[CONTRIBUTION FROM THE DEPARTMENT OF RESEARCH IN ORGANIC CHEMISTRY, MELLON INSTITUTE]

REINVESTIGATION OF ALLOXANTIN AND DIALURIC ACID

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That administration of sufficient aqueous alloxan solution to animals causes an experimental diabetes is well established (1), but the mechanism of its action is still obscure. It is important to know whether its two reduction products are also diabetogenic, but the reports in the literature are contradictory. Thus, some authors (2, 3, 4) state that alloxantin solution is diabetogenic; others claim that it is not (1, 5). Similarly, dialuric acid is (2, 4, 6, 7) and is not (1, 5, 8) diabetogenic. In order to find out whether these conflicting reports are ascribable to idiosyncrasies in the test animals or to use of chemically unsatisfactory injection solutions of the respective pyrimidines, we have reinvestigated the preparation and properties of alloxantin dihydrate and dialuric acid monohydrate.

According to Nightingale (9) and others (10–13), treatment of a fairly concentrated aqueous solution of alloxan with excess hydrogen sulfide affords a mixed precipitate of crystalline alloxantin dihydrate plus free sulfur. We find that, if the precipitate formed is exposed to atmospheric oxygen (during filtration, drying, and removal of sulfur by recrystallization from water), the organic crystals do indeed consist, partly or solely, of alloxantin dihydrate. This compound is, however, an artifact resulting from aerial oxidation during these processes, because, if the initial precipitate is freed from sulfur by washing with carbon disulfide (in an oxygen-free atmosphere) and is then dried in an oxygen-free atmosphere, the product is crystalline dialuric acid monohydrate.

Consequently, it is obvious that, in order to prepare alloxantin dihydrate directly, one molar proportion of hydrogen sulfide should be added to two molar proportions of alloxan in solution; a simple method of achieving this is described. Recrystallization should be conducted in a non-oxidizing, non-reducing atmosphere (*e.g.*, nitrogen); otherwise, the product may be contaminated with alloxan monohydrate or dialuric acid monohydrate, respectively.

This situation has, in some instances, led to incorrect descriptions of the physical properties of the two reduction products. Some of their physical properties are now recorded. The two products are readily distinguished by their different x-ray powder diagrams. The infrared spectra also provide a quick and satisfactory means for identifying samples; incidentally, these spectra throw some light on the structure of crystalline alloxan monohydrate and dialuric acid monohydrate.

In addition to the possibility that, in the experiments on diabetogenic potency, the ease of reaction with atmospheric oxygen (of the moist crystals during isolation for use) led to employment of initially impure samples of alloxantin dihydrate and dialuric acid monohydrate, it should also be borne in mind that, even were pure samples of these compounds used, aerial oxidation during preparation of the injection solutions could account for the conflicting results observed.

Apparently, only Brückmann and Wertheimer (2) took precautions to exclude oxygen from the injection solutions. It should be noted that a significant difference between the action of solutions of pure alloxan and of alloxan contaminated with alloxantin has been observed (14). Our results suggest that the conflicting results of the biological experiments are not caused by idiosyncrasies of the test animals.

EXPERIMENTAL

Preparation of dialuric acid monohydrate from alloxan monohydrate. The chosen volume of a freshly-prepared aqueous solution of alloxan monohydrate of any suitable concentration (*e.g.*, 5 or 10%) was placed in a separatory-funnel, and moist hydrogen sulfide was passed in until there was no further gain in weight. Carbon disulfide was now added, and the stream of hydrogen sulfide continued for about 5 minutes. As soon as all the free sulfur had dissolved, the funnel stopcock was gradually opened and the suspension filtered, under slight hydrogen-sulfide pressure, by means of a Büchner funnel with sintered-glass septum (and Büchner flask), both preflushed with hydrogen sulfide. As much liquid as possible was now removed under slight pressure of hydrogen sulfide, the colorless crystals were washed on the filter (hydrogen sulfide atmosphere) with carbon disulfide, and the funnel plus crystals (deliberately left reeking with carbon disulfide and hydrogen sulfide) dried in the vacuum-desiccator (Desiguard) over soda-lime and phosphorus pentoxide under a high vacuum (with trap cooled in Dry Ice-chloroform). Allowing for the product remaining in solution (*ca.* 12.6 g. per liter at 25°), the yield of dialuric acid monohydrate was quantitative. A further crop could be readily isolated by cooling the aqueous filtrate to 0° and filtering as above, or by evaporating to dryness under diminished pressure (nitrogen atmosphere).

Because of a tendency to decompose in boiling aqueous solution, recrystallization is best obviated by starting with recrystallized alloxan monohydrate and using clean glassware. If, however, purification is necessary, 42.5 g. may be recrystallized from 250 ml. of hot water exactly as for the recrystallization of alloxantin dihydrate, except that an atmosphere of hydrogen sulfide is used. Prolonged boiling should be avoided. [According to Biltz and Damm (15), the solubility of dialuric acid monohydrate is about 170 g. per liter of boiling water. However, we have prepared a solution of 288 g. per liter of boiling water saturated with hydrogen sulfide; this is too concentrated for convenient recrystallization.] Recrystallization may also be effected by preparing a saturated solution at room temperature and cooling to 1° in ice.

The behavior of the compound on heating at 2° per minute (Al block; initial temperature, 150°) was examined simultaneously with that of a sample of alloxantin dihydrate. The dialuric acid monohydrate appeared unchanged at 200°, turned faintly pink at 203 to 206°, and gradually became reddish-brown (229 to 232°) and then purplish-black at about 270°. Even at 300°, there was no true melting or observable gas evolution. [According to the literature, dialuric acid monohydrate turns red at 180° (15, 16) or 188–189° (17). It melts at 212° (17) or melts with decomposition at 214–215° (15, 16). A specimen whose elementary analysis corresponded to that of the anhydrous compound melted at 224° (18).]

Anal. Calc'd for $C_4H_6N_2O_5$: C, 29.64; H, 3.73; N, 17.28.
Found: C, 29.70; H, 3.65; N, 16.85.

It was practically insoluble in cold or hot chloroform, carbon tetrachloride, or benzene; very sparingly soluble in cold and but sparingly soluble in hot dry ether or ethyl acetate; sparingly soluble in cold but fairly soluble in hot pyridine; fairly soluble in cold but soluble in hot absolute ethanol, acetone, or glacial acetic acid; and readily soluble in cold absolute methanol. On boiling, the glacial acetic acid solution remained clear and colorless, but the pyridine solution became yellow colored and then very slowly changed to pink and, eventually, pale purple.

Under low-power magnification (36x), a binocular microscope revealed the crystals of dialuric acid monohydrate to be thin needles, definitely dissimilar morphologically from those of alloxantin dihydrate.[1] Under a polarizing microscope at a magnification of 300x, only one habit was seen, *viz.*, long, colorless needles. These showed, with plane-polarized light, extinction that was either parallel or inclined at an angle of less than 10°. The ends of the needles appeared rounded, although they might well consist of two faces meeting at a large obtuse angle. [Böeseken, *et al.* (19) described the crystals as "bundles of clear, yellow needles," but also stated that dialuric acid is colorless. Other authors (15, 16, 20–22) gave descriptions which seemingly agree with that of alloxantin dihydrate crystals.]

In order to check these points, larger crystals were grown. A saturated solution [*ca.* 21 g. per liter; *cf.*, Bailey, *et al.* (7)] was prepared under reflux at 46° (bath temp., 50°) in the presence of carbon disulfide and hydrogen sulfide. The bath (of large volume) was now allowed to cool very slowly to room temperature while a very slow stream of hydrogen sulfide was passed over the aqueous surface. The crystals were then filtered off, washed with carbon disulfide, and dried under a high vacuum; wt., 8.9 g. In bulk, these crystals exhibited an extremely pale yellow color; single crystals appeared colorless. To make sure that no chemical change had occurred, the infrared absorption spectrum was determined;[1] it agreed with that obtained with the microcrystals. On microscopic examination,[1] it was found that the habit varied somewhat from elongated prismatic to needle-like, the largest prismatic specimens being *ca.* 0.1 × 0.4 × 2.0 mm. in size, and the largest needle-like crystals being about the same length but only 0.1 to 0.2 mm. in the larger diameter. The crystals of both types were lath-like; one dimension of the cross-section was about 4 or 5 times the other. The larger size of these crystals permitted a closer examination of the ends. These were now seen to be limited by two clean-cut faces which made obtuse angles with each other and with the needle axis. With plane-polarized light and crossed Nicols, the crystals displayed extinction parallel to their long axes. Zero- and first-level precession photographs[1] taken about the needle axis both showed the diffraction symmetry C_2 (a two-fold axis); taken about an important zone axis ($u0w$) perpendicular to the needle axis, such photographs showed the respective symmetries C_{2l} and C_l (the former is a line of symmetry combined with a two-fold axis, and the latter is a line of symmetry). These observations indicate the crystal class to be *monoclinic* (C_{2h}, C_2, or C_s). Hence the needle axis is the monoclinic b axis. The observed extinctions include ($h0l$), present only with $(h + l) = 2n$, and probably ($0\ k\ 0$), present only with $k = 2n$. These denote the presence respectively of an n glide plane with component $(a/2 + c/2)$, and a [010] screw axis with component $b/2$. These symmetry elements uniquely characterize the space group C_{2h}^5, *i.e.*, $P2_1/n$. [However, since insufficient ($0\ k\ 0$) reflections were observed to establish positively the second class of extinctions, there is some possibility that the space group is C_{2h}^4, *i.e.*, $P2/n$.] The structure can be described in terms of a unit cell with the dimensions: $a_0 = 12.84$, b_0 3.68, and $c_0 = 13.08$ Å; the axial angle, β, is 95.5°, and the volume, V, = 615×10^{-24} cm^3. Assuming a density[2] of 1.812, the number of molecules per unit cell is found to be 4. [Beck (23) gives the density of alloxan monohydrate at −5° as 1.839.]

Direct preparation of alloxantin dihydrate (only) from alloxan monohydrate. Distilled water (1000 g.) was placed in a 1,500-ml. Erlenmeyer flask and saturated with moist hydrogen sulfide at room temperature. It was cooled in ice to 1.5° and again saturated with hydrogen sulfide (7 g.). Then a freshly-prepared solution of 65.7 g. of alloxan monohydrate in 329 ml. of distilled water was rapidly added, and the flask quickly stoppered and cooled in ice. Sulfur was immediately liberated. After keeping this mixture overnight in the refrigerator, colorless crystals had separated, the odor of hydrogen sulfide had disappeared, and the aqueous layer was no longer opalescent. Carbon disulfide (100 ml.) was added, and the

[1] Determination made in the Department of Research in Chemical Physics, Mellon Institute.

[2] Dr. G. A. Jeffrey, private communication.

mixture was swirled and then filtered with slight suction (under nitrogen atmosphere). The crystals were washed with carbon disulfide and dried in the vacuum-desiccator; wt. (dry), 62.5 g. The alloxantin in the aqueous mother liquor was recovered by evaporation to dryness under diminished pressure (nitrogen atmosphere), making the yield quantitative.

Since the solubility of alloxantin dihydrate has been reported as *ca.* 60 g. in 1,000 g. of boiling water (35) and 62.5 g. in 1,000 ml. of boiling water (25), the crystalline product was recrystallized as follows. A 4-mm. stopcock was sealed onto a 2-liter round-bottomed flask (neck uppermost) at a point halfway up the wall. Distilled water (400 ml.) was placed in the flask (stopcock horizontal and closed), deaerated by boiling under reflux, and then allowed to cool somewhat while moist nitrogen was passed in through the condenser. Nitrogen was now passed in through the side arm, the condenser was removed, 25 g. of dry, crude alloxantin dihydrate was added, the stopcock was closed and the condenser replaced, and (with nitrogen admitted through the condenser) the suspension was boiled under reflux until the crystals had just dissolved. The heat source was removed, the condenser was quickly replaced by a rubber stopper through which passed nitrogen inlet and outlet tubes, and the flask was tilted through 90° so that the stopcock was underneath. The stopcock stem was rapidly inserted in one hole of a two-holed rubber stopper (bearing an inlet tube, and attached to a Büchner funnel and flask as previously described), giving a modified Schmidlin apparatus (24). Funnel and flask were preflushed with nitrogen, and the hot solution was now filtered under nitrogen pressure applied at the neck of the 2-liter flask. The receiver was cooled in ice, while the nitrogen stream was continued, and then stoppered and kept overnight in the refrigerator. The colorless crystals were filtered off under nitrogen and dried in the vacuum-desiccator; yield (dry), 22.5 g. [*cf.* (25)].

The substance exhibited the following behavior on heating at 2° per minute (initial temperature, 150°): it turned pink at 165° and yellow at 204°; moisture evolution was evident at 215°; melting started at 220°, with development of a brown color; and gas evolution with complete melting occurred at 235°. [According to the literature, alloxantin dihydrate slowly loses its water of crystallization on heating at 110° (26) or 120° (9, 27–29). It is said to redden at 192° and melt with decomposition at 220° (30); melt with decomposition at 234–238° (9); melt at 235° (31); melt with reddening and decomposition at 243–245° (32); melt at 245° (33); turn yellow at 225° and decompose at 253–255° (34, 35).]

Anal. Calc'd for $C_8H_{10}N_4O_{10}$: C, 29.82; H, 3.13; N, 17.39.
Found: C, 29.62; H, 3.20; N, 17.39.

It was practically insoluble in cold or hot dry ether, chloroform, carbon tetrachloride, benzene, or ethyl acetate; very sparingly soluble in cold and sparingly soluble in hot acetone or absolute ethanol; sparingly soluble in cold but soluble in hot absolute methanol or glacial acetic acid; and fairly soluble in cold and soluble in hot pyridine. On boiling, the acetic acid solution became yellow colored. On keeping at room temperature for a few minutes, the cold pyridine solution became purple colored; development of this color was much accelerated by heating.

Under low-power magnification (36x), a binocular microscope revealed the crystals of alloxantin dihydrate as prismatic or tabular in form, mixed habits being observed.[1] The interfacial angles commonly noted were approximately right angles. Under a polarizing microscope at a magnification of 300x it was seen that only the larger crystals, mostly prismatic in habit, actually displayed interfacial angles of 95° to 100°. The majority of the crystals were much smaller and were thin plates in the form of oblique parallelograms. Under plane-polarized light, *all* the crystals examined showed oblique extinction, indicating the crystal symmetry to be monoclinic or triclinic. These observations agree well with those in the literature (28, 36; *cf.*, 12, 26, 29, 34, 37, 38, 39). Several twinned crystals were also noted; their appearance agreed with the earlier descriptions (28, 36).

The infrared absorption spectrum[1] of alloxantin dihydrate is shown in Figure 1, curve B. This shows excellent agreement with the curve published by Randall, *et al.* (40).

X-ray diffraction examination of alloxantin dihydrate and dialuric acid monohydrate crystals.[1] The samples were prepared for diffraction analysis by grinding for a few minutes in an agate mortar. The fine portion was then placed inside a thin-walled parlodion tube

TABLE I

X-RAY POWDER DIAGRAMS

ALLOXANTIN DIHYDRATE		DIALURIC ACID MONOHYDRATE	
d(Å.)	I	d(Å.)	I
9.4	vw	9.5	w
7.8	vw	8.6	vw
6.7	vw	7.9	vw
6.3	ms	7.0	vw
		6.4	m
5.6	ms	5.7	vw
5.2	ms	5.2	vw
4.9	vw		
4.6	vw		
4.4	vw		
4.1	vvw	4.7	w
3.8	vw	3.8	vw
		3.51	mw
		3.40	mw
3.31	mw	3.26	vw
3.21	vw	3.17	s
3.08	vw	3.10	vw
3.00	s	3.05	mw
2.90	mw	2.95	vvw
2.83	mw	2.78	vw
2.74	m	2.74	vw
2.66	vw	2.68	vw
2.58	vw	2.60	vw
2.48	ms	2.51	vvw
2.43	mw	2.47	w
2.26	w	2.41	vw
2.18	w	2.37	vw
2.14	w	2.15	w
2.09	w	2.06	vvw
2.03	w	2.03	vvw
1.87	vw	1.95	vvw
1.84	vw	1.86	vw
1.80	vw	1.77	vvw
1.78	vw	1.68	vvw
1.75	vw	1.66	vvw
1.65	vw	1.62	vvw
1.64	vvw	1.59	vw
1.61	vw		

d(Å.) = interplanar spacing in Ångstrom units; I = estimated relative intensity; m = medium; s = strong; v = very; w = weak.

(0.6 mm. inside diameter) and sealed off from the air with parlodion solution. The specimens prepared in this way were rotated in a Debye-Scherrer camera of 114.6-mm. diameter, and "powder" diagrams were prepared using CrK α radiation and exposures of 5 hours.

The diffraction patterns were both of good quality, and quite different (see Table I), indicating the two materials to be structurally distinct species. The interplanar (d) spacings observed in the two patterns range from moderate to small values, and no very long spacings are present. This is indicative of moderate unit-cell dimensions.

Infrared absorption spectra of alloxan monohydrate, alloxantin dihydrate, and dialuric acid monohydrate. The infrared absorption spectra were recorded[1] on a Baird spectrophotometer; the wave-length accuracy of this instrument is better than ±0.1 micron. All spectra (see Fig. 1) were obtained for suspensions of the solid samples in Nujol.

Alloxan monohydrate gave a curve (A, Fig. 1) which agreed excellently with that of Randall, *et al.* (41), except for the following minor points: their 6.5 μ

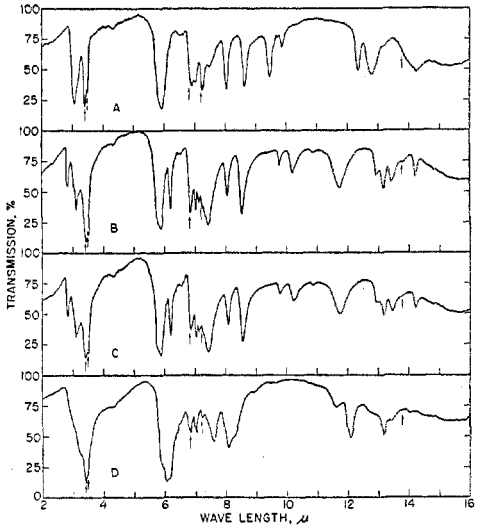
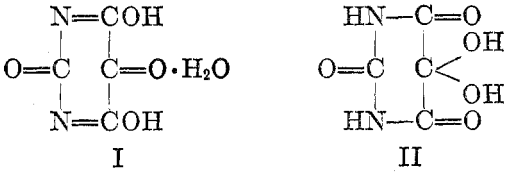


FIGURE 1. INFRARED ABSORPTION SPECTRA. A. Alloxan monohydrate; B. Alloxantin dihydrate; C. Commercial "Dialuric acid"; D. Dialuric acid monohydrate. (Arrows indicate Nujol bands)

band was sharp, where ours is diffuse; their 9.7 μ band was much more definite than ours. Two possible structural formulas for this compound are

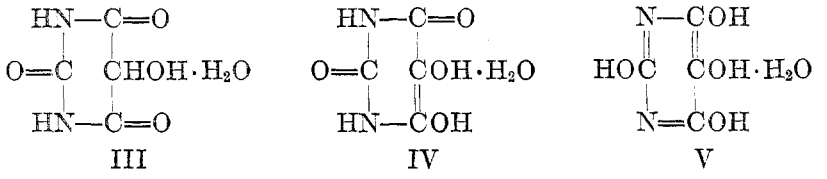


For the following reasons, it was concluded that II probably represents the structure of crystalline alloxan monohydrate. (a) Since the spectrum shows bands at 1730 cm^{-1} (5.78 μ) and 1690 cm^{-1} (5.92 μ), structure II (having the —CO—NH—CO— grouping) is favored. Structure I, with α,α′conjugated carbonyls absorbing at <1670 cm^{-1} (5.99 μ), and C=N absorptions expected below 1680 cm^{-1} (5.95 μ), cannot account for the 1730 cm^{-1} band. (b) Structure II has N—H groups, and the N—H deformation is expected at 1510–1580 cm^{-1}

(6.62–6.33 μ). The spectrum shows a band at 1535 cm^{-1} (6.51 μ), favoring structure II. [Both formulas have OH groups, and should exhibit the OH deformation. This probably accounts for the 1058 cm^{-1} (9.45 μ) band.]

Curve B (Figure 1) records the infrared absorption spectrum of *alloxantin dihydrate*. It is seen that it differs from those of alloxan monohydrate and dialuric acid monohydrate, but it does not provide sufficient evidence to permit deduction of the structure of the crystalline compound.

Three possible structures for *dialuric acid monohydrate* are



The observed infrared absorption spectrum (D, Fig. 1) indicates that V represents the structure of the crystalline compound. This conclusion is based on the following points. (a) No absorption maxima were observed in the 1785–1725 cm^{-1} (5.60–5.80 μ) region, whereas structures III and IV (containing the —CO—NH—CO— group) should give rise to bands in this region and at 1710–1670 cm^{-1} (5.85–5.99 μ). (b) Consequently, C=O is presumed to be absent; and so, only structure V will account for the multiplicity of bands in the 1700–1600 cm^{-1} (5.88–6.25 μ) region. (The complexity observed in this region is obviously the result of several overlapping bands, and the characteristic frequencies for C=O, C=N, and C=C occur in this region.)

The ultraviolet absorption spectrum of dialuric acid in acid aqueous solution is in agreement with this conclusion; the spectrum (42) has a single sharp band at 270 mμ. Structure V should give rise to a relatively sharp band at 265–275 mμ (characteristic of an "aromatic" diazine ring), whereas III and IV would be expected to display broad bands centering at about 280 mμ, characteristic of carbonyl groups.

The sample of dry, crystalline dialuric acid monohydrate was now deliberately exposed to the laboratory air for one week, and its infrared absorption spectrum again determined. Only minor changes in the spectrum were observed, and no evidence for the formation of alloxantin dihydrate could be found; this confirms Hill's observations (16).

The infrared absorption spectrum of a sample of commercial "dialuric acid" is recorded (C, Fig. 1); it proved to be identical in every detail with that of authentic, recrystallized alloxantin dihydrate.

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SUMMARY

1. Improved procedures for the preparation of dialuric acid monohydrate and alloxantin dihydrate, respectively, have been devised.

2. Some physical properties of these compounds are recorded. Structures for crystalline alloxan monohydrate and dialuric acid monohydrate have been deduced from their infrared absorption spectra.

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