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CYANOGENESIS IN *SORGHUM VULGARE*—I.
AN IMPROVED METHOD FOR THE ISOLATION OF DHURRIN; PHYSICAL PROPERTIES OF DHURRIN*

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Abstract—An improved method for the isolation of dhurrin is described. Steps not included in the original procedure of Dunstan and Henry are: (*a*) removal of sugars by yeast fermentation, (*b*) deionization with ion-exchange resins, and (*c*) cellulose column chromatography. A paper chromatographic study of *Sorghum* extracts showed that dhurrin is probably the only cyanogenetic glycoside present. Dhurrin has m.p. 163–165°; $[\alpha]_D^{28}$ −64° in H_2O, −65° in ethanol; and pK_a 8·93. The u.v. and i.r. spectra of dhurrin, and the physical constants of its pentaacetate are reported. A modification of the pyrazolone colorimetric determination of cyanide, suitable for the assay of cyanogenetic materials, is described.

INTRODUCTION

IN SEARCH of the poisonous substance in *Sorghum vulgare*, Pers., Dunstan and Henry in 1902 isolated dhurrin and obtained evidence which characterized the compound as *p*-hydroxymandelonitrile β-D-glucoside.[1] They reported that the preparation had no definite melting point and probably contained water of hydration. Subsequent to the work of Dunstan and Henry, there appears to have been only one attempt (unsuccessful) to isolate dhurrin in crystalline form.[2] The effects of environmental and genetic factors on the cyanogenetic glycoside content of *Sorghum* varieties (particularly Sudan grass) have been studied extensively,[3-5] but always by assay of the cyanide released from the tissue under autolyzing conditions. More recently, Conn and colleagues[6,7] and Gander[8,9] have studied the biosynthesis of dhurrin. These workers have separated the glucoside on paper chromatograms and identified it (in solution) by conversion to the expected degradation products. The possible occurrence of more than one cyanogenetic glycoside in *Sorghum* does not seem to have been investigated.

Since Dunstan and Henry provided little information about the chemical and physical properties of dhurrin, it seemed of importance, in view of the continued interest in the compound, to have crystalline material once more available. The present paper describes an

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1 W. R. DUNSTAN and T. A. HENRY, *Phil. Trans.* **A199**, 399 (1902).
2 J. J. WILLAMAN, *J. Biol. Chem.* **29**, 37 (1917).
3 F. T. BOYD, O. S. AAMODT, G. BOHSTEDT and E. TRUOG, *J. Am. Soc. Agron.* **30**, 569 (1938).
4 P. G. HOGG and H. L. AHLGREN, *J. Agric. Res.* **67**, 195 (1943).
5 I. T. CARLSON, *Agron. J.* **50**, 302 (1958).
6 T. AKAZAWA, P. MILJANICH and E. E. CONN, *Plant Physiol.* **35**, 535 (1960).
7 JANE KOUKOL, P. MILJANICH and E. E. CONN, *J. Biol. Chem.* **237**, 3223 (1962).
8 J. E. GANDER, *Plant Physiol.* **35**, 767 (1960).
9 J. E. GANDER, *J. Biol. Chem.* **237**, 3229 (1962).

improved procedure for the isolation of pure crystalline dhurrin, and the careful determination of several of its physical properties. A search was also made for congeners which might occur along with dhurrin in the *Sorghum* plant.

RESULTS AND DISCUSSION

Crude extracts of *Sorghum vulgare* (see Experimental) were examined by paper chromatography, which usually revealed five or more bands that absorbed in the u.v., and three or more that reduced silver nitrate. Only one band was both u.v.-absorbing (short-wave) and silver-reducing. Its R_f value was identical with that of isolated dhurrin, and it contained 90 per cent or more of the cyanogenetic material of the crude extracts which were applied to the paper. It thus almost certainly represents the dhurrin present in these extracts. In the absence of any other identifiable cyanide-yielding band it may be concluded that the extracts contained no major quantity of cyanogenetic glycoside differing markedly from dhurrin in R_f, such as a corresponding disaccharide derivative. But the presence of analogs more closely resembling dhurrin in structure cannot be ruled out.

The syrupy residues from the crude *Sorghum* extracts were extracted with ethyl acetate, and many attempts were made to crystallize dhurrin from the ethyl acetate solutions, as described by Dunstan and Henry. The solutions did indeed deposit crystals, but these invariably consisted of salts of inorganic ions, and further attempts to purify yielded only syrups. Analyses of the crude extracts showed that they were of a very low degree of purity, containing less than 10 per cent dhurrin. In addition to salts, they also contained much sugar, principally glucose. A yeast treatment to remove fermentable sugars and a resin deionization step were therefore introduced at this point to purify the extracts, and chromatography on cellulose powder was substituted for ethyl acetate extraction as a means of separating the dhurrin from the purified extracts. This modified procedure consistently gave crystalline dhurrin in acceptable yield.

The determination of dhurrin in chromatographic eluates, extracts, etc. was accomplished by treating the samples with sweet almond emulsin in Conway dishes[10] to release hydrogen cyanide, which was trapped in alkali. The cyanide was measured by a modification of the pyrazolone method.[11] A variation of this assay has also been used in this laboratory to determine cyanide (dhurrin) in Sudan-grass tissue. The range of the assay is 0·05–0·6 μg of HCN. It is thus a hundred-fold more sensitive than the picrate method.[12] which has been extensively used in studies on the cyanogenetic potential of Sudan grass.

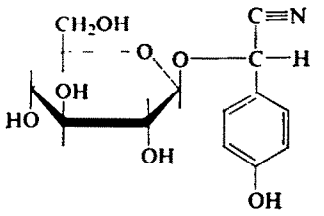
The structure assigned to dhurrin by Dunstan and Henry was confirmed by analysis of the crystalline glucoside. On hydrolysis, it gave glucose, HCN, and *p*-hydroxybenzaldehyde in a molar ratio of essentially 1:1:1. Elemental analysis gave values which fit the theory for anhydrous dhurrin, and in addition the substance maintained a constant weight on prolonged drying in vacuum at 60°. Our preparation thus contained no water of hydration.

In contrast to the observation of Dunstan and Henry, that dhurrin turns brown when heated above 100° and decomposes completely at 200°, our preparation had a fairly well defined melting point of 163–165°.

The complete stereochemistry of dhurrin appears to be established as a result of recent work by Towers, McInnes and Neish, on its diastereomer taxiphyllin (from species of *Taxus*

[10] M. FELDSTEIN and N. C. KLENDSHOJ, *Can. J. Med. Technol.* **17**, 29 (1955).
[11] J. EPSTEIN, *Analyt. Chem.* **19**, 272 (1947).
[12] P. G. HOGG and H. L. AHLGREN, *J. Am. Soc. Agron.* **34**, 199 (1942).

and *Phyllanthus*; formerly called phyllanthin).[13,*] The optical rotations of the acetates suggest that taxiphyllin is related to the long-known prunasin (D-mandelonitrile β-D-glucopyranoside),[14,15] and dhurrin to sambunigrin (L-mandelonitrile β-D-glucopyranoside),[14,15] but the argument is weakened by the seemingly anomalous rotation of free taxiphyllin which is claimed[13] to be essentially the same as that of free dhurrin. The NMR spectra,[13] however, convincingly show that dhurrin is *p*-hydroxy-L-mandelonitrile β-D-glucopyranoside (I)



(I)

[*p*-hydroxy-(*S*)-mandelonitrile β-D-glucopyranoside in the absolute system of Cahn, Ingold and Prelog]. Taxiphyllin is the corresponding derivative of *p*-hydroxy-D-mandelonitrile.

The u.v. absorption spectra of dhurrin are shown in Fig. 1. In acidic and neutral solutions, $\lambda_{max} = 230\ m\mu$, $\epsilon = 1{\cdot}07 \times 10^4$. In alkaline solution there is a bathochromic shift to $\lambda_{max} = 255$

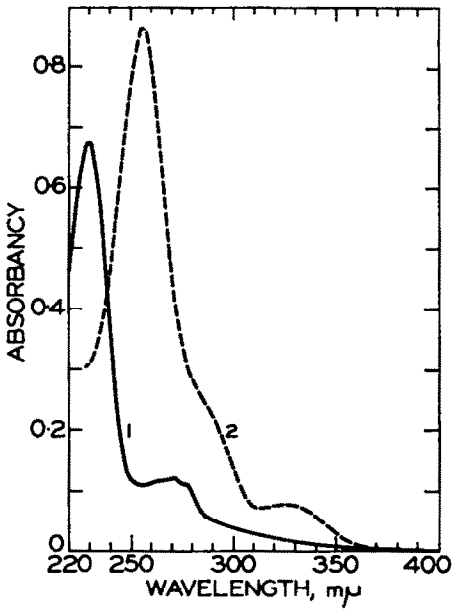


FIG. 1. ULTRAVIOLET ABSORPTION SPECTRA OF DHURRIN IN WATER SOLUTION
Curve 1: Undissociated dhurrin, in H_2O or 0·067 N HCl solution. Curve 2: Dhurrin anion, in 0·067 N NaOH solution.

* We thank Dr. Towers for making his data available to us before publication.

[13] G. H. N. TOWERS, A. G. MCINNES and A. C. NEISH, *Tetrahedron* **20**, 71 (1964).
[14] W. KARRER, *Konstitution und Vorkommen der Organischen Pflanzenstoffe*, p. 949, Birkhäuser-Verlag, Basel and Stuttgart (1958).
[15] F. K. BEILSTEIN, *Handbuch der Organischen Chemie*, Vol. XXXI (4th Ed.), pp. 238 and 239.

mμ, $\epsilon = 1{\cdot}51 \times 10^4$, characteristic of phenols with electron-attracting groups in the *para* position.[16] This effect, in dhurrin, must be attributed to the cyano and glucosyloxy groups attached to the benzyl carbon.

The fact that dhurrin and its anion have well-separated absorption maxima made possible the spectrophotometric determination of the dissociation constant of the phenolic function. On the basis of concordant results in three different buffer systems (see Experimental) pK_a at 25° was calculated to be $8{\cdot}93 \pm 0{\cdot}04$. Dhurrin is thus a somewhat weaker acid than *p*-hydroxybenzaldehyde, which has pK_a 7·66.[17] This is to be expected, since the electron-attracting groups of dhurrin are one carbon removed from the aromatic ring, whereas the dissociation of *p*-hydroxybenzaldehyde gives a resonant anion in which part of the charge is borne by the carbonyl oxygen.

In the course of our work on the isolation of dhurrin, we observed that it is decomposed when passed through a column of Dowex 1 (OH^-) resin. This behavior suggested that dhurrin might be an alkali-sensitive glucoside. Further experimentation, which has been confirmed in the literature,[6] showed this to be the case. The half life of dhurrin in solution at pH's above 11 is about 21 min at room temperature. We have studied the kinetics of this alkali decomposition in some detail. The results, together with a proposed mechanism for the reaction, will be published elsewhere.

EXPERIMENTAL

Reagents

Sweet almond emulsin was purchased from Nutritional Biochemicals Corporation. *p*-Hydroxybenzaldehyde, from the Matheson Company, was decolorized and recrystallized from hot water until the melting point was constant. 3-Methyl-1-phenyl-2-pyrazoline-5-one ("pyrazoline"), 3,3'-dimethyl-1,1'-diphenyl-4,4'-bi-2-pyrazoline-5,5'-dione ("bipyrazoline"), and chloramine-T were Eastman Organic Chemicals.

Preparation of Crude Extracts

The procedure was essentially the same as that of Dunstan and Henry,[1] except that freshly cut whole *Sorghum* plants were used instead of dry material. These were extracted with (usually) 10 volumes of boiling 95% ethanol as soon as possible to minimize enzymic decomposition of dhurrin. The ethanol was removed from the extract *in vacuo*, the residue was taken up in water (volume one-fifth that of the ethanol used), and insoluble matter was removed by filtration on a bed of Celite. The aqueous filtrate was treated with neutral lead acetate until no further precipitate formed, and this precipitate was filtered off by gravity. Excess lead was precipitated with hydrogen sulfide, the lead sulfide was filtered off by gravity, and the filtrate was concentrated to a syrup in a rotary vacuum evaporator at 60

Paper Chromatography

Most of the chromatographic work was done with extracts of Sudan grass (var. Sweet Sudan) which had been subjected to a lead acetate precipitation. A few samples were examined which had not been so treated, but these showed no noteworthy differences from lead-treated extracts. With *n*-butanol:water, 50:9 (v/v), on Whatman No. 1 paper the dhurrin-containing band was 0·1-0·15 R_f units wide; average R_f (center of band) was 0·50. Methyl

[16] L. DOUB and J. M. VANDENBELT, *J. Am. Chem. Soc.* **69**, 2714 (1947).
[17] *The Merck Index* (7th Ed.), p. 538, Merck and Co.

ethyl ketone–water and *n*-propanol–water gave less distinct separation. "Mineralights" (UV-Products, Inc., S. Pasadena, Calif.) were used to detect u.v. absorption. Ammoniacal silver nitrate was applied to pilot strips, and eluates from the remaining portions of the papers were analyzed for cyanogenetic glycoside as described below.

Analysis of Extracts

Conway cells of 6·6 cm diameter were used. In the center chamber was placed 2·0 ml of 0·1 N sodium hydroxide, and in the outer chamber 1·0 ml of sample (1–12 μg dhurrin), 1·0 ml of 0·1 N (sodium) acetate buffer of pH 5·2, 1·0 ml of 0·1% emulsin solution (mixed with the sample after covering), and 2 drops of toluene. Diffusion time was 48 hr.

Duplicate aliquots of the alkali–cyanide solutions containing 0·05–0·6 μg HCN (from 0·58–6·9 μg dhurrin) were pipetted into test tubes in an ice bath. The following additions were then made, and the contents of each tube mixed well: 0·1 N NaOH to bring the volume to 1·0 ml; 0·15 M NaH_2PO_4, 1·0 ml; and 0·14% chloramine-T, 0·5 ml. Two to five min after the addition of chloramine-T each tube received 1·0 ml of pyridine–pyrazolone reagent (85 mg of "pyrazoline":"bipyrazoline", 5:1 w/w, in 25 ml of pyridine A.R., freshly made up).[18] After 90 min at room temperature the absorbancies were read at 620 mμ.

For estimating the cyanide (dhurrin) content of Sudan grass tissue, a seedling or piece of leaf (5–20 mg) was quickly weighed, with care to avoid injury to the tissue, then placed in the outer chamber of a Conway cell and treated with several drops of chloroform. After 48 hr the alkali–cyanide solution from the center well was analyzed as described in the preceding paragraph.

Isolation

The largest batch of material worked up consisted of 14 kg of fresh *S. vulgare* var. Leoti* harvested when the plants were about 25 cm high. Successive extractions with 80 and 60 l. of ethanol, then concentration, yielded 2 l. of extract containing 42 g of dhurrin. The extract was diluted with 16 l. of warm water for the lead acetate precipitation; evaporation of the filtrate gave 850 ml of thick syrup.

This syrup was dissolved in 2·6 l. of water, 4 g of starch-free baker's yeast was added, the solution was incubated at 37° until no free glucose could be detected by glucose oxidase paper (Tes-tap, Eli Lilly Co.), and the yeast was removed by filtration. The filtrate was deionized on columns of Dowex 50 (H^+) (220 g) and Amberlite IR-45 (OH^-)† (200 g). Concentration of the effluents gave 77 ml of thick syrup containing 22·4 g of dhurrin. The recovery in the lead acetate step was about 85 per cent; in the fermentation step, 90 per cent; in the deionization, 70 per cent.

The deionized syrup was chromatographed in portions of 7 ml (2 g of dhurrin) on Whatman cellulose powder, which had been washed on a Büchner several times with warm water, then with *n*-butanol:water, 9:1 (v/v), until the wash liquid was free of u.v.-absorbing impurities. The column (packed as a slurry) was 9·8 × 47 cm; elution was with *n*-butanol:water, 9:1, at 1 ml per min. The cellulose had to be rewashed and repacked after each run. The dhurrin band, eluate volume (approx.) 2·1 l. (beginning) to 3·2 l. (end) contained a yellow pigment which served as an indicator for it. The pooled fractions were reduced to a dry foam in a rotary vacuum evaporator at 60°, and just enough warm, dry *n*-butanol was added drop by

* A "high-cyanide" variety of sorghum.

† The use of a *weakly basic* anion-exchange resin is essential.

[18] K. JORGENSEN, *Acta Chem. Scand.* 9, 548 (1955).

drop to each residue to collapse the foam. The solutions thus obtained gave yellowish crystals on standing, and deposited additional crystals after a period in the refrigerator. In a typical run, the harvest of crude dhurrin, m.p. 154–160° (dec.), was about 1 g, or 50 per cent of the amount present in the deionized syrup charged to the column.

For purification the crude dhurrin was dissolved in water, the solution was decolorized with active carbon, and the filtrate was evaporated in vacuum to a syrup. White rectangular crystals (0·86 g, m.p. 161–163°, dec.) were obtained by dissolving the syrup in warm dry *n*-butanol and placing the solution in the refrigerator. Another recrystallization from the same solvent gave 0·84 g of pure dhurrin, m.p. 163–165° (dec.), $[\alpha]_D^{28}$ −64° (*c* = 2·4, water), and −65° (*c* = 2·3, ethanol). The analytical data are given in the accompanying table.

TABLE 1. COMPOSITION OF DHURRIN ISOLATED BY CHROMATOGRAPHY

(Found:* C, 54·35; H, 5·81; N, 4·82. Calc. for $C_{14}H_{17}O_7N$ (mol. wt. 311·28): C, 54·02; H, 5·51; N, 4·50%.)

Product	Method of analysis	Theoretical (%)	Found (%)	Found (mole per 311·28 g)
Glucose	Anthrone[19]	57·9	56·9	0·98
HCN	Pyrazolone	8·7	8·5	0·98
p-Hydroxybenzaldehyde	Spectrophotometric†	39·2	36·7	0·94

* Micro-Tech. Laboratories, Skokie, Ill.

† Absorption at 330 mμ after treatment with 0·1 N NaOH, 25°, 3 hr.

The i.r. spectrum of dhurrin (KBr pellet) has bands at 3100–3450 (strong) (OH), 1600 and 1620 (medium) (aromatic ring), and many strong and medium bands in the range 1000–1400 cm^{-1} corresponding to O–H deformation and C–O stretching. The C≡N stretching band at 2250 cm^{-1} is very weak, because of the many oxygenated groups in the molecule.[20] There is much more detail in the region 800–900 cm^{-1} than in the spectra of the methyl glucopyranosides, making it difficult to distinguish the band or bands which would indicate the configuration at the anomeric center.

Dissociation Constant

One ml of $1·8 \times 10^{-5}$ M dhurrin was mixed with 2 ml of buffer in a cuvette (1 cm path length) and the absorbancy was read within 30 sec to minimize error from the decomposition of dhurrin, which is rapid at the higher pH values. A Beckman model DU spectrophotometer was used. Because of absorption by the buffer, readings could not be taken at 230 mμ (undissociated dhurrin) in bicarbonate–carbonate; only the dhurrin anion (255 mμ) was measured in this system. The dissociation curves (Fig. 2) were visually fitted to the plotted points, each of which represents the average of three measurements. Both curves are essentially superimposable on theoretical curves calculated by the Henderson–Hasselbalch equation, and both correspond to p*K* 8·93 ± 0·04.

[19] D. L. MORRIS, *Science* **107**, 254 (1948).

[20] L. J. BELLAMY, *The Infra-red Spectra of Complex Molecules* (2nd Ed.), p. 265. Methuen and Co. (1958).

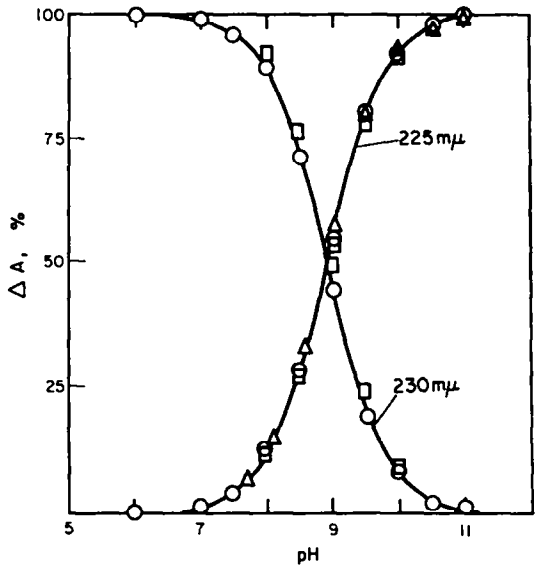


FIG. 2. DISSOCIATION CURVES OF DHURRIN.

230 mμ curve, dhurrin free acid vs. pH; 255 mμ curve, dhurrin anion vs. pH. One hundred per cent ΔA is the difference between the absorbancy where there is no dissociation (pH 1·3) and that at complete dissociation (pH 12·7). Buffers (the number given with each buffer system is the table number by which it is listed in reference 21): ○, sodium phosphate–borate (5 universal, universal buffer with citrate and diethylbarbiturate omitted); □, sodium borate with added KCl (5*Z*); △, sodium bicarbonate–carbonate (5*M*, I = 0·1).

Dhurrin Pentaacetate

According to Towers[13] dhurrin pentaacetate has m.p. 132–132·5° and $[\alpha]_D^{20}$ −50° in ethanol. A sample was prepared by treatment of dhurrin with pyridine–acetic anhydride at room temperature for 2 days, after which the reagent was evaporated off *in vacuo*. The oily residue was crystallized from ethanol–water. We found m.p. 132·5–133°, $[\alpha]_D^{25}$ −46° (c = 0·56, ethanol). (Found: C, 55·68; H, 5·42. Calc. for $C_{24}H_{27}O_{12}N$ (mol. wt. 521·46): C, 55·28; H, 5·22%.) The i.r. spectrum (KBr pellet) shows the expected acetyl bands at 1760 cm^{-1} (strong) (C=O) and 1430 (medium) and 1370 (medium) (C—H bending). The C≡N band is scarcely detectable. The bands for aromatic ring are shifted to slightly lower wave numbers (1610 and 1520) as compared to dhurrin; and the spectrum of the acetate has somewhat less detail in the region 1000–1400 cm^{-1}.

Acknowledgements—The authors are greatly indebted to Dr. Paul Drolsom of the Department of Agronomy for growing the *Sorghum* used in the isolation of dhurrin.

[21] C. LONG, *Biochemists' Handbook*, pp. 36, 40 and 41, D. Van Nostrand Co. (1961).