[CONTRIBUTION FROM CHEMISTRY DEPARTMENT, IOWA STATE COLLEGE]

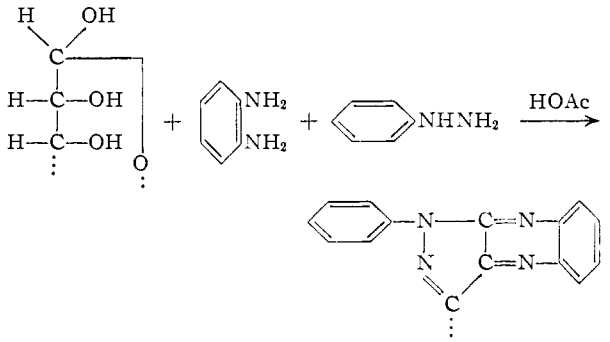
1-Phenyl-flavazole Derivatives of Starch Dextrins[1]

BY PHILIP NORDIN AND DEXTER FRENCH

RECEIVED OCTOBER 18, 1957

The singly branched dextrins containing from four to seven glucose units, obtained by the extended action of salivary amylase on waxy maize starch, were isolated and their 1-phenyl-flavazole derivatives prepared. The individual dextrin flavazoles all reacted with amyloglucosidase to yield a singly branched flavazole derivative containing four glucose units. A structure for this derivative based on partial acid hydrolysis experiments is proposed. The advantage of this method of structure determination is the ease of purification of derivatives.

1-Phenyl-flavazole derivatives of reducing sugars were first described by Ohle.[2,3] The flavazole reaction is a condensation process whereby a reducing sugar condenses with *o*-phenylenediamine and phenylhydrazine



The resulting yellowish-orange derivative is called a flavazole. The phenylhydrazine in addition to becoming part of the flavazole molecule, serves as the condensing agent, being reduced to aniline and ammonia. Generally five moles of phenylhydrazine are used per mole of reducing sugar.

Flavazoles have been used to determine the type of linkage in disaccharides. If positions 4 and 5 of the reducing sugar residue are unsubstituted, periodate oxidation of the disaccharide flavazole will yield 1-phenyl-flavazole aldehyde.[2,4,5]

In the present paper, flavazole derivatives of dextrins obtained from the action of salivary amylase on waxy maize starch are described. They have been used to tag or identify the reducing end of the dextrin which was then partially degraded by an amyloglucosidase preparation to yield fragments of lower D.P. number. A particular fragment containing four glucose units was isolated from the digest and partially hydrolyzed with acid. Identification of the degradation products by their characteristic R_f values allowed a tentative formulation of the fragment.

The techniques described under the Experimental section offer a number of advantages as a means of characterizing the starch dextrins: (a) The flavazoles can be chromatographed in the same solvent as is used for sugars. Thus chromatography can be used to purify a flavazole preparation as well as to identify sugar and flavazole fragments after a degradation treatment. The introduction of a flavazole unit increases greatly the mobility of the sugars. (b) The flavazoles are colored and they can be detected on chromatograms without the use of developing strips. They fluoresce strongly under ultraviolet light, revealing even trace amounts.

Experimental

Enzymes.—The salivary amylase used was freshly collected and filtered.

A commercial enzyme preparation "dextrinase" of microbial origin was used. It was high in amyloglucosidase activity as shown by its rapid hydrolysis of the linear dextrins maltotriose, maltotetraose and maltoheptaose to glucose and maltose. Maltose was in turn hydrolyzed to glucose but at a slower rate.

Although this enzyme preparation had some transglucosidase activity (*i.e.*, traces of isomaltose were produced by its action on maltose) it could not be observed with linear dextrin flavazoles as substrates. Also since observations with this enzyme were confined to the initial reaction, transglucosidase activity as a source of error can be ruled out.

Branched Dextrins.—Branched dextrins were obtained by the extended action of salivary amylase on waxy maize starch.[6] Chromatographic analysis showed that the linear dextrins had been converted to glucose and maltose. Singly branched dextrins contained four, five, six and seven glucose units,[7] and for convenience are referred to as B_4, B_5, B_6 and B_7. The flavazole derivatives of these compounds are referred to as B_4^f, B_6^f, etc. Doubly branched dextrins contained nine, ten, eleven and twelve glucose units[7] and are referred to as BB_9, BB_{10}, BB_{11} and BB_{12}. Enriched fractions of the singly branched dextrins were obtained by first treating with bakers' yeast to ferment the glucose and most of the maltose. The residue was adsorbed on a carbon column and fractionally eluted with ethyl alcohol.[8] The singly branched dextrins were thus obtained free from glucose, maltose and multiply branched dextrins and were partially resolved into the individual components.

Panose-coupled Products.—The panose-coupling reaction[9] was used to prepare a mixture of oligosaccharides which contained the panose configuration at the reducing ends.

Maltotriose and Maltotetraose.—Maltotriose and maltotetraose were obtained by carbon chromatography[10] of a salivary amylase–amylodextrin digest. The conversion was carried to the achromic point.[11]

Flavazole Derivatives.—The flavazole reaction is generally carried out in glacial acetic acid out of contact with the

(1) Journal Paper No. J-3283 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Proj. No. 1116. Supported by a grant from the Corn Industries Research Foundation. Presented in part before the Division of Biological Chemistry of the American Chemical Society, Sept., 1953.

(2) H. Ohle and G. Melkonian, *Ber.*, **74**, 279 (1941).

(3) H. Ohle and M. Hielscher, *ibid.*, **74**, 13 (1941); H. Ohle and G. Melkonian, *ibid.*, **74**, 398 (1941); H. Ohle and R. Liebig, *ibid.*, **75**, 1536 (1942); H. Ohle and J. Kruyff, *ibid.*, **77**, 507 (1944).

(4) G. Neumüller, *Arkiv Kemi, Mineral. Geol.*, **21A**, No. 19, 1 (1945).

(5) D. French, G. M. Wild and W. J. James, THIS JOURNAL, **75**, 3664 (1953).

(6) G. M. Wild, Ph.D. thesis, Ames, Iowa, Iowa State College Library, 1953.

(7) D. French and G. M. Wild, THIS JOURNAL, **75**, 2612 (1953).

(8) R. L. Whistler and D. F. Durso, *ibid.*, **72**, 677 (1950).

(9) D. French, M. L. Levine, E. Norberg, P. Nordin, J. H. Pazur and G. M. Wild, *ibid.*, **76**, 2387 (1954).

(10) W. J. Whelan, J. M. Bailey and (in part) P. J. P. Roberts, *J. Chem. Soc.*, 1298 (1953).

(11) J. H. Pazur, D. French and D. W. Knapp, *Proc. Iowa Acad. Sci.*, **57**, 203 (1950).

air, one mole of *o*-phenylenediamine and five moles of phenylhydrazine being required per mole of reducing sugar.[4] Since the amount of carbohydrate available was frequently on the order of a few milligrams, an excess of reagents was used. The reaction mixture was heated in a boiling water-bath in a sealed tube for six hours.

The flavazoles of the mono- and disaccharides are insoluble in water and crystallize out as the reaction proceeds. The derivatives of the higher dextrins, however, were soluble in the reaction mixture and had to be purified by paper chromatography.

Paper Chromatography.—Eaton and Dikeman No. 613 filter paper was used. The solvent systems were water-saturated butanone (solvent 1) and a solution consisting of three parts water, four parts pyridine and six parts 1-butanol by volume (solvent 2). Reducing sugars were located by the alkaline copper spray reagent followed by a phosphomolybdic acid solution.[12] The flavazoles were located by an ultraviolet lamp (long wave length). For purification the crude mixture was applied in a streak near the bottom of the paper. After chromatography the appropriate bands were cut out and extracted with methanol-water and concentrated to dryness.

Reaction with Dextrinase.—Reactions of panose-coupled products, starch dextrins and flavazole derivatives with dextrinase were carried out on a spot plate. Samples were withdrawn and applied directly to filter paper. In order to ensure that the proper time range was covered, the experiments were repeated several times varying the amount of dextrinase and the time.

Partial Acid Hydrolysis.—All partial acid hydrolysis experiments with flavazole derivatives were to a degree α = 0.25 to 0.30, where $\alpha = Nkt$ is the degree of hydrolysis of the α-1,4-bonds, N is the molar concentration of acid, k is the reaction rate constant (0.067 at 100°)[13] and t is the time in minutes. The degree of hydrolysis for an α-1,6-bond is $\alpha/4$.[14] The α-1,4-bond adjacent to the flavazole unit is also apparently more resistant than other α-1,4-bonds. This has been observed for maltose flavazole, maltotriose flavazole as well as for the dextrin flavazoles. The products were identified by their R_f values and a comparison of the concentration of the products was made when it was obvious from the size of the spots that significant differences existed.

The flavazoles were heated at 100° in 0.1 N H_2SO_4 for the time required to obtain a degree of hydrolysis of 0.25 to 0.30. The mixture was then neutralized with $Ba(OH)_2$, filtered, concentrated and examined by chromatography.

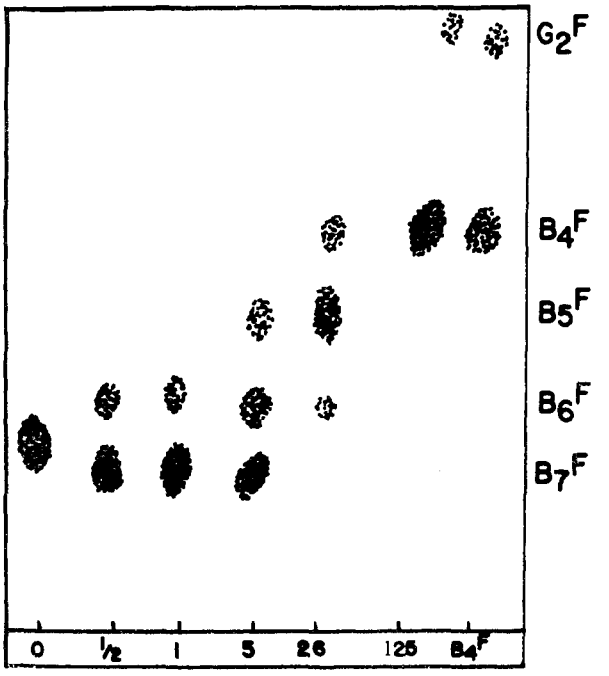


Fig. 1.—Reaction of B_7^F with dextrinase; reaction time given in minutes; B_4^F, standard, after treatment with dextrinase.

Results

Chromatography of flavazole preparations was tried in two solvents, the R_f's of which are recorded in Table I. In the chromatography of the crude dextrin flavazoles a heavy dark band moved with the solvent front. This band contained the excess *o*-phenylenediamine and phenylhydrazine. Dextrin flavazoles were prepared from quite pure B_4 and B_5; B_6 and B_7 were mixtures.

TABLE I

R_f VALUES OF 1-PHENYL-FLAVAZOLE DERIVATIVES

1-Phenylflavazole deriv.	R_f Solvent 1	R_f Solvent 2
G_1^f	0.90	0.97
G_2^f	.60	.90
G_3^f	.45	.80
G_4^f	.25	.70
B_3^f	.40	.75
B_4^f	.20	.65
B_5^f	..	.55
B_5^f	..	.40
B_7^f	..	.30

The branched derivatives B_5^f to B_7^f were all attacked by dextrinase to yield a B_4^f as a comparatively stable product. This is illustrated in Fig. 1 with B_7^f. Similar results were obtained with the other derivatives. The degradation went rapidly by stages to the same stable B_4^f by hydrolysis of glucose units from the non-reducing end of the chain. B_4^f was attacked very slowly by the enzyme mixture to yield maltose flavazole. Partial acid hydrolysis yielded isomaltose and maltose flavazole as the two main products. Small amounts of glucose and traces of flavazoles conforming in R_f to maltotriose flavazole and glucose flavazole were also observed.

The B_4^f obtained from the salivary amylase limit dextrins behaved differently in its reaction to dextrinase in that initially a small amount of panose flavazole was produced. The main portion, however, was resistant. After treatment some of the starting material was reisolated and a portion treated with fresh dextrinase and the remainder subjected to partial acid hydrolysis (see above). The behavior in both instances was identical to that of the B_4^f obtained by dextrinase from the higher dextrins.

Dextrinase reacted with the panose-coupled products and with the salivary amylase dextrins in the same manner as when these dextrins were derivatized as the flavazole. With the panose-coupled products, panose was a comparatively stable end product and with salivary amylase dextrins two stable products were found, one conforming in R_f to a B_4 and the other to a BB_7. The B_4 was isolated and converted to the flavazole. It was found to be identical in R_f and in its reaction with acid to the B_4^f produced by the action of dextrinase on the dextrin flavazoles.

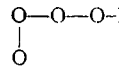
(12) D. French, D. W. Knapp and J. H. Pazur, THIS JOURNAL, **72**, 5150 (1950).

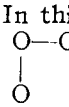
(13) W. Kuhn, *Ber.*, **63**, 1503 (1930).

(14) M. L. Wolfrom, E. N. Lassettre and A. N. O'Neill, THIS JOURNAL, **73**, 595 (1951).

Discussion

The results from the acid hydrolysis experiments were consistent only with this structure for B_4[15]



taking into account the resistance of the α-1,6-bond and the α-1,4-bond adjacent to the flavazole unit. Only this structure could yield isomaltose and maltose flavazole as the main products.[16] It has been proposed that amyloglucosidase operates by removal of glucose units from the non-reducing end of a starch chain.[16] The stable B_4 presumably represents the reducing end of the dextrins. Dextrinase removed glucose units readily until it was partially obstructed by the α-1,6-link. Thus, except for the minor component of B_4, the salivary amylase dextrins at advanced stages of hydrolysis have a uniform arrangement at the reducing end of the chain. In the case of the panose-coupled products the reaction is analogous. In this case, however, the panose configuration,  , is found at the reducing end.

It should be noted that with this enzyme preparation the α-1,6-link was by-passed, although slowly. It would be interesting to determine whether a pure amyloglucosidase preparation, if one were available, would be completely arrested at the α-1,6-link. The action patterns of the amyloglucosidase from *A. niger*,[16] *Rhizopus delemar*[17] and *Clostridium acetobutylicum*[18] have been compared with that of β-amylase. β-Amylase, however, for all practical purposes is arrested before the α-1,6-link is reached.[19] A pure amyloglucosidase preparation, therefore, could be of considerable value in structure determination.

(15) O– signifies a glucose unit with its reducing group; —O–O— signifies 2 glucose units bonded with an α-1,4-linkage;  signifies 2 glucose units bonded with an α-1,6-linkage. O–F signifies a glucose unit which has been converted into its 1-phenyl-flavazole derivative.

(16) R. W. Kerr, F. C. Cleveland and W. J. Katzbeck, THIS JOURNAL, **73**, 3916 (1951).

(17) L. L. Philips and M. L. Caldwell, *ibid.*, **73**, 3559, 3563 (1951).

(18) D. French and D. W. Knapp, *J. Biol. Chem.*, **187**, 463 (1950).

(19) R. Summer and D. French, *ibid.*, **222**, No. 1, 469 (1956).

AMES, IOWA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING, STANFORD UNIVERSITY]

The Deamination of 2-Amino-3-phenylbutane-1-C^{14} with Nitrous Acid[1]

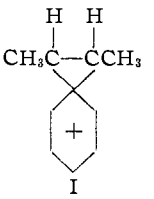
BY WILLIAM A. BONNER AND DENNIS D. TANNER

RECEIVED SEPTEMBER 9, 1957

2-Amino-3-phenylbutane-1-C^{14} (IV) has been synthesized by the reaction sequence: 3-phenyl-2-butanol-1-C^{14} → 3-phenyl-2-butanone-1-C^{14} → oxime → IV. The radiochemical structure of IV was established by degradation of the 3-phenyl-2-butanol-1-C^{14} to iodoform of identical radioactivity assay, and by the known lack of rearrangements occurring during conversion of a carbinol such as 3-phenyl-2-butanol to an amine such as IV. 2-Amino-3-phenylbutane-1-C^{14} (IV) was deaminated by the action of nitrous acid at 0° in aqueous solvent. A reaction product consisting primarily of a mixture of carbinols was obtained. By the use of vapor–liquid partition chromatography, alumina column chromatography and infrared spectrophotometry, this mixture was shown to consist principally of a phenyl migration product, 3-phenyl-2-butanol (V), a hydrogen migration product, 2-phenyl-2-butanol (VI), and a methyl migration product, 1-phenyl-2-methyl-1-propanol (VII). Small amounts of olefinic and ketonic products were also noted, but were not characterized. The crude deamination reaction mixture was also degraded with sodium hypoiodite. Of the various carbinol products identified, only the phenyl migration product, 3-phenyl-2-butanol-1,4-C^{14} (V), could yield iodoform under these conditions. The iodoform obtained in this degradation showed a radioactivity assay exactly half as high as that noted for the 3-phenyl-2-aminobutane-1-C^{14} precursor (IV). This observation accords with the theoretical concept of a symmetrical phenonium ion intervening as a reaction intermediate in the formation of the phenyl migration product V.

Introduction

The concept of bridged, non-classical carbonium ion reaction intermediates, originally introduced[2] in 1941 by Lane and Wallis to account for the stereochemical results of the Wolff rearrangement, has been extended and amplified in recent years, particularly by the researches of Cram[3] and Roberts.[4] Perhaps the most compelling evidence for the "symmetrical phenonium ion" intermediate (I) hypothesis is to be found in the earlier investigations of Cram, wherein the stereochemical consequences of various solvolytic reactions in the 3-phenyl-2-butyl tosylate system, and closely related systems, have been described.[3] The data of these investigations were logically and economically rationalized by the postulation of bridged non-classical structures, such as I, intervening as the cation of ionic pairs[3] as intermediates in such solvolytic processes.



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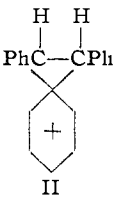

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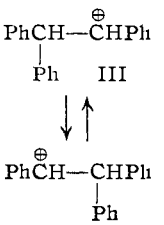

In order to test or extend these hypotheses, Collins and Bonner have more recently investigated similar solvolytic reactions in the 1,2,2-triphenylethyl system, using carbon skeletons

(1) We are indebted to the National Science Foundation for its generous support of a portion of this research.

(2) J. F. Lane and E. S. Wallis, THIS JOURNAL, **63**, 1674 (1941).

(3) D. J. Cram and co-workers, *ibid.*, **71**, 3863, 3875 (1949); **74**, 2129, 2137, 2159, 5839 (1952); **75**, 339, 3189 (1953).

(4) J. D. Roberts and co-workers, *ibid.*, **74**, 5943 (1952); **75**, 2069, 5759 (1953); **77**, 5558 (1955).