[CONTRIBUTION FROM THE IOWA AGRICULTURAL EXPERIMENT STATION]

Studies on the Schardinger Dextrins. VII. Co-substrate Specificity in Coupling Reactions of *Macerans* Amylase[1,2]

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RECEIVED NOVEMBER 2, 1953

In coupling reactions of *macerans* amylase a cycloamylose molecule is simultaneously opened and coupled to a co-substrate molecule, in such a way that there is formed a linear amylose fragment terminated at the reducing end by a group characteristic of the co-substrate used. Subsequent redistribution reactions lead to the formation of an homologous series of compounds. The series includes the co-substrate and higher members contain additional D-glucose units linked to each other and to the co-substrate by α-1,4-D-glucosidic bonds. Effective co-substrates include D-glucose, D-gluco-oligosaccharides, glucosides and compounds configurationally similar to D-glucose. Coupling reactions present a method for synthesis of model amylose chains in which the reducing group is labeled or substituted by any group for which the corresponding co-substrate is available.

In a previous communication from this Laboratory[3] evidence was presented that *Bacillus macerans* amylase[4] is capable of effecting a glycosidic exchange reaction between maltose and cyclohexaamylose[5] which results in the formation of higher molecular weight saccharides. The concept of glycosidic exchange, first suggested by Cori,[6] has been amplified and illustrated by Hehre.[7] In the meantime, it has been found[8] that *macerans* amylase acts upon individual linear amyloöligosaccharides to redistribute the D-glucose residues among the members of an homologous series. Thus amyloheptaose which is formed as the initial coupled product in the reaction between D-glucose and cyclohexaamylose, rapidly undergoes redistribution (homologizing) reactions leading to the formation of D-glucose, maltose and all higher members of the homologous series of linear amyloöligosaccharides. Some of the linear oligosaccharides in turn may be reconverted into Schardinger dextrins (cycloamyloses) so that eventually a state is approached in which the cyclic and linear compounds are in equilibrium with each other

$$\alpha + G_n \rightleftarrows G_{(n+6)} \quad (1)$$

$$\beta + G_n \rightleftarrows G_{(n+7)} \quad (2)$$

$$\gamma + G_n \rightleftarrows G_{(n+8)} \quad (3)$$

$$\xrightarrow{\text{coupling}} \quad \xleftarrow{\text{cyclization}}$$

$$G_n + G_m \rightleftarrows G_{(n+x)} + G_{(m-x)} \quad (4)$$

homologizing

where G_n, G_m, etc., represent amylose fragments of n, m, etc., glucose units. The fragments may be terminated at the reducing end by some group other than an α-1,4-linked glucose unit.

In the present paper the coupling reaction has been extended to include a variety of co-substrates such as oligosaccharides, glycosides and substances configurationally related to D-glucose. No attempt has been made to exhaust the list of potential co-substrates, but it appears that in order to function facilely a co-substrate must contain the D-glucopyranose arrangement unsubstituted except at the glucosidic position.

Effective co-substrates in *macerans* coupling reactions include D-glucose, D-glucoheptulose, polygalitol, maltose, maltobionic acid, cellobiose, turanose, sucrose, planteose, melezitose, oligosaccharide I,[9] oligosaccharide II,[9] panose, 6′-(α-D-glucopyranosyl)-maltose,[10] isomaltose[11] and the glucosides α-methyl-D-glucopyranoside, α-phenyl-D-glucopyranoside, aucubin and phlorizin. Configurational or structural changes of the D-glycopyranose unit, other than at the position 1, greatly retard or prevent coupling reactions (melibiose, raffinose, D-sorbitol, D-gluconic acid, D-mannose, D-galactose, D-arabinose, D-xylose, D-fructose, 2-amino-D-glucose).

In the initial experiments, coupling reactions were followed by observing the increase in optical rotation (Fig. 1). However, even without added co-substrate there is a gradual increase in rotation when *macerans* amylase acts upon cyclohexaamylose,[11] which masks rotational changes with slow-acting co-substrates. More definitive (albeit less quantitative) results have been obtained by examining the composition of the enzyme digests by paper chromatography. Coupled products, if present, are detected by a method which is sufficiently characteristic for the coupled series that there is no ambiguity as regards the nature of the products. For example, coupling to radio-D-glucose gives radio-oligosaccharides, which can be located by radioautography (Fig. 2). Coupling to sucrose, planteose, melezitose or raffinose gives non-reducing oligosaccharides, which can be located by the phloroglucinol spray for ketoses (Fig. 3). Coupling to polygalitol (1,5-anhydro-D-sorbitol) gives a series of non-reducing compounds which darken ammoniacal silver nitrate. Coupling to aucubin (a glucoside) gives a series of compounds giving a characteristic blue-green color with phloroglucinol–hydrochloric

(1) Journal Paper No. J-2397 of the Iowa Agricultural Experiment Station, Ames, Iowa. Proj. 1116. Supported by a grant from the Corn Industries Research Foundation.

(2) Presented in part before the Division of Biological Chemistry of the American Chemical Society, Sept., 1952.

(3) D. French, J. Pazur, M. L. Levine and E. Norberg, THIS JOURNAL, **70**, 3145 (1948).

(4) E. B. Tilden and C. S. Hudson, *J. Bact.*, **43**, 527 (1942); THIS JOURNAL, **61**, 2900 (1939).

(5) D. French and R. E. Rundle, *ibid.*, **64**, 1651 (1942).

(6) C. Cori, *Federation Proc.*, **4**, 226 (1945).

(7) E. J. Hehre, *Adv. Enzymology*, **11**, 297 (1951). See especially Fig. 8 and pages 322–324.

(8) E. Norberg and D. French, THIS JOURNAL, **72**, 1202 (1950).

(9) J. H. Pazur, *J. Biol. Chem.*, **199**, 217 (1952).

(10) S. C. Pan, L. W. Nicholson and P. Kolachov, THIS JOURNAL, **73**, 2547 (1951); D. French, *Science*, **113**, 352 (1951); M. L. Wolfrom, A. Thompson and T. T. Galkowski, THIS JOURNAL, **73**, 4093 (1951).

(11) W. S. McClenahan, E. B. Tilden and C. S. Hudson, *ibid.*, **64**, 2139 (1942).

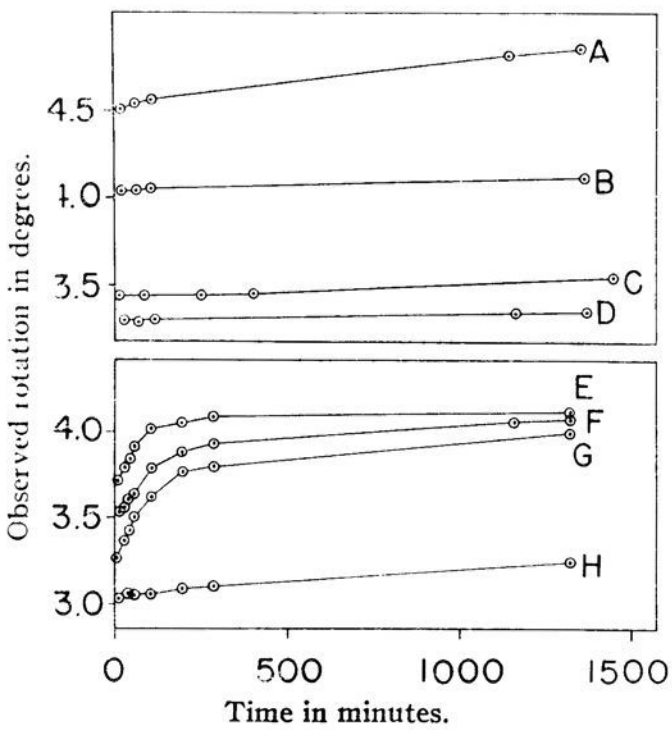

Fig. 1.—Optical rotatory change during coupling reactions of *macerans* amylase: A, cycloheptaamylose and maltose; B, cycloheptaamylose and sucrose; C, cycloheptaamylose and D-glucose; D, cycloheptaamylose alone (blank); E, cyclohexaamylose and methyl α-D-glucopyranoside; F, cyclohexaamylose and sucrose; G, cyclohexaamylose and D-glucose; H, cyclohexaamylose alone (blank).

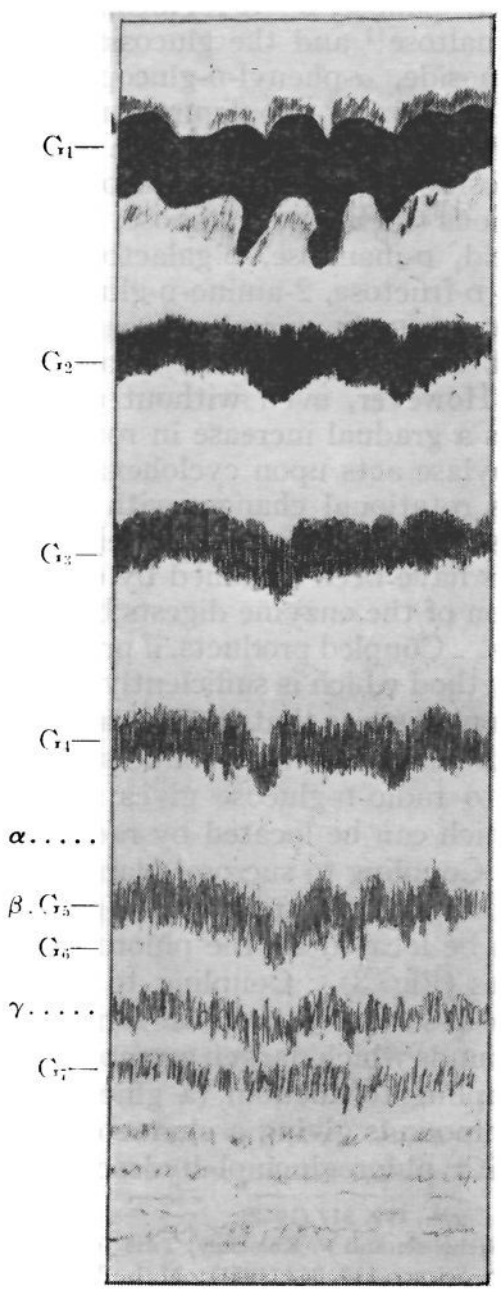

Fig. 2.—Radioautograph of papergram separation of coupling reaction mixture from radioactive glucose: 3 ascents on Whatman #1 paper using water:pyridine:butanol, 3:4:6 by volume as the developing solvent; G_1, G_2, G_3, etc., represent glucose, maltose, amylotriose, etc.; α,β,γ indicate the positions to which cyclohexaamylose, cycloheptaamylose and cycloöctaamylose move.

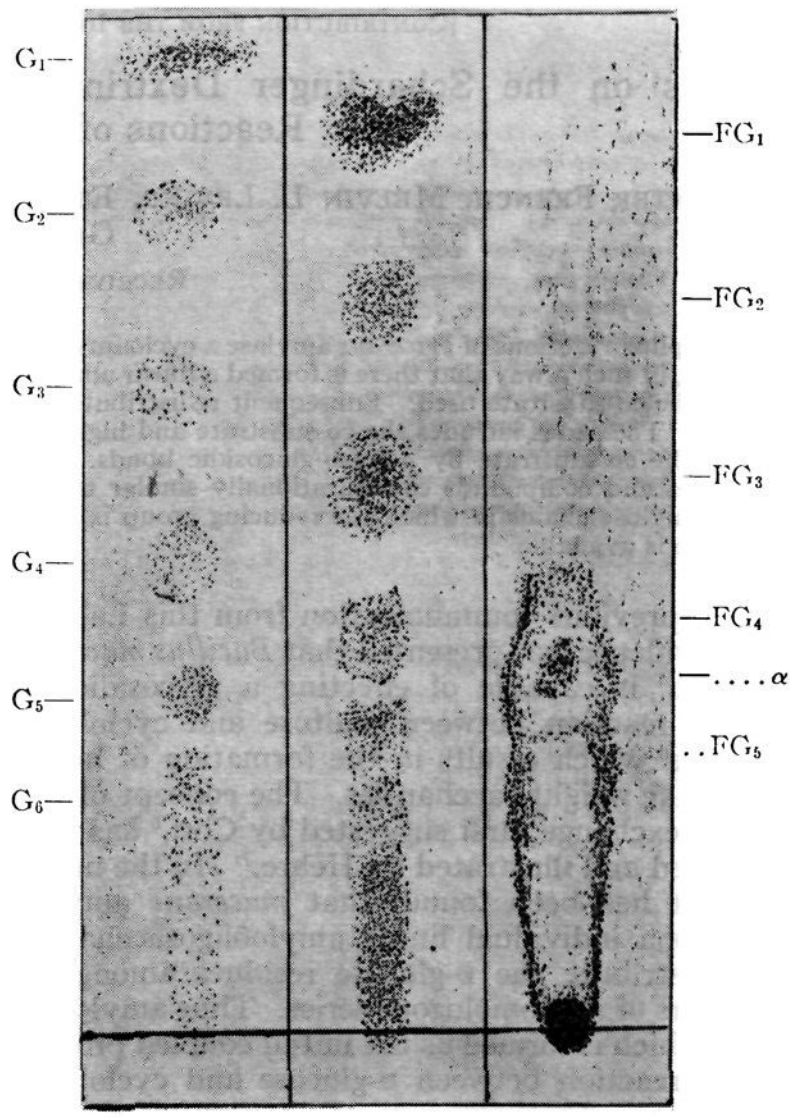

Fig. 3.—Coupling products of sucrose: a, reference series of linear amyloöligosaccharides, reducing spray; b, sucrose coupled series, urea–hydrochloric acid spray; c, sucrose coupled series, glucose-1-phosphate–phosphorylase spray, followed after 10 minutes by iodine spray; G_1, G_2, G_3, etc., represent glucose, maltose, amylotriose, etc.; FG_1, FG_2, FG_3, etc., represent sucrose, α-maltopyranosyl β-D-fructofuranoside, α-amylotripyranosyl, β-D-fructofuranoside, etc.; α represents cyclohexaamylose.

acid. Perhaps the most sensitive test for coupling is the formation of a series of compounds, other than the ordinary linear amyloöligosaccharides, which have the ability to react with D-glucose-1-phosphate and potato phosphorylase to give iodine-staining products[12] (Fig. 3). This test is applicable to any series if the individual members can be definitely distinguished by chromatographic character from the linear amyloöligosaccharides, or if such linear compounds can be first destroyed (as by alkali treatment) without destroying the desired series. In coupling reactions with isomaltose[13] or panose,[10] it is apparent that the reducing saccharides produced belong to homologous series[14] of the amylose type containing a single 1,6-linkage per molecule. These coupled products can be recognized in that they have considerably lower papergram mobilities than corresponding members of the linear series.

The exact nature of the coupled products has been more rigorously tested in a few cases. With these, coupling has been uniformly found to result in products containing the regular amylose chain

(12) D. French and G. M. Wild, THIS JOURNAL, **75**, 4490 (1953).

(13) M. L. Wolfrom, L. W. Georges and I. L. Miller, *ibid.*, **71**, 125 (1949).

(14) D. French and G. M. Wild, *ibid.*, **75**, 2612 (1953).

with the co-substrate moiety at the reducing terminus. In the case of coupling with radioglucose as co-substrate, the reaction leads to formation of radioamylooligosaccharides in which the radioactivity resides exclusively in the reducing glucose unit. The radiomaltose was converted into its 1-phenylflavazole,[15] which upon acid hydrolysis gave radioactive D-glucose-1-phenylflavazole and inactive D-glucose. In another test, the radioamylotriose was treated very extensively with β-amylase, a procedure which is known to remove maltose from the non-reducing end of starch chains,[16] and the products were separated by paper chromatography. All the radioactivity was found in the D-glucose fraction, again confirming that the radioactivity was exclusively in the reducing unit of the original trisaccharide. Moreover, the Schardinger dextrins present at the conclusion of the coupling reaction were completely free of radioactivity, as would be expected since their formation proceeds only from the non-reducing terminus of a starch chain, without changing significantly the total number of reducing end-groups.[4,11]

Coupling to sucrose leads to non-reducing fructose-containing oligosaccharides which are easily recognized as a regular homologous series.[14] Mild acid hydrolysis or treatment with yeast invertase gives D-fructose together with fructose-free amylooligosaccharides. Similarly, coupling with planteose[17] as co-substrate leads to a different series of non-reducing fructose-containing oligosaccharides, which on mild acid hydrolysis give amyloöligosaccharides plus planteobiose[17] as the exclusive fructose-containing compound. By way of contrast, melezitose affords coupled products which on acid hydrolysis give both the normal amylose series together with a parallel fructose-containing series, of which the first member is turanose.

The regularity of the starch chain in any coupled product is indicated by the regularity of the chromatographic series,[14] by the fact that the coupled products are attacked by the ordinary starch-splitting enzymes, and by the uniformity with which the products act as primers for phosphorylase synthesis.[12] Moreover, in all reactions, *macerans* amylase appears to act exclusively by glycosidic exchange of α-1,4 bonds. Since all bonds in cyclohexaamylose are α-1,4 bonds,[18] then all bonds in the coupled product chain would be of this type.

The coupling reaction provides a method by which irregularities may be incorporated into the reducing terminus of an amylose chain fragment. For example, the coupling product of cyclohexaamylose and sucrose is an amylose chain terminated by a fructofuranoside group. This molecule could be modified further through action of amylases or other biological or chemical agents. Coupling with panose gives doubly-coupled compounds which are of interest as models of branching in starch. These models are of great potential value in studying the effect of branching on the action of the hydrolytic amylases or phosphorylase. Further, demonstration of reversibility in coupling systems invites measurement[19] of the equilibrium constants for the reactions involved (*i.e.*, Eq. 1–4).

It is interesting to note that coupling reactions are far more rapid with α- than with β- or γ-dextrins. The α-dextrin which is initially formed in the *macerans* amylolysis of starch may be almost completely converted[20] into β-dextrin in the presence of a precipitant such as toluene or trichloroethylene. In this case the residual open-chain dextrins act as co-substrate. β- and γ-dextrins thus formed do not participate in rapid reverse reactions and hence once they are formed they are not rapidly reconverted. The variation of α- and β-dextrin yields from amylose and amylopectin is likewise explicable on the basis of the paucity of co-substrate groupings in amylose residues available for coupling reactions, in contrast to the relatively numerous end-groups derived from amylopectin molecules. The formation and gradual disappearance[21] of Schardinger dextrins, on treating starch with crude *macerans* amylase containing appreciable hydrolytic activity, is also readily interpreted by considering that the cyclic compounds undergo eventual coupling to give linear substrates for hydrolytic breakdown to fermentable sugars.

Acknowledgment.—We are indebted to Dr. N. K. Richtmyer for a gift of polygalitol, to Dr. M. L. Wolfrom for the isomaltose used, to Dr. S. C. Pan for a generous sample of 6′-(α-D-glucopyranosyl)-maltose, to Dr. J. C. Sowden for the D-glucoheptulose and to Dr. A. R. Trim for a sample of aucubin. The experiments with radioactive glucose were carried out in coöperation with Dr. S. Aronoff.

Experimental

Digests.—The *macerans* amylase digests were set up as before[3] except that in those cases where the work was done on a semi-micro scale the amounts of substrates were 2–20 mg.

Optical Rotatory Effects.—About 0.25 g. of the Schardinger dextrin (cyclohexaamylose or cycloheptaamylose) was treated with an approximately equimolar amount of the co-substrate and 20 units[20] of *macerans* amylase in a total volume of 25 ml. at room temperature. The observed rotations (2 dm. tube) are recorded for a few typical experiments in Fig. 1.

Paper Chromatography of Macerans Amylase Digests.—After several hundred conversion periods[20] samples of semi-micro digests were withdrawn and examined by multiple-ascent paper chromatography.[22,23] Coupled products, if present, were located by use of a selective spray reagent where one was known. The spray test for reducing sugars was used to locate coupled products from glucose, maltose, isomaltose, cellobiose, panose, etc. Duplicate chromatograms or portions of the same chromatogram were sprayed with a mixture of potato phosphorylase and 0.1 *M* glucose-

(15) G. Neumüller, *Arkiv Kemi, Mineral., Geol.*, **21A**, No. 19 (1946).

(16) M. L. Caldwell and M. Adams in J. A. Anderson, "Enzymes and Their Role in Wheat Technology," Interscience Publishers, Inc., New York, N. Y., 1946, p. 38.

(17) D. French, W. J. James and B. Young, THIS JOURNAL, **75**, 709 (1953).

(18) K. Freudenberg, E. Schaaf, G. Dumpert and T. Ploetz, *Naturwiss.*, **27**, 850 (1939).

(19) J. H. Pazur and D. French, paper presented before the Division of Biological Chemistry, American Chemical Society, Chicago, Ill., Sept., 1950, Abstracts, p. 51C.

(20) D. French, M. L. Levine, J. H. Pazur and E. Norberg, THIS JOURNAL, **71**, 353 (1949).

(21) E. Kneen and L. D. Beckord, *Arch. Biochem.*, **10**, 41 (1946); K. Myrbäck and L. G. Gjörling, *Arkiv Kemi, Mineral. Geol.*, **20A**, No. 5 (1945).

(22) A. Jeanes, C. S. Wise and R. J. Dimler, *Anal. Chem.*, **23**, 415 (1951).

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

1-phosphate, followed after 10 min. by dilute iodine spray, to test for formation and find location of "priming" oligosaccharides.[12] The Schardinger dextrins, especially cyclohexaamylose, markedly inhibited phosphorylase[24] and depending on the relative amount present gave either a white zone of inhibition (low concentration) or a wide zone of inhibition with central blue-violet area of the cyclohexaamylose–iodine–potassium iodide complex (high concentration). High primer concentrations were found to reduce the extent of inhibition (see Fig. 1). With co-substrates containing keto groups (sucrose, turanose, planteose, melezitose, D-glucoheptulose) coupled products were revealed by spraying with urea or phloroglucinol in hydrochloric acid, followed by heating in the oven at 100° for about 5 min. Aucubin coupled products gave blue-green colors with the phloroglucinol–hydrochloric acid spray. Ammoniacal silver nitrate was found to be a satisfactory spray reagent for coupled products from polygalitol.

By comparing spot positions and characters with controls of the ordinary amylose series and suitable blanks it was possible to ascertain whether, and to what extent, coupling reactions had occurred.

Coupling with Radioactive Glucose.—A sample of radioactive glucose (2 mg., 0.5 mc.) was treated with cyclohexaamylose (10 mg.) and *macerans* amylase (1.25 units[4]) for 500 min. at room temperature. The whole of the digest was transferred to a sheet of filter paper and chromatographed. A radioautograph of the resulting chromatogram (Fig. 2) showed that radioactive oligosaccharides containing at least 10 D-glucose units were produced and clearly resolved. By sectioning the chromatogram individual radiooligosaccharides were obtained for structure analysis and other work. No evidence was seen for any radioactivity in the Schardinger dextrin bands. Cyclohexaamylose appeared particularly well separated midway between G_4 and G_5. It was eluted and definitely identified by means of the iodine test.[4] In non-radioactive experiments, cycloheptaamylose was shown to have nearly the same R_f as G_5 while cyclooctaamylose fell between G_6 and G_7.

Structure of Coupled Products Derived from Radioactive Glucose.—The resolved chromatogram (Fig. 2) was autographed, sectioned and individual saccharides up to G_7 were isolated. G_2 was converted into the 1-phenylflavazole derivative by heating with 10 mg. of carrier G_2 and the required proportions of reagents[15] in a sealed capillary tube at 100° for 3 hours. Maltose-1-phenylflavazole was isolated from the reaction mixture by paper chromatography, and shown to be radioactive. Acid hydrolysis gave radioactive glucose-1-phenylflavazole and inactive glucose (separated by paper chromatography). Isolated G_3 and higher oligosaccharides were cleaved by salivary amylase forming radioactive saccharides such as G_1, G_2 and G_3. Action of soybean β-amylase on G_3 and G_5 gave inactive G_2 and radioactive G_1.

Structure of Coupled Products Derived from Sucrose.—A *macerans* amylase digest of sucrose and cyclohexaamylose was treated with 0.1 *N* NaOH at 100° for 30 min. (to remove traces of reducing oligosaccharides) and neutralized. Paper chromatography then showed the presence of a series of fructose-containing compounds of which the tetrasaccharide was a poor primer and higher members were good primers for phosphorylase (Fig. 3). Partial hydrolysis ("inversion") by 0.1 *N* hydrochloric acid at 70° for 5 min. or by yeast invertase, produced fructose and the normal series of reducing amyloöligosaccharides; no fructose-containing oligosaccharides were present.

Coupled Products Derived from Panose.—Panose[10] gave a series of coupled products which differed from other coupling series in that there were two different distinctly resolved tetrasaccharide components, and the pentasaccharide and higher components appeared to be mixtures of isomers. Partial acid hydrolysis of the tetrasaccharides and their flavazoles indicated the faster moving component to be

α-D-Glu *p* 1 → 4 α-D-Glu *p* 1 → 4D-Glu
α-D-Glu *p* 1 → 6——┘

and the slower moving component to be α-D-Glu *p* 1 → 4 α-D-Glu *p* 1 → 6 α-D-Glu *p* 1 → 4 D-Glu.

The lowest component capable of priming phosphorylase was the pentasaccharide. However, when the mixture was treated with either salivary amylase or β-amylase, the ability of the pentasaccharide to prime phosphorylase was largely destroyed, though a significant amount of reducing pentasaccharide remained. Action of an amyloglucosidase[25] on the panose coupling mixture converted it entirely into glucose and panose.

(24) D. E. Green and P. K. Stumpf, *J. Biol. Chem.*, **142**, 355 (1942).

(25) "Dextrinase," a microbial enzyme sold by the Delta Chemical Works, 23 W. 60th Street, New York 23, N. Y.

AMES, IOWA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, PRESIDENCY COLLEGE, CALCUTTA]

Bitter Constituents of the Seeds of *Corchorus olitorius* L., "Corchorgenin"—A New Cardiac-active Aglycone

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RECEIVED SEPTEMBER 1, 1953

A new cardiac-active aglycone, $C_{28}H_{32}O_6$, m.p. 227° (uncor.), $[\alpha]^{21}D$ +90° (ethanol), has been isolated by chromatography of the bitter principles from seeds of *Corchorus olitorius* L. grown in West Bengal (India) and studied. Its pharmacological activity in cats, indicates that it is more potent than either of the isomeric genins, corchortoxin of *Corchorus capsularis* or strophanthidin of *Strophanthus kombe*.

During the last few years there has been a renewed interest in the chemistry of the bitter principles of jute (*Corchorus*[1]) seeds and different bitter principles have been reported. The occurrence of a bitter principle as a brown amorphous powder under the name of "corchorin" in the seeds of *C. capsularis* L. was first reported by Tsuno,[2] but our present knowledge regarding the isolation, chemistry and pharmacological properties of corchorin is mainly due to Sen.[3] He described corchorin as a glycoside, $C_{22}H_{36}O_8$, m.p. 174–175°, $[\alpha]D$ +33.4° (ethanol), which yielded glucose and corchogenin, $C_{16}H_{26}O_3$, m.p. 112–114°, on acid hydrolysis. Corchogenin according to Ruzicka[4] may be a sesquiterpene or sesquiterpenoid. Sen also isolated another bitter principle "corchoritin,"[5] $C_{12}H_{18}O_3$, m.p. 218–

(1) The two important species of *Corchorus* L. (*Tiliaceae*), *viz.*, *C. capsularis* L. and *C. olitorius* L. are grown in West and East Bengal which produce about four-fifths of the world's total jute output; the latter species is widely used as a pot-herb. The seeds of the two species can be differentiated morphologically; those of *C. capsularis* are relatively larger in size, irregular in shape and coppery red when mature, whereas the seeds of *C. olitorius* are somewhat triangular and greyish-green or bluish-black in color. In the present investigation, the seeds of *C. olitorius* L. collected from the Hooghly District (West Bengal) have been employed.

(2) Tsuno, *Monatsh. Tierheilk*, **6**, 455 (1896).

(3) N. K. Sen, *J. Indian Chem. Soc.*, **7**, 83 (1930); *Indian J. Physiol. Allied Sci.*, **2**, 1 (1948).

(4) L. Ruzicka, *Ann. Rev. Biochem.*, **1**, 594 (1932).

(5) N. K. Sen, *J. Indian Chem. Soc.*, **8**, 651 (1931).