outlet tube. The mixture was warmed to 35–38°, and 1.7 g. (0.026 mole) of sodium azide was added slowly with vigorous stirring. The reaction was slightly exothermic; and by controlling the rate of addition of the azide, the reaction temperature was maintained at 38–40°. After the addition was complete, the suspension was stirred for one-half hour and allowed to stand for 1.5 hours more. The reaction mixture was then added to 50 ml. of water; after shaking, the aqueous layer, which contains the amine salt, was separated. To this solution was added an excess of aqueous sodium hydroxide; subsequent steam distillation of the free amine into excess 10% hydrochloric acid gave a solution of the amine hydrochloride. The solvent was evaporated under reduced pressure. Recrystallization of the residue from methanol–ethyl acetate afforded a 52% yield of the pure hydrochloride, m.p. 267–270°.

(B).—The amine was also prepared by reduction of the dihydro nitro adduct (IV) with iron and acetic acid.[10] The melting point of the hydrochloride, 268–269°, was undepressed by that of the material described above. The melting point (200–203°) of the urea derivative from (A) also showed no depression upon admixture with the same derivative from (B), m.p. 200–202.5°. The melting point reported for the urea derivative is 203°.[10]

MADISON, WISCONSIN

[CONTRIBUTION FROM THE DEPARTMENT OF BACTERIOLOGY AND IMMUNOLOGY, HARVARD MEDICAL SCHOOL, AND THE DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY]

The Stereochemical Specificity of the Oxidation of Cyclitols by Acetobacter Suboxydans[1,2]

BY BORIS MAGASANIK, ROBERT E. FRANZL AND ERWIN CHARGAFF

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The study of the oxidation of cyclitols by *Acetobacter suboxydans* has been extended to two new desoxyinositols, related to *epi*-inositol (I), one a racemic mixture (VI + VII), the other the corresponding levorotatory isomer (VI). Only the dextrorotatory desoxyinositol (VII) was found to be attacked. The structure of the resulting monoketone (VIII) was determined. These results permit the stricter definition of the rules predicting the type of cyclitol hydroxyls that can be oxidized by the enzyme system. The specific steric requirements for oxidation can be defined by the statement that *only polar hydroxyl groups are oxidized* and that *the carbon atom in meta position to the one carrying the polar hydroxyl group (in counterclockwise direction, if north polar; clockwise, if south polar) must carry an equatorial hydroxyl group.*

In previous communications[3] the oxidative action of *Acetobacter suboxydans* on various polyhydroxy derivatives of cyclohexane, belonging to the inositol and quercitol series, has been described. There exists a considerable body of physical evidence for the chair form of the cyclohexane ring[4]; and when this formulation was extended to its polyhydroxy derivatives, it could be shown[3b,c,d] that only those hydroxyl groups that were located in a polar plane could be oxidized by the biological system, a conclusion borne out by more recent observations[5] on the oxidation of *d*- and *l*-viburnitol by the same organism. It has, however, already been pointed out[3b] that, while none but polar hydroxyl groups were attacked, not all polar hydroxyl groups were so treated. The present paper describes further studies, leading to a more stringent definition of the rules predicting which polar hydroxyl groups can be oxidized.

The compounds discussed here are listed in Table I in two sets of terms based on the numbering systems proposed by us[3b] and by Fletcher, *et al.*,[6] respectively.

(1) This work was supported in part by grants from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council and from the Nutrition Foundation.

(2) A portion of the material was presented at the American Chemical Society Meeting in Washington, D. C., September, 1948 [B. Magasanik and E. Chargaff, Abstracts, 114th Meeting, American Chemical Society, 35 C (1948)].

(3) (a) E. Chargaff and B. Magasanik, *J. Biol. Chem.*, **166**, 379 (1946); (b) B. Magasanik and E. Chargaff, *ibid.*, **174**, 173 (1948); (c) B. Magasanik and E. Chargaff, *ibid.*, **175**, 929 (1948); (d) B. Magasanik and E. Chargaff, *ibid.*, **175**, 939 (1948).

(4) O. Hassel, *Tids. Kjemi, Bergvesen Met.*, **3**, 32 (1943); *C. A.*, **39**, 2244 (1945); R. S. Rasmussen, *J. Chem. Phys.*, **11**, 249 (1943); C. W. Beckett, K. S. Pitzer and R. Spitzer, THIS JOURNAL, **69**, 2488 (1947); F. D. Rossini and K. S. Pitzer, *Science*, **105**, 647 (1947).

(5) T. Posternak, *Helv. Chim. Acta*, **33**, 350, 1594 (1950).

(6) H. G. Fletcher, Jr., L. Anderson and H. A. Lardy, *J. Org. Chem.*, **16**, 1238 (1951).

TABLE I

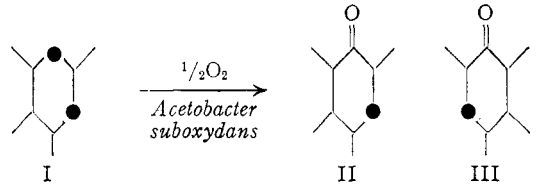
POLYHYDROXY DERIVATIVES OF CYCLOHEXANE

No.	Compound Designations previously employed	Numbering system: Present authors[3b]	Numbering system: Fletcher, *et al.*[6]
I	*epi*-Inositol	Cyclohexane-(1,2,3,4,5)-*cis*-hexol	*epi*-Inositol
II	*l-epi*-Inosose	Cyclohexane-(1,3,4,5) *cis*-pentol-2-one	D-2-Keto-*epi*-inositol
III	*d-epi*-Inosose	Cyclohexane-(1,2,3,5) *cis*-pentol-4-one	L-2-Keto-*epi*-inositol
IV	*meso*-Inosose	Cyclohexane-(1,3,5) *cis*-pentol-2-one	2-Keto-*myo*-inositol
V	Desoxyscyllitol	Cyclohexane-(1,3,5) *cis*-4,6-pentol	2-Desoxy-*myo*-inositol
VI		Cyclohexane-(1,3,4,5) *cis*-6-pentol	D-2-Desoxy-*epi*-inositol
VII		Cyclohexane-(1,2,3,5) *cis*-6-pentol	L-2-Desoxy-*epi*-inositol
VIII		Cyclohexane-(1,3,5) *cis*-6-pentol-2-one	D-2-Keto-4-desoxy-*epi*-inositol
IX	*meso*-Inositol	Cyclohexane-(1,2,3,5) *cis*-hexol	*myo*-Inositol
X	*d*-Viburnitol	Cyclohexane-(1,2,5) *cis*-4,6-pentol	L-1-Desoxy-*myo*-inositol
XI	*d*-Inositol	Cyclohexane-(1,2,5) *cis*-hexol	D-Inositol
XII	*d*-Inosose	Cyclohexane-(1,2,5) *cis*-pentol-3-one	L-1-Keto-*myo*-inositol
XIII	*l*-Viburnitol	Cyclohexane-(1,4,5) *cis*-2,6-pentol	D-1-Desoxy-*myo*-inositol
XIV	*l*-Inositol	Cyclohexane-(1,2,4) *cis*-hexol	L-Inositol
XV		Cyclohexane-(1,2,4) *cis*-pentol-6-one	D-1-Keto-*myo*-inositol
XVI	*d*-Quercitol	Cyclohexane-(1,2,5) *cis*-3,6-pentol	L-2-Desoxy-*muco*-inositol

For the purposes of the following discussion the spatial constellations of the compounds are of greater significance than their planar projections. The constellations of the substances, numbered as in Table I, will therefore be presented schematically: north polar hydroxyls are indicated by full

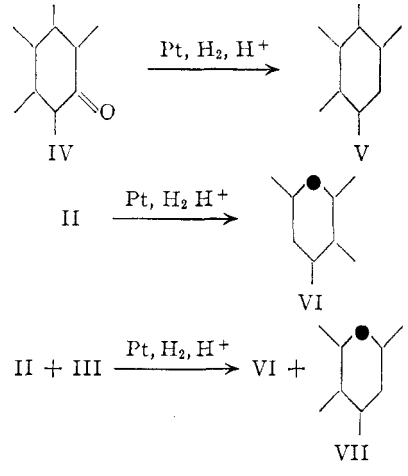
circles, south polar hydroxyls by open circles, equatorial hydroxyls by lines; the hydrogen atoms are not shown. The planar structures have been given in previous publications.[3b,c,d]

The oxidation of I by *Acetobacter suboxydans* yields optically active II,[3a,b,7] only one of the two polar hydroxyl groups located in the same plane having been attacked. While II is thus shown to resist further oxidation, the *d,l*-compound III + II is found to consume 0.25 mole of oxygen when subjected to the action of the microörganism. This indicates that the dextrorotatory isomer III



can be further oxidized, presumably to a diketone.[3b] This difference in susceptibility to oxidation made it appear of interest to study the action of *Acetobacter suboxydans* on the pair of optically isomeric desoxyinositols, derived from *epi*-inositol (I) by the alternate replacement of each of the two polar hydroxyl groups by hydrogen.

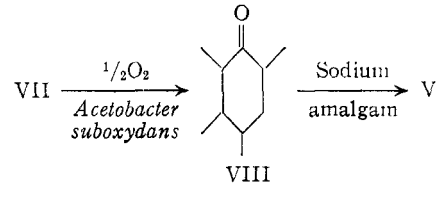
By the catalytic reduction, under acidic conditions,[5,8] of IV the desoxy compound V was prepared. The same procedures led from II to the levorotatory desoxy compound VI and from racemic II + III to racemic VI + VII.



These compounds were exposed to resting cells of *Acetobacter suboxydans* in the Warburg apparatus, as described previously.[3b] Compounds V and VI were not attacked by the organism; the racemic desoxyinositol VI + VII was oxidized with the uptake of 0.25 mole of oxygen. When the oxidation of VI + VII was carried out with resting bacteria on a preparative scale by the technique described previously,[3c,d] the reaction mixture was found to reduce Benedict solution in the cold. Paper chromatography revealed the presence of a single strongly reducing component. The reaction fluid yielded an insoluble phenylhydrazone; and from the filtrate of the latter an optically active desoxyinositol could be isolated which proved identical with compound VI, prepared by the acidic reduction of II. These results showed clearly that only one of the desoxy isomers, compound VII, was susceptible to oxidation by *Acetobacter suboxydans*, yielding a monoketone.

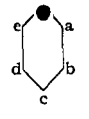
The attempt to recover this oxidation product by the cleavage of its phenylhydrazone with benzaldehyde in the usual manner[3b,c] afforded a crude monoketone preparation which failed to yield analytically pure material even after repeated crystallization from various solvents. The recrystallized product was shown by paper chromatography to contain two strongly reducing components. The major, faster moving component was identical with the one mentioned before as solely present in the original oxidation mixture. The other compound, contaminating the monoketone, must therefore have been an artifact introduced in the course of the formation or the cleavage of the phenylhydrazone.

The structure of the product of the biological oxidation of VII was determined by its reduction, without prior isolation, to a mixture of desoxyinositols. This was accomplished by treating the reaction mixture, obtained by the action of the resting bacteria on VI + VII, with sodium amalgam. One of the resulting desoxyinositols, isolated by virtue of its sparing solubility, proved identical with the desoxyscyllitol V obtainable, as mentioned before, from IV.

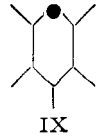


The structure shown as VIII is thus established for the monoketo compound; the oxidation of VII has taken place in agreement with the previously stated rule[3] that only polar hydroxyl groups can be oxidized by *Acetobacter suboxydans*.

The pairs of enantiomorphs of which, in each case, one member is oxidized by *Acetobacter suboxydans* are contrasted in Table II. It can be seen that all compounds susceptible to attack possess an equatorial hydroxyl group in position d relative to the location of the oxidizable polar hydroxyl group.



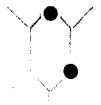
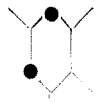
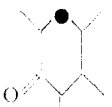
The replacement of the equatorial hydroxyl in d by a polar hydroxyl (compare structure Ib in Table II), by oxygen (II) or by hydrogen (VI) prevents the biological oxidation. Corresponding changes in position b are without effect. The validity of this generalization may be confirmed and its scope extended by considering the cyclitols studied previously.[3,5] *meso*-Inositol (IX) has five


IX

(7) T. Posternak, *Helv. Chim. Acta*, **29**, 1991 (1946).
(8) T. Posternak, *ibid.*, **24**, 1045 (1941).

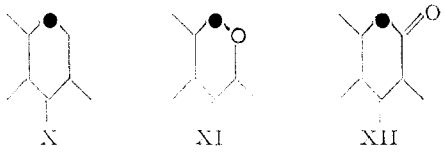
TABLE II

SELECTIVE OXIDATION OF POLAR HYDROXYL GROUPS BY *Acetobacter Suboxydans*

Oxidized[a]		Not oxidized[a]	
	Ia		Ib
	III		II
	VII		VI

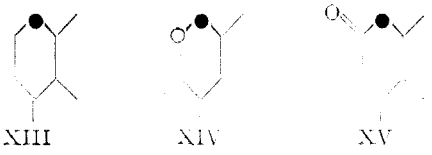
[a] The structures (numbered as in Table I) are so arranged as to place the polar hydroxyl groups under comparison at the top of the hexagon. For this reason, *epi*-inositol (I) is shown in two arrangements (Ia and Ib).

equatorial hydroxyl groups. The replacement of the equatorial hydroxyl in position a by hydrogen (X), by a south polar hydroxyl group (XI) or by oxygen (XII) does not interfere with the biological oxidation of the north polar hydroxyl.



X XI XII

Similarly, oxidation of the polar hydroxyl group may occur when the equatorial hydroxyl in position e is replaced by hydrogen (XIII) or by a south polar hydroxyl (XIV). The same is presumably also true of substitution by oxygen, since the monoketone XV may be assumed to be the intermediate in the oxidation of XIV to a diketone.[3b,c]



XIII XIV XV

The behavior of *d*-quercitol (XVI)[3d] shows that the equatorial hydroxyls in a and e may both be

XVI

replaced in that manner without effect on the oxidation. The influence of substitution of the equatorial hydroxyl group in position c cannot be appraised at present, since no compounds of appropriate structure were available for study.[9]

(9) Pinitol, a monomethyl ether of *d*-inositol (XI), previously shown not to be oxidized by *Acetobacter*,[3a,b] apparently carries the equatorial methoxy group in this position (A. B. Anderson, D. L. MacDonald and H. O. L. Fischer, Abstracts, XIIth International Congress of Pure and Applied Chemistry, New York, September, 1951, page 82). It is, however, possible that this relatively large substituent would prevent enzymatic oxidation regardless of its position.

The steric specificity of the oxidation of inositols and desoxyinositols by *Acetobacter suboxydans* may be defined as follows: (a) *Only polar hydroxyl groups are oxidized.* (b) *The carbon in meta position to the one carrying the polar hydroxyl group (in counter-clockwise direction, if north polar; clockwise, if south polar) must carry an equatorial hydroxyl group.*[9a]

Two points of contact between the substrate and the enzyme responsible for the specific oxidation are thus demonstrated. For an asymmetric synthesis, however, such as the formation of II from I, three points of contact are required.[10] This third point may be the equatorial hydroxyl group in position c, which all the compounds studied had in common. Alternatively, it may be represented by the equatorial hydrogen atom on the carbon carrying the polar hydroxyl or the polar hydrogen atom in position d, whose configurations are defined by the indispensable locations of the corresponding hydroxyls.

Further studies will be required before it will be possible to state to what extent the rules derived here for the inositols and desoxyinositols may be applied to straight-chain polyhydroxy compounds or to cyclic diols and triols,[11] substances having less rigid structures, in which the relative positions of the hydroxyl groups may change more easily.

Experimental

Material.—The cultivation of *Acetobacter suboxydans* and the preparation of compounds II, II + III and IV have been described in a previous publication.[3b] Desoxyscyllitol (V), m.p.[12] 233–234°, and the corresponding pentaacetate, m.p. 191–192°, were prepared from IV, as described by Posternak.[7]

***d,l*-Desoxyinositol (VI + VII).**—Because of the low solubility of II + III, this *d,l*-compound is best added in several portions during the course of the reaction. In a typical experiment, 5 g. of II + III was dissolved in 150 cc. of 0.1 *N* hydrochloric acid and shaken with 3 g. of platinum oxide in an atmosphere of hydrogen. Three additional 5-g. portions of II + III were added, each after the uptake of about 1600 cc. of hydrogen by the preceding portion. The reaction was finally allowed to proceed until no more hydrogen was absorbed. A total of 7780 cc. of hydrogen was consumed in 33 hours, corresponding to about three moles of hydrogen per mole of II + III (after correction for the reduction of the catalyst), instead of two moles required theoretically for the reduction to desoxyinositol. The addition of methanol to the heavy sirup, obtained after the evaporation *in vacuo* of the filtrate from the catalyst, resulted in the deposition of 10.9 g. (59% of the theoretical yield) of white, crystalline material, melting between 198 and 204°. Two recrystallizations from aqueous methanol yielded 6.2 g. (34%) of micro-crystalline *d,l*-desoxyinositol (VI + VII), melting at 214–215°.

Anal.[13] Calcd. for $C_6H_{12}O_5$ (164.16): C, 43.90; H, 7.37. Found: C, 43.82; H, 7.43.

(9a) NOTE ADDED IN PROOF.—It has recently been observed by one of us (R. E. F.) that *muco*-inositol (G. Dangschat and H. O. L. Fischer, *Naturwiss.*, **27**, 756 (1939)), a sample of which was most generously supplied by Dr. H. O. L. Fischer of the University of California, Berkeley, Calif., consumed 1 mole of oxygen when treated with *A. suboxydans*. This may be considered as additional support for the rules formulated here, since in this isomer, which is cyclohexane-(1,2,4,5)-*cis*-hexol with north polar hydroxyls 2 and 4 and south polar hydroxyl 3, the north polar hydroxyl in position 4 would be expected to be unavailable for oxidation.

(10) A. G. Ogston, *Nature*, **162**, 963 (1948).

(11) T. Posternak and F. Ravenna, *Helv. Chim. Acta*, **30**, 441 (1947).

(12) The melting points, reported without correction, were determined with an electrically heated stage (Fisher–Johns).

(13) We are indebted to Miss R. Rother for several microanalyses; others were carried out by the Elek Micro Analytical Laboratories, Los Angeles, Cal.

The pentaacetate of VI + VII, prepared by the treatment of the *d,l*-desoxyinositol with acetic anhydride in the presence of anhydrous zinc chloride, formed needles melting after recrystallization from ethanol at 113–115°.[14]

Anal. Calcd. for $C_{16}H_{22}O_{10}$ (374.34): C, 51.33; H, 5.92. Found: C, 51.28; H, 5.98.

l-Desoxyinositol (VI).—A solution of 356 mg. (0.002 mole) of *l-epi*-inosose (II) in 10 cc. of 0.1 *N* hydrochloric acid was shaken with 150 mg. of platinum oxide in an atmosphere of hydrogen. The consumption of 163 cc. of hydrogen (2.6 moles per mole of II, after correction for the reduction of the catalyst) was completed in 3 hours. The catalyst was removed by filtration and the filtrate evaporated *in vacuo*, when a light sirup was obtained, which was dried *in vacuo* over phosphorus pentoxide. The addition of methanol resulted in the deposition of 130 mg. (40%) of white crystals melting between 174 and 179°. The crude material was purified by five recrystallizations from aqueous methanol. The microcrystalline *l*-desoxyinositol obtained melted sharply at 194°. The substance was levorotatory in water (*c* 1.01, *l* 2 dm., α_D −0.11° ± 0.01°); $[\alpha]_D^{27}$ −5.4 ± 0.4°.

Anal. Calcd. for $C_6H_{12}O_5$ (164.2): C, 43.9; H, 7.4. Found: C, 44.0, H, 7.3.

Action of Acetobacter Suboxydans; Oxygen Consumption.—The determinations were carried out by allowing a bacterial suspension containing about 1.5 mg. of bacterial nitrogen to act on about 10 micromoles of the desoxyinositols in a total volume of 3 cc. of *M*/15 phosphate buffer of *p*H 6.0 at 36° in the presence of air. The uptake of oxygen was 0.24 mole per mole of *d,l*-desoxyinositol (VI + VII). Desoxyscyllitol (V) and *l*-desoxyinositol (VI) were not oxidized.

Enzymatic Oxidation of *d,l*-Desoxyinositol (VI + VII). A. Isolation of *l*-Desoxyinositol (VI).—To a solution of 6.2 g. (0.038 mole) of VI + VII in 75 cc. of *M*/15 phosphate buffer of *p*H 6.0, 25 cc. of a suspension of washed *Acetobacter suboxydans* (about 0.5 g. dry weight) was added. The mixture was shaken under oxygen at 37°. Oxygen uptake ceased after 250 cc. of oxygen (0.26 mole per mole of substance) had been consumed in 3 hours. The bacteria were removed by centrifugation in the cold (4000 r.p.m., 30 min.) and filtration through infusorial earth. The filtrate reduced Benedict solution in the cold.

The reducing material could be demonstrated by ascending paper chromatography of 0.01-cc. volumes of the filtrate, with *n*-butanol–diethylene glycol–water (3:1:1, by volume) as the solvent. The dried paper was sprayed with a reagent composed of two volumes of 0.4% aqueous potassium ferricyanide and one volume of 1.6% aqueous sodium carbonate containing 0.025% sodium cyanide. The treated papers were dried in a stream of warm air and sprayed with a solution of 5 g. of ferric sulfate in one liter of 6.5% aqueous phosphoric acid. This treatment caused the appearance of a single blue spot with an R_F value of 0.43. *meso*-Inosose (IV) treated in the same manner has an R_F value of 0.25.[15]

The volume of the filtrate was reduced *in vacuo* to about 30 cc. A solution of 3 cc. of phenylhydrazine in 6 cc. of 50% acetic acid was added to the chilled concentrate and the resulting insoluble phenylhydrazone removed by filtration. The red filtrate was extracted repeatedly with ethyl ether and the aqueous phase evaporated to dryness *in vacuo*. The gummy residue was suspended in 50 cc. of ethanol and the mixture boiled under reflux for 3 hours. The semi-crystalline brown residue was collected by filtration, dissolved in a small amount of water and decolorized with activated charcoal. To the filtrate, diluted with 10 volumes of ethanol, ethyl ether was added in portions until no more of a yellow oil separated. The supernatant solution was decanted from the oily deposit and chilled. Crystallization, which began after about 12 hours, was allowed to continue for 8 weeks. At the end of this period 500 mg. of white crystals, melting at 187–189°, was collected. This material did not reduce Benedict solution; it melted, after two recrystallizations from methanol, sharply at 194°. The admixture of *l*-desoxyinositol (VI) did not lower the melting point.

B. Reduction to Desoxyscyllitol (V).—A solution containing 460 mg. (0.0028 mole) of VI + VII in 10 cc. of phosphate buffer was shaken with washed bacteria (0.1 g. dry weight) in an atmosphere of oxygen at 37°; 18 cc. of oxygen (0.26 mole per mole of substrate) was consumed in 2 hours. The bacteria were removed, as described before; the filtrate was acidified with acetic acid and shaken with 2-g. portions of 2.5% sodium amalgam. The reaction mixture was kept neutral by the addition of acetic acid. After 16 g. of sodium amalgam had been added, the mixture was no longer reducing. The dry evaporation residue of the filtrate was refluxed for 5 minutes with 5 cc. of acetic anhydride in the presence of zinc chloride. The excess reagent was decomposed by the addition of 4 volumes of water. The chilled mixture deposited 460 mg. of crystalline material, which was extracted with 5 cc. of boiling ethanol. The insoluble residue (190 mg.), recrystallized twice from ethanol, yielded 16 mg. of the pentaacetate of desoxyscyllitol (V), melting at 191–192°. The admixture of an authentic specimen of this derivative produced no lowering of the melting point.

Thirty mg. of this pentaacetate, obtained in another experiment, was dissolved in a small volume of boiling ethanol and treated with 1 cc. of a 0.5 *N* solution of barium hydroxide in methanol. The precipitate that formed was dissolved by the addition of water and the solution was heated in a boiling water-bath for 15 minutes. The required amount of sulfuric acid was added to precipitate barium as the sulfate which was removed by filtration through activated charcoal. From the filtrate 9 mg. of desoxyscyllitol (V) was obtained, melting at 234–236°. The mixed melting point with V prepared from IV was also 234–236°.

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(14) The *d,l*-desoxyinositol (VI + VII) and its pentaacetate have, after the communication of our preliminary results,[2] been independently described by E. L. May and E. Mosettig, *J. Org. Chem.*, **14**, 1137 (1949).

(15) Sugars that reduce Benedict reagent only after being heated do not give colored spots in this procedure.