termination with an authentic specimen (m.p. 186–187° dec.).

The sublimation residue, after recrystallization from ethyl acetate, yielded 582 mg. (55%) of colorless crystals, m.p. 187–188°, identified as succinic acid by a mixed melting point determination with an authentic specimen (m.p. 187.5–188.0°).

The solution of steam-volatile acids obtained above was neutralized with sodium hydroxide solution, concentrated to a small volume on the steam-bath, and acidified to congo red with sulfuric acid. The solution was rapidly steam distilled, the distillate neutralized with 0.1 *N* sodium hydroxide solution, and the neutral solution evaporated to dryness and the *p*-phenylphenacyl ester prepared. It melted at 111° and a mixed melting point determination with authentic *p*-phenylphenacyl acetate, m.p. 111°, showed no depression.

The distillation residue was extracted with ether in a continuous extractor, and the ether solution was dried and freed of solvent. The crystalline residue sublimed completely at 90–95° (15 mm.). Recrystallization from Skellysolve B gave 845 mg. (65%) of colorless feathery needles, m.p. 107°, identified as *N*-isobutyloxamic acid by a mixed melting point determination with a synthetic sample, m.p. 107°.

BELTSVILLE, MARYLAND

[CONTRIBUTION FROM THE NORTHERN UTILIZATION RESEARCH BRANCH[1a]]

The Structure of α-Amylase Modified Waxy-Corn Starch[1b]

BY R. L. LOHMAR

RECEIVED APRIL 5, 1954

Graded hydrolysis of waxy-corn starch by malt α-amylase produced a series of modified amylopectins which were characterized by their reducing power, the amount of formic acid produced on periodate oxidation and their convertibility by β-amylase. From these determinations, the average chain length and the average inner- and outer-branch lengths were calculated. These calculated values decreased as the extent of hydrolysis progressed. Hence, the modified products are best represented as polymers differing from the parent amylopectin in molecular weight and in the average length of both inner and outer branches. It is concluded further that the initial hydrolysis by malt α-amylase is not confined to the outer branches, but that the longer inner branches are attacked simultaneously. Similar studies on modified β-limit dextrins confirmed this conclusion.

It is known that when branched starch substances are hydrolyzed by malt α-amylase, the 1,4-glucosidic linkages are broken and the 1,6-glucosidic linkages at branch points are stable.[2,3] Our most precise knowledge of the mode of attack by the various α-amylases comes from studies of the low-molecular-weight products liberated from the branched substrates in the early[4] or in the final[3] stages of hydrolysis. There appear to have been few investigations of the residual polysaccharide produced during the early stages of hydrolysis.[5] Myrbäck[6] has contended that malt α-amylase hydrolyzes most rapidly those 1,4-linkages near the middle of the linear portions of the molecule and that long chains are more susceptible to attack than short chains. That is, the 1,4-linkages in the neighborhood of branch points (or end groups) are cleaved at a lower rate than those farther away. His picture is based almost wholly on data obtained from studies of the sugars and oligosaccharides that are split away from the polysaccharide substrate. Kinetic studies by Schwimmer[7] support the concept of dependence of rate of hydrolysis on the number of anhydroglucose units in a chain, provided that the chain is composed of less than about ten units. It is evident that assessment of the structure of the modified amylopectin remaining after slight hydrolysis would serve to test Myrbäck's theory.

We have treated waxy-corn starch with graded amounts of malt α-amylase to produce a series of modified amylopectins. These modified amylopectins were precipitated by addition of alcohol to 60% concentration and characterized by their reducing power, by the amount of formic acid produced from them on periodate oxidation and by their convertibility by β-amylase (Table I).

TABLE I

PROPERTIES OF MODIFIED WAXY-CORN STARCHES

α-Amylase, units/g.	Yield, %	Reducing power[a]	Non-reducing end group, %	β-Convertibility, %
0	..	..	4.8	55
1.5	81	13.7	6.2	55
2.5	78	17.4	6.7	52
5.0	65	33.8	8.5	50
10.0	62	38.8	9.2	44

[a] Maltose hydrate equivalent, mg./g.

The percentage non-reducing end group and the average chain length (the number of anhydroglucose units per non-reducing end group) were calculated from the formic acid production. Some of our materials have appreciable reducing power and it is necessary to correct the formic acid values for the two moles of acid arising from each mole of reducing end group.[8] The proportion of anhydroglucose units in the outer branches is given by the "β-amylase convertibility"—the fraction of the molecule removed as maltose on treatment with β-amylase. The product of convertibility and average chain length then gives the average number of anhydroglucose units removed from the outer branches. To obtain the average outer-branch length, there must be added to this product a constant value to allow for the "stubs," at branch points, that β-amylase is not able to digest. Follow-

(1) (a) One of the Branches of the Agricultural Research Service, U. S. Department of Agriculture, article not copyrighted. (b) Presented before the Division of Carbohydrate Chemistry at the 125th National Meeting of the American Chemical Society, Kansas City, Mo., March, 1954.

(2) K. Myrbäck, *Advances in Carbohydrate Chem.*, **3**, 251 (1948).

(3) K. H. Meyer and W. F. Gonon, *Helv. Chim. Acta*, **34**, 308 (1951).

(4) R. Bird and R. H. Hopkins, *Biochem. J.*, **56**, 86 (1954).

(5) K. Myrbäck and R. Lundén, *Arkiv. Kemi Mineral. Geol.*, **23A**, No. 7, 1 (1946).

(6) K. Myrbäck and L. G. Sillén, *Svensk Kem. Tidskr.*, **56**, 60 (1944); K. Myrbäck, *Arch. Biochem.*, **14**, 53 (1947).

(7) S. Schwimmer, *J. Biol. Chem.*, **186**, 181 (1950).

(8) K. H. Meyer and P. Rathgeb, *Helv. Chim. Acta*, **31**, 1545 (1948); A. L. Potter and W. Z. Hassid, THIS JOURNAL, **70**, 3488 (1948).

ing Illingworth, Larner and Cori[9] we have used the value of two units. Recently, Peat, Whelan and Thomas[10] have provided evidence that the "stub" must be two or three units long.

The method used to calculate the average inner-branch length is dependent on the molecular size of the amylopectin. There is one less inner branch than there are outer branches in the amylopectin molecule. When the molecule is sufficiently large to have a negligible reducing power, this difference may be ignored, and the average inner-branch length is equal to the average chain length minus the average outer-branch length. However, with smaller molecules this tacit assumption of equal numbers of inner and outer branches may lead to spurious results. To compensate for the difference in number of outer and inner branches, the number of chains in such molecules is calculated from the average chain length and the degree of polymerization (DP). The number of inner branches is one less than the number of chains. The calculation of the inner-branch length of the modified amylopectin produced by treatment of waxy-corn starch with 1.5 units α-amylase (Table I) serves to illustrate the manner of calculation:

Average chain length: $100/6.2 = 16.1$
Average outer-branch length: $(0.55 \times 16.1) + 2 = 10.8$
Degree of polymerization: $(1000/13.7) \times (2/0.9) = 160$
Chains per molecule: $160/16.1 = 10$
Average inner-branch length, adjusted: $(160 - 108)/(10 - 1) = 5.8$

It should be pointed out that, with either method of calculation, the branch terminating in the reducing end group is included with the inner branches.

Although the average branch lengths are given to the first decimal place, it is emphasized that these are average and approximate values. The average values are given in this form in order to convey an approximate idea of the fine structure of the compounds under examination. Trends are considered to be of more significance than the actual values.

The results of such analysis of the four modified amylopectins and their parent waxy-corn starch, listed in Table I, are given in Table II.

TABLE II

STRUCTURE OF MODIFIED AMYLOPECTINS

α-Amylase, units/g.	Chain length	Branch length: Outer	Branch length: Inner, By diff.	Branch length: Inner, Adjusted
0	20.8	13.5	7.3	..
1.5	16.1	10.8	5.3	5.8
2.5	14.9	9.7	5.2	6.0
5.0	11.8	7.9	3.9	4.8
10.0	10.9	6.8	4.1	5.2

Substantiation of the values obtained for the inner-branch lengths can be obtained by examining the β-amylase limit dextrins of the materials listed in Tables I and II. The limit dextrins were subjected to the same analysis as their antecedent modified amylopectins. The β-convertibility being zero, the average outer-branch length was taken to be 2. The results of the analysis are shown in Table III. It is seen that the inner-branch lengths found for the β-limit dextrins agree with those calculated for their antecedents. Better agreement is found when the adjusted values are compared. It is to be noted that the reducing power of each β-limit dextrin is about half, rather than twice, that of its antecedent. It is presumed that the β-amylase conversion of the smaller fragments of the modified amylopectin had reduced them to such size that they were soluble in the 60% alcohol used for isolation. This would result in the removal of fractions whose antecedents contributed substantially to the reducing power of the modified amylopectin. Experiments are underway to test this proposition.

TABLE III

STRUCTURE OF β-LIMIT DEXTRINS

Reducing power[a]	Non-reducing end group, %	Chain length	Inner-branch length: By diff.	Inner-branch length: Adjusted
0	10	10	8	..
7.1	13.3	7.5	5.5	5.7
9.7	13.2	7.6	5.6	5.9
18.4	14.1	7.1	5.1	5.4
22.8	14.8	6.8	4.8	5.4

[a] Maltose hydrate equivalent, mg./g.

Finally, a β-limit dextrin of waxy-corn starch was modified with graded amounts of malt α-amylase in the same fashion as was used for waxy-corn starch. The results of the analysis are given in Tables IV and V.

TABLE IV

PROPERTIES OF MODIFIED β-LIMIT DEXTRINS

α-Amylase, units/g.	Yield, %	Reducing power[a]	Non-reducing end group, %	β-Convertibility, %
0	..	..	10.0	0
0.25	98	10.7	12.1	7
0.5	92	25.0	13.7	11
1.0	80	50.7	16.3	11
2.5	71	98.9	20.1	11

[a] Maltose hydrate equivalent, mg./g.

TABLE V

STRUCTURE OF MODIFIED β-LIMIT DEXTRINS

α-Amylase units/g.	Chain length	Branch length: Outer	Branch length: Inner, By diff.	Branch length: Inner, Adjusted
0	10.0	2	8	..
0.25	8.3	2.6	5.7	6.0
0.5	7.3	2.8	4.5	5.9
1.0	6.1	2.6	3.5	4.0
2.5	5.0	2.5	2.5	3.2

Discussion

Examination of the structures of the modified amylopectins and β-limit dextrins shows that both the inner- and outer-branch lengths decrease as the degree of enzyme attack progresses. It is evident that both inner and outer branches are attacked simultaneously by malt α-amylase in the early stages of hydrolysis. Myrbäck's[6] contention that the longer inner branches are attacked preferentially adequately accounts for the decrease in the average inner-branch length. These longer inner

(9) B. Illingworth, J. Larner and G. T. Cori, *J. Biol. Chem.*, **199**, 631 (1952).

(10) S. Peat, W. J. Whelan and G. T. Thomas, *J. Chem. Soc.*, 4546 (1952).

branches may contain as many as ten,[11] certainly more than six,[10,12] units. Schwimmer's kinetic studies[7] are also in agreement with the results reported in this paper. Each scission of an inner branch results in the formation of a "new" outer branch. Differentiation between these "new" outer branches and the original outer branches (unaltered or shortened) is not possible with the techniques described here. However, it does appear probable that the decrease in outer-branch length is real and not an artifact arising from dilution by "new" outer branches.

The presence of short inner branches in the modified β-limit dextrins (Table V) lends further support to the hypothesis of preferential attack of the longer branches. The inner branches referred to here include the reducing end of the molecule. Hence, the average length of the remainder of the inner branches may be greater or less than that calculated for inner branches, depending on the number of units in the branch terminating in the reducing end group. Two extreme examples relating to the structure of a product similar to the third modified β-limit dextrin in Table V may be cited. In a molecule of DP 44, chain length of 6.1, convertibility of 11% and a reducing branch 2 units long, the assignment could be: 7 outer branches averaging 2.7 units and 5 inner segments averaging 4.6 units. Alternatively, if the reducing branch contained 10 units, the inner segments would average 3 units. In either case, the average length of inner segments is substantially less than for the original β-limit dextrin. The presence in amylopectin of inner branches as short as 3 to 5 units also has been suggested by the work of French,[13] who found doubly branched dextrins of DP 9 to 13 among the products of the hydrolysis of amylopectin by salivary α-amylase.

The data on β-limit dextrin modifications thus definitely indicate the presence in amylopectin of shorter-than-average inner branches which are relatively resistant to malt α-amylase hydrolysis. It should be noted, on the other hand, that the results on the amylopectin modifications are not definitive in themselves. Decreases in outer- and inner-branch lengths similar to those observed can be calculated for a homogeneous amylopectin structure in which all inner branches are of the same length, provided that certain assumptions are made concerning the action of the α-amylase.[14] As an example, it can be assumed that all cleavages occur in the middle of branches and that 1.5 times as many outer branches are cleaved as inner branches (an arbitrary value which could reflect a lower affinity of enzyme for the inner branches). If a modified amylopectin analogous to the second one in Table I (2.5 α-amylase units/g.) is formed by cleavage of 30 outer branches (small fragments discarded) and 20 inner branches of each 100 chains (20.8 units long), the resulting product would have an average outer-branch length of 10.1 and an average inner-branch length of 5.5, or adjusted, 6.6. Such calculations, however, cannot be made to yield values comparable to those obtained for the β-limit dextrin modifications (Table V).

(11) M. A. Swanson, *J. Biol. Chem.*, **172**, 825 (1948).

(12) M. A. Swanson and C. F. Cori, *ibid.*, **172**, 814 (1948).

(13) D. French and G. M. Wild, Abstracts of Papers, 122nd Meeting, American Chemical Society, 5R (1952).

(14) The author is indebted to Dr. R. J. Dimler for pointing out this alternative hypothesis.

Experimental

Materials.—Waxy-corn starch was a commercial product. It sorbed 2.2 mg. of iodine per g. when titrated potentiometrically according to the procedure of Bates, French and Rundle,[15] as modified by Wilson, Schoch and Hudson.[16]

β-Amylase-limit dextrin of waxy-corn starch was prepared by the action of wheat β-amylase on the same waxy-corn starch used for the α-amylase studies. The dextrin was generously supplied by Dr. R. J. Dimler of this Laboratory. The preparation will be described elsewhere.

Malt α-amylase was similar to that used by Dimler, Bachmann and Davis.[17] It had an activity of 2,700 SKB[18] units/g.

Wheat β-amylase was prepared by modification of the method of Ballou and Luck.[19] The centrifuged aqueous extract was held at *p*H 3 for 30 minutes at 0°, prior to precipitation of the β-amylase, to inactivate the α-amylase. The potency was determined by the method of Kneen and Sandstedt.[20]

95% Ethanol was used for all precipitations.

Analytical methods. Reducing Power.—The reducing powers of the polysaccharides were determined by Meyer's[21] modification of the Sumner dinitrosalicylic acid method. The dialysis was omitted. Transmittance was read at 525 mμ, using 16-mm. cuvettes.

β-Amylase Convertibility.—Approximately 0.2000 g. (dry basis) polysaccharide was weighed into a 25-cc. volumetric flask. The solid was dissolved in water and 1 cc. of *p*H 4.8 acetic acid–acetate buffer and 1 cc. wheat β-amylase (1 unit/cc.) were added. The volume was brought to 25 cc. with water and toluene was added. After 24 hours, aliquots were removed and the reducing power was determined by the dinitrosalicylic acid method. Analysis was repeated at 48 hours. Analysis of the enzyme showed that it had negligible reducing power. Results were corrected for the reducing power of the substrate and are expressed as percentage of the theoretical yield of maltose hydrate.

The products obtained by the action of α-amylase on the limit dextrins showed progressively increased conversions over a period of 168 hours. Conversions were rerun on solutions that had been autoclaved before the addition of buffer. On these samples the 24-hour values were not increased at 48 or 72 hours. Evidently some α-amylase had been adsorbed on the polysaccharide.

Periodate Oxidation.—The oxidations were carried out using sodium metaperiodate at 25° as described by Jeanes and Wilham[22] in their work on dextrans except that the liberated formic acid was titrated with 0.01 *N* $Ba(OH)_2$ instead of NaOH. The values used were obtained after 72 hours oxidation.

Degradation with α-Amylase.—Waxy-corn starch (100 g., air-dry) was gelatinized in 2,500 cc. of water on the steam-bath with rapid stirring. The *p*H was 4.9. The mixture was cooled to 60° and put in a thermostated bath at 60°. The enzyme, in 100–250 cc. of water, was added and the mixture was stirred mechanically. After 10 minutes the product was precipitated with 4.3 liters of ethanol under vigorous agitation. The mixture was allowed to cool to room temperature and the supernatant was siphoned or decanted from the gummy precipitate. The precipitate was dissolved in 500 cc. of water and clarified by supercentrifugation. The polysaccharide was precipitated in a finely divided form by slowly adding the clarified solution to 8–9 volumes of vigorously agitated ethanol.

After the precipitate had settled, the supernatant was

(15) F. L. Bates, D. French and R. E. Rundle, THIS JOURNAL, **65**, 142 (1943).

(16) E. J. Wilson, Jr., T. J. Schoch and C. S. Hudson, *ibid.*, **65**, 1380 (1943).

(17) R. J. Dimler, R. C. Bachmann and H. A. Davis, *Cereal Chem.*, **27**, 488 (1950).

(18) R. M. Sandstedt, E. Kneen and N. J. Blish, *ibid.*, **16**, 712 (1939).

(19) G. A. Ballou and J. M. Luck, *J. Biol. Chem.*, **139**, 233 (1941).

(20) E. Kneen and R. M. Sandstedt, *Cereal Chem.*, **18**, 237 (1941).

(21) K. H. Meyer, G. Noelting and P. Bernfeld, *Helv. Chim. Acta*, **31**, 103 (1948).

(22) A. Jeanes and C. A. Wilham, THIS JOURNAL, **72**, 2655 (1950).

siphoned off and the precipitate was further dehydrated with ethanol. It was then filtered on a büchner funnel under dental dam to exclude moisture and dried over calcium chloride in a vacuum desiccator. The sample was then exposed to 65% R.H. for several days to displace adsorbed ethanol. Degradations of β-amylase-limit dextrin (*p*H, 6.5–6.6) were carried out in the same fashion.

β-Amylase-limit Dextrins.—The conversions were carried out on 4% solutions of the modified amylopectins at *p*H 4.7, unbuffered. After 45 hours, the solutions were heated to inactivate the enzyme and the dextrins were precipitated with 1.5 volumes of ethanol. The precipitated gums were washed several times with 60% ethanol, dissolved in water and reprecipitated in flocculent form in 8 volumes of ethanol. The dextrins were isolated and humidified in the same fashion as the modified amylopectins.

Acknowledgment.—We wish to thank Mr. J. C. Rankin for the periodate oxidations, Mrs. Marjorie Austin for some of the enzyme preparations and Mr. W. L. Deatherage for the iodine-sorption measurement.

PEORIA, ILLINOIS

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, CHEMICAL DIVISION, MERCK & CO., INC.]

Benzotriazines. II. Synthesis of 3-Amino-7-halo-1,2,4-benzotriazine-1-oxides

BY F. J. WOLF, R. M. WILSON, JR., K. PFISTER, 3RD, AND M. TISHLER

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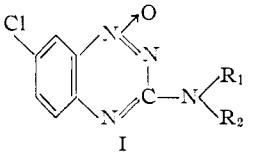
A series of 7-halo-1,2,4-benzotriazine-1-oxides bearing variously substituted 3-amino radicals has been prepared and examined for antimalarial activity.

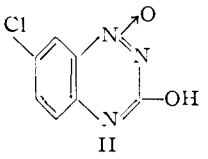
The antimalarial activity of substituted 3-amino-1,2,4-benzotriazines has been described.[1] Since the most active compounds were those with a halogen in position 7, and preliminary experiments indicated that activity was retained by replacement or modification of the amino group in position 3, a series of 7-halogen benzotriazines (I) containing substituted amino groups in this position was



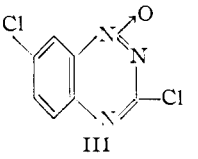
I

prepared and tested for antimalarial activity. In general, the resulting compounds were found to be somewhat less active than the unsubstituted amine. The most active compounds, in which R_1 is benzyl or methyl and R_2 is hydrogen, have about one-tenth the activity of the parent substance. Side chains giving enhanced activity in the plasmoquin and atebrin series were not efficacious.

Although various methods for the preparation of 1,2,4-benzotriazines have been described, none appeared suitable for the preparation of substituted 3-amino derivatives. It appeared probable that 3,7-dichloro-1,2,4-benzotriazine-1-oxide (III) could be readily aminated yielding the desired compounds. However, some difficulty was encountered in obtaining this dichloro compound. When refluxed with thionyl chloride, 3-hydroxy-1,2,4-benzotriazine-1-oxide (II) is recovered unchanged. On refluxing with phosphorus oxychloride or mixtures of phosphorus oxychloride and phosphorus pentachloride, a low yield (about 20%) of the desired 3-chloro compound is obtained. However, when phosphorus oxychloride and dimethylaniline[2] are used, an excellent yield of the dichloro compound is obtained.

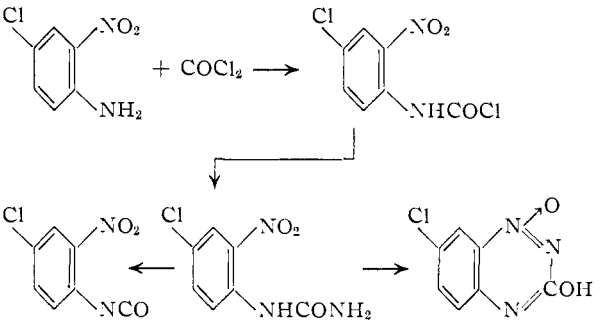


II



III

In order to provide sufficient quantities of the hydroxy compound, which had previously been prepared in 50% yield by diazotization of the corresponding amine, a new method of synthesis was developed utilizing conditions for ring closure similar to those described by Arndt[3] for the preparation of other benzotriazine compounds. The requisite ureide was obtained by treatment of the corresponding nitroaniline with phosgene and then ammonia. Depending on the conditions used, the ureide could be converted to the corresponding isocyanate or to the required 3-hydroxy-1,2,4-benzotriazine-1-oxide, ring closure being the favored reaction at high concentration of sodium hydroxide.



The chloro compound reacted readily with a variety of amines using temperatures of 60–70° for the amination. These amines are bright yellow in color and although lower melting than the parent compound were obtained in the crystalline state with one exception. The amines used were either available or prepared by standard literature procedures.

It is of considerable interest that when 7-halo-3-amino-1,2,4-benzotriazine-1-oxide or the desoxy compound was refluxed with benzylamine, 7-halo-

(1) For the previous publication in this series, see F. J. Wolf, K. Pfister, 3rd, R. M. Wilson, Jr., and C. A. Robinson, THIS JOURNAL, **76**, 3551 (1954).

(2) J. Baddiley and A. Topham, *J. Chem. Soc.*, 678 (1944).

(3) F. Arndt, *Ber.*, **46**, 3522 (1913); F. Arndt and B. Eistert, *ibid.*, **60**, 2598 (1927); F. Arndt and T. Tschenscher, *ibid.*, **56**, 1988 (1923).