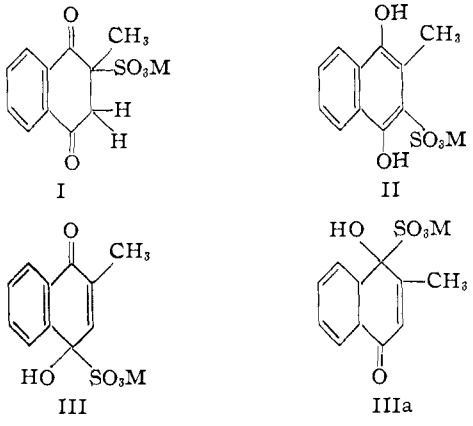

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sized by the method of Baker, *et al.*[2] The recrystallized active salt was dried for five days in a vacuum desiccator (P_2O_5).

The infrared spectrum of this material, while exhibiting a very weak absorption in the 3 μ region probably due to a trace of moisture in the potassium bromide used in the pellet, very clearly indicates lack of hydroxyl in the sample.

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Hydrolysis of Isomaltotriose by Oligo-1,6-glucosidase

BY JOSEPH LARNER

RECEIVED JULY 18, 1955

The enzymatic hydrolysis of isomaltose, "panose" and branched α-amylase dextrins by oligo-1,6-glucosidase from hog intestinal mucosa has been reported.[2,3] When coupled to the hexokinase, Zwischenferment, TPN system, the enzyme has no activity on dextran.[3] The present report demonstrates the enzymatic hydrolysis of isomaltotriose (6-α-isomaltopyranosyl-D-glucose),[4] the next higher homolog of isomaltose.

Hydrolysis of isomaltotriose has been followed by increase in reducing power, paper chromatography and by coupling with the hexokinase, Zwischenferment, TPN system. Incubation of 4.0 mg. of isomaltotriose with 120 units[2] of oligo-1,6-glucosidase at 30° in the absence of buffer resulted in a 60.2% hydrolysis in 120 minutes. Aliquots of the reaction mixture were deproteinized by the $Ba(OH)_2$-$ZnSO_4$ method of Somogyi[5] and reducing power determined by the method of Nelson.[6] The more alkaline reagent 60 of Shaffer and Somogyi[7] and a boiling time of 30 minutes were used. Paper chromatographic analysis at 22.0, 29.3, 39.8, 52.9 and 60.2% hydrolysis revealed only isomaltose and glucose as products, when about 100 γ of total sugar was applied to each spot.

An experiment in which the rate of hydrolysis of equimolar amounts of isomaltose and isomaltotriose are compared is presented in Table I. The results are expressed both as amount of reducing sugar (as glucose) appearing per time period as well as percentage of hydrolysis. In terms of appearance of reducing sugar, isomaltotriose is hydrolyzed more rapidly than isomaltose. Since, on a molar basis, isomaltose has one susceptible linkage for each two of isomaltotriose, isomaltose is more rapidly hydrolyzed when compared in terms of percentage of hydrolysis. When calculated in terms of linkages hydrolyzed, 1.5 linkages of isomaltotriose[8] were hydrolyzed for each linkage of isomaltose during the 25 minute time period, and 1.9 during the 90-minute period.

TABLE I

HYDROLYSIS OF ISOMALTOSE AND ISOMALTOTRIOSE BY OLIGO-1,6-GLUCOSIDASE

Reaction mixture contained 100 units oligo-1,6-glucosidase, 1.98 μmoles isomaltotriose, or 2.22 μmoles isomaltose; total volume, 2.2 ml.

Substrate	Time, min.	Δ Reducing sugar, as glucose, μg./2.2 ml.	Hydrolysis, %
Isomaltose	25	172	44.8
	90	358	92.2
Isomaltotriose	25	224	33.8
	60	448	67.5
	90	566	85.4

In the presence of hexokinase, Zwischenferment, and TPN formation of glucose could be conveniently followed by the increase in optical density at 340 mμ (Fig. 1). With equimolar amounts of substrates, the rate of TPN reduction with isomaltotriose was 80% that of isomaltose (slope of curve). When calculated in terms of linkages hydrolyzed, 1.6 linkages of isomaltotriose were hydrolyzed for each linkage of isomaltose in good agreement with the results of Table I.

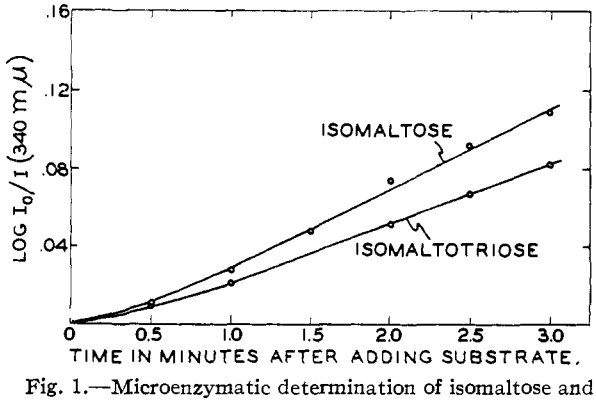

Fig. 1.—Microenzymatic determination of isomaltose and isomaltotriose hydrolysis by oligo-1,6-glucosidase. The reaction mixture (3.0 ml. volume) contained 41 units oligo-1,6-glucosidase and 1 μmole of either isomaltose or isomaltotriose. All other components as previously described.[3]

K_m values have been determined for the two substrates using the hexokinase Zwischenferment sys-

(1) Supported in part by a grant from the Graduate College, University of Illinois, Urbana, Illinois.

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(4) We gratefully acknowledge the gifts of isomaltose and isomaltotriose from Dr. A. Jeanes, Northern Utilization Research Branch, Peoria, Illinois.

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(8) It is not known which of the two linkages of isomaltotriose the enzyme hydrolyzes most readily.

tem. At *p*H 6.85, K_m values have been determined by plotting *s versus s/v* and determining the negative *y* intercept. For both substrates a value of 7×10^{-4} mole/l. has been found.

These results indicate that under these conditions at equimolar concentrations the enzyme hydrolyzes isomaltotriose more readily than isomaltose. The chain length specificity of the enzyme is therefore raised to include the trisaccharide. With the availability of suitable substrates it would be of interest further to delineate the chain length specificity of the enzyme.

The ability of digestive enzyme to hydrolyze isomaltotriose brings to mind the interesting possibility of the occurrence of two adjacent α-1,6-linkages of starch. Such "double" branch points could conceivably arise from the action of a branching enzyme in which an α-1,6-linked disaccharide or oligosaccharide such as isomaltose or "panose" served as cosubstrate or acceptor of the linear segment transferred in the branching reaction. Such a reaction in which maltose acts as a cosubstrate has been described in the case of *Polytomella coeca* branching enzyme.[9]

Experiments testing the specificities of branching enzymes from potato, liver, broad bean and wrinkled pea with maltose, isomaltose and "panose" as cosubstrates are in progress and thus far have shown no stimulation of the branching reaction by these saccharides. Because of their relation to the mechanism of action of branching enzymes, these experiments will be reported separately.

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The Diels–Alder Reaction of Cyclopentadiene with Nitroölefins. Tertiary Nitro Adducts

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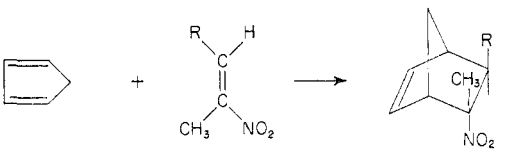
RECEIVED JULY 14, 1955

Cyclopentadiene is known to undergo Diels–Alder reactions with several unsubstituted and 2-substituted nitroölefins: nitroethylene[2] in 61[3] to 66%[4] yield, 1-nitropropene in 55[2] to 59%[5] yield, 1-nitropentene in 72% yield[2] and β-nitrostyrene in 88[6] to 95%[7,8] yield, giving adducts containing secondary nitro groups. Nightingale and Janes[9] report, however, that all efforts to condense the longer chain 2-substituted nitroölefin, 1-nitroheptene, with cyclopentadiene were unsuccessful. Also, they report that 3-nitro-3-hexene, 2-nitro-4-methyl-2-pentene and 2-nitro-4-ethyl-2-hexene, which would yield tertiary nitro adducts, did not add to cyclopentadiene. Similarly, it has been found[10] that β-methyl-β-nitrostyrene and β-ethyl-β-nitrostyrene do not react with cyclopentadiene in refluxing toluene solution, the only product being dicyclopentadiene.

The Diels–Alder reaction of cyclopentadiene with a nitroölefin may be regarded as involving a competition between two dienophiles, the nitroölefin and cyclopentadiene itself. Whether or not a nitroölefin adduct is formed will depend upon the relative reactivity of the nitroölefin and cyclopentadiene, acting as a dienophile, except at temperatures sufficiently high so that the formation of dicyclopentadiene is reversible. The great tendency of the simplest non-aromatic nitroölefins to polymerize is a complicating factor. Refluxing the simplest 2,2-disubstituted nitroölefin, 2-methyl-1-nitropropene, which shows very little tendency to polymerize, with dicyclopentadiene at 150–180° gave the nitro adduct in 10–15% yield (not isolated in pure form),[11] but in our hands under the mild conditions of refluxing with cyclopentadiene on the steam-bath, dicyclopentadiene was the only product isolated.

In an attempt to obtain tertiary nitro adducts, we have carried out reactions between cyclopentadiene and the simplest 1- and 1,2-disubstituted nitroölefins. 2-Nitropropene reacted vigorously with cyclopentadiene and, after considerable purification, a waxy solid adduct, m.p. 104°, was obtained in 27% yield. 2-Nitro-2-butene also reacted, but without vigor, and another waxy solid adduct, m.p. 84.5–86.5°, was obtained in 41% yield.



R = H or CH_3

To our knowledge, these are the first examples of the formation of tertiary nitro compounds by the Diels–Alder reaction. The tertiary nitro adducts have considerably higher melting points than those of the secondary nitro adducts previously reported.

Experimental

Melting points were determined on a calibrated thermometer.

5-Nitro-5-methylbicyclo[2,2,1]-2-heptene (With Paul E. Swartzentruber).—The procedure[2,5] for the preparation of 5-*endo*-nitro-6-*exo*-methylbicyclo[2,2,1]-2-heptene was adapted for use here.

Previously cooled freshly cracked cyclopentadiene (87.8 g., 1.33 moles), glacial acetic acid (65 cc.), 2-nitropropene[12] (90.6 g., 1.04 moles) and hydroquinone (0.1 g.) were mixed at about 10°, where no apparent reaction took place. The solution was warmed gently to 30° and heating was discon-

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(9) D. Nightingale and J. R. Janes, THIS JOURNAL, **66**, 352 (1944).

(10) With Paul Melnychyn and Gerald R. Modig in these laboratories.

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(12) (a) B. M. Vanderbilt and H. B. Hass, *Ind. Eng. Chem.*, **32**, 34 (1940); (b) G. D. Buckley and C. W. Scaife, *J. Chem. Soc.*, 1471 (1947).