvate of crystallization.

Experimental

Preparation of Crystalline Benzylpenicillinic Acid Diisopropyl Etherate.—A number of variations of the following method of preparation of the crystalline acid have been used quite successfully so that the details given below are not in any sense critical.

An aqueous solution containing 250 mg. of sodium benzylpenicillinate in 5 cc. of water was acidified to a *p*H of 2–2.5 using 0.5 *N* hydrochloric acid and was immediately extracted with 5 cc. of diisopropyl ether (peroxide free). After separation of the phases the diisopropyl ether phase was dried for a brief time over anhydrous sodium sulfate, filtered and the filtrate set aside at room temperature. In about twenty minutes crystals were observed to separate. After standing several hours the large needle-like crystals were isolated by filtration and washed on the filter with liberal amounts of diisopropyl ether in which the crystalline acid is quite insoluble. By saturation of the aqueous phase with sodium chloride and further extraction with diisopropyl ether additional yields of the crystalline acid were obtained. These were further increased by crystallization at 0–5°. By combining all the crystalline crops an over-all yield of at least 70% was obtained. Recrystallization of the crystalline acid was effected by dissolving 100 mg. of the acid in 2 cc. of diethyl ether followed by addition of 8 cc. of diisopropyl ether whence in excess of 90% of the initial weight was recovered after crystallization at 5°.

Anal. Calcd. for $C_{22}H_{32}N_2O_5S$: C, 60.6; H, 7.34; N, 6.43. Found: C, 60.9; H, 7.20; N, 6.28.

Isolation of Crystalline Benzylpenicillinic Acid Diisopropyl Etherate from Crude Sodium Penicillinate.—One gram of crude amorphous sodium penicillin, having a bioassay of 540 μ/mg., was dissolved in 100 cc. of water and then inactivated by the addition of 5.0 cc. of 1 *N* lithium hydroxide (*p*H about 12). After standing at room temperature for two hours the *p*H was readjusted to 7 with 5.0 cc. of 1 *N* hydrochloric acid. Bioassay showed this solution to be inactive. One gram of pure, crystalline sodium benzylpenicillinate was added to the inactivated solution so that its bioassay was 150,000 μ/cc. and therefore corresponded to a 1.7% solution of 830 μ/mg. of crude sodium benzylpenicillinate. Addition of 9 cc. of 1 *N* hydrochloric acid reduced the *p*H of this system to 2.5 and it was immediately extracted with two 100-cc. portions of diisopropyl ether. The aqueous layer was then saturated with sodium chloride and again extracted with one 100 cc. and one 50-cc. portion of diisopropyl ether. The four ether extracts were combined, filtered and stored in a refrigerator for about seventeen hours at 5°. After filtration and washing 0.715 g. of crystalline benzylpenicillinic acid diisopropyl etherate was obtained. This is 58% of the theoretical amount based on the sodium benzylpenicillinate used.

Separation of Crystalline Benzylpenicillinic Acid Diisopropyl Etherate from Penicillin K.—To a solution containing 70 mg. of sodium benzylpenicillinate and 30 mg. of ammonium *n*-heptylpenicillinate in 2 cc. of water was added 3.0 cc. of 0.1 *N* hydrochloric acid and the resulting acidic solution (*p*H 2–2.5) was then saturated with sodium chloride. After extracting with two 5-cc. portions of diisopropyl ether, the ether extracts were combined and after seeding and standing overnight at 5° a yield of 50 mg. of crystalline benzylpenicillinic acid diisopropyl etherate was obtained. This corresponds to 60% of the theoretical amount of benzylpenicillin (calcd. as free acid) used. An N-ethyl piperidine assay of this product showed it to be better than 90% pure benzylpenicillinic acid.

Separation of Crystalline Benzylpenicillinic Acid Diisopropyl Etherate from Penicillin F.—A 2.0-g. sample of a crude N-ethylpiperidinium benzylpenicillinate, which had been found to contain 18% of N-ethylpiperidinium Δ^2-pentenylpenicillinate (by degradative assay), was dissolved in 200 cc. of water, acidified to *p*H 2.5 with 4.5 cc. of 1 *N* hydrochloric acid, and extracted with 200 cc. of diisopropyl ether. After saturation of the aqueous layer with sodium chloride it was again extracted with one 200 cc. and one 100-cc. portion of diisopropyl ether. The combined ether extracts were filtered and after storage at 5° for twenty hours 0.99 g. of 98% (by N-ethylpiperidine assay) pure benzylpenicillinic acid diisopropyl etherate

was obtained. This is 62% of the N-ethyl piperidinium benzylpenicillinate known to be present in the original material.

Decomposition of Benzylpenicillinic Acid Diisopropyl Etherate in Chloroform.—One gram of the crystalline acid was dissolved in 100 cc. of chloroform and allowed to stand about forty-three hours at 23°, during which time an amorphous precipitate formed. After filtering and washing with chloroform 270 mg. of dry solid was thus obtained. After crystallization from ethanol (in which the solid was initially easily soluble until crystallization took place) an examination of its ultraviolet and infrared absorption spectra gave ample evidence of its identity as benzylpenillic acid, the spectra of our isolation product being everywhere superposable upon those given by an authentic sample of benzylpenillic acid.

Summary

1. A crystalline form of benzylpenicillinic acid has been described.

2. Possible uses as a means of isolating benzylpenicillin from the other known penicillin homologs and as a primary standard of purity for benzylpenicillin have been indicated.

RAHWAY, NEW JERSEY RECEIVED MARCH 9, 1948

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NEW YORK STATE COLLEGE FOR TEACHERS]

A Spectrophotometric Study of the Reaction of Ferric Iron and Citric Acid

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Although it is well known that citric acid and ferric iron form a soluble complex and this fact is sometimes used in analytical separations,[1] a search of the literature indicates no systematic study of this reaction has been made. Although Bobtelsky and Semichen[2] have concluded that the reaction of solutions of ferric chloride and trisodium citrate produces two complexes of the types $Fe_2(citr)_3^{-3}$ and $Fe_3(citr)_2^{+3}$, the validity of the conclusion may be questioned inasmuch as their solutions contained chloride which also forms a complex with ferric ion.[3,4]

More recently Bobtelsky and Jordan[5] have claimed that citric acid and ferric iron form a complex in which the ratio of citric acid to iron is 2:3. Their statement is based on "conductimetric, photometric and thermometric" titrations. The end-point of these titrations is located graphically and appears to involve considerable uncertainty. These authors give no quantitative data except in graph form. However, even if the method employed by these authors is validated, their conclusions in this particular case still are subject to question since they used ferric sulfate as a source of ferric iron for the titration in question. We have established, in work to be described in a later paper, that ferric iron and sulfate ion themselves form a complex. Moreover, the solution in question undoubtedly contained appreciable quantities of the primary hydrolysis product of ferric ion, namely, $FeOH^{++}$, since it has been shown[6,7] that even in solutions of high acidity considerable quantities of this ion are present. It would appear that the solutions employed by Bobtelsky and Jordan in this connection contained either mixtures of iron complexes or mixed iron complex or both. For these reasons one is inclined to question the validity of their conclusions.

Accordingly, the present investigation was undertaken, which established not only the composition of the complex but its dissociation constant as well.

By the method used it was shown that the reaction was

$$Fe^{+3} + H\ citr^{-2} \rightleftharpoons Fe(H\ citr)^{+} \qquad (1)$$

There was no evidence to indicate that there are any higher complexes formed between the reactants.

The ratio of iron to citrate in the complex was established by the method of continuous variations proposed by Job[8] and elaborated by Vosburgh and Cooper.[9]

Solutions of ferric nitrate and citric acid were mixed in various ratios so that the total moles of iron plus citric acid was constant throughout the series, as was also the volume of the solutions. The extinction coefficient of each solution was measured at various wave lengths between 325 and 485 mμ. The difference between the observed extinction coefficient and that calculated on the assumption of no reaction between iron and citric acid was plotted against that ratio M_{Fe} (M_{Fe} plus $M_{citric\ acid}$). The resulting curves show sharp maxima at $R = 0.5$ for all wave lengths employed. This indicates that the only complex formed in this solution is that in which the ratio of iron to citrate is unity.

The ionic state of the citrate which forms the complex was established by noting the effect of changes in concentration of hydrogen ion on the stability of the complex. From the above the indicated reaction is

$$Fe^{+3} + H_3citr \rightleftharpoons Fe(H_xcitr)^{+x} + (3 - x)H^{+} \qquad (2)$$

(1) H. A. Fales and F. Kenny, "Inorganic Quantitative Analysis," The Century Company, New York, N. Y., 1939, p. 345.

(2) M. M. Bobtelsky and A. E. Semichen, *Compt. rend.*, **208**, 646 (1939).

(3) H. E. Bent and C. L. French, THIS JOURNAL, **63**, 568 (1941).

(4) E. Rabinowitch and W. H. Stockmayer, *ibid.*, **64**, 335 (1942).

(5) M. Bobtelsky and J. Jordan, *ibid.*, **69**, 2286 (1947).

(6) A. B. Lamb and A. G. Jacques, THIS JOURNAL, **60**, 967, 1215 (1938).

(7) W. C. Bray and A. V. Hershey, *ibid.*, **56**, 1889 (1934).

(8) P. Job, *Ann. chim.*, [10] **9**, 113 (1928).

(9) W. C. Vosburgh and G. R. Cooper, THIS JOURNAL, **63**, 437 (1941).