when a solution of benzophenone in pyridine containing lithium chloride is electrolyzed between aluminum electrodes is explainable on the same basis as cited above.

It was noted that the anolyte following electrolysis of solutions of benzophenone between aluminum electrodes was incapable of corroding massive aluminum but did attack a magnesium rod. This behavior, in our opinion, is merely a reflection of the difference in reactivity of the two metals toward the ketyl radical-ion (the precursor of the pinacolate ion) generated in the anolyte by the action of bipositive aluminum on benzophenone. The fact that neither magnesium nor aluminum is attacked by the catholyte after electrolysis is consistent with our interpretation of the mechanism for the formation of pinacol in the catholyte, provided the assumption is made that lithium pinacolate is not dissociated appreciably. Evidence for the small degree of disssociation of this salt has been obtained by magnetic susceptibility measurements.[21,22]

The one puzzling feature of the investigation was the fact that V_i-values for aluminum substantially lower than 3 were obtained in sodium iodide–pyridine solutions in the absence of benzophenone. Attempts were made to isolate a salt of lower valent aluminum but without success.[23] However the anolyte solution following electrolysis possessed reducing power as demonstrated by its ability to reduce benzophenone to the conjugate use of benzopinacol.

Acknowledgment.—The authors are indebted to the Office of Ordnance Research, U. S. Army, for a research grant which has made this investigation possible.

(21) S. Sugden and F. L. Allen, *J. Chem. Soc.*, 440 (1936).

(22) E. Muller and W. Janke, *Z. Elektrochem.*, **45**, 380 (1939).

(23) It is of interest that the only lower valent aluminum compound reported to have been isolated in the solid state is AlI. This compound was formed by the use of an electrodeless discharge produced in the vapor of AlI_3 at a temperature below 50° (W. C. Schumb and H. H. Rogers, THIS JOURNAL, **73**, 5806 (1951)).

LAWRENCE, KANSAS

[CONTRIBUTION FROM THE BIOLOGY DEPARTMENT, BROOKHAVEN NATIONAL LABORATORY, UPTON, L. I., N. Y.]

Competitive Inhibition by Substrate during Enzyme Action. Evidence for the Induced-fit Theory[1,2]

BY JOHN A. THOMA AND D. E. KOSHLAND, JR.

RECEIVED NOVEMBER 9, 1959

The Schardinger dextrins, cyclohexa- and cycloheptaamylose and the internal segments of the starch molecule act as competitive inhibitors of sweet potato beta-amylase. These facts are shown to add support for the induced-fit theory of specificity and to suggest that this new type of competitive inhibition by substrate exists in other enzyme systems.

Introduction

Beta-amylase hydrolyzes only the penultimate linkage of its normal substrate, a linear starch chain. Since the linear portions of the starch can be considered to be a chain of "maltose units," this means that the enzyme has a way of distinguishing the terminal "maltose unit" from all the other "maltose units." This type of subtle distinction is typical of enzymes and hence studies on beta-amylase were performed to clarify the action of this enzyme in particular and the specificity pattern of enzymes in general.

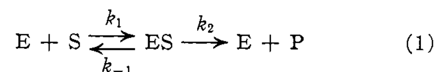
The terminal maltose unit differs from the internal maltose units by the presence of an unsubstituted hydroxyl at the C4 position. It would be desirable, therefore, to test the binding affinity of compounds which had maltose units but no free C4 hydroxyl. Accordingly, cyclohexa- and cycloheptaamylose, which are molecules in this category, were investigated and found to be competitive inhibitors.

This ability of the cycloamyloses to form complexes with beta-amylase suggested that binding of the internal segments of starch chains would occur. Such "competitive inhibition" by the internal segments would be obscured in a kinetic treatment of hydrolysis because the inhibitor is a part of the substrate itself. The *relative* amount of inhibition, however, is a function of the chain length of the substrate and variation of polymer length could provide evidence that such inhibition occurs.

These facts can be shown to be inconsistent with a template-type specificity pattern[3] but to be readily explainable by an induced-fit specificity theory[4] of enzyme action.

Theoretical

Inhibition by Internal Segments of a Polymer Chain.—The conventional equations for competitive inhibition are



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P \qquad (1)$$

$$E + I \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} EI \qquad (2)$$

$$K_m = \frac{k_{-1} + k_2}{k_1} \qquad (3)$$

$$K_i = \frac{k_{-3}}{k_3} \qquad (4)$$

$$\frac{1}{v} = \frac{1}{V_m}\left[1 + \frac{K_m}{(S)}\left(1 + \frac{(I)}{K_i}\right)\right] \qquad (5)$$

Inhibition by internal segments of a homogeneous polymeric chain cannot be demonstrated with

(1) A preliminary note of some of this work has been published. Abst. Amer. Chem. Soc. Meeting, Sept. 1959, p. 40c.

(2) Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

(3) E. Fischer, *Ber.*, **27**, 2985 (1894).

(4) D. E. Koshland, Jr., *Proc. Natl. Acad. Sci., U. S.*, **44**, 98 (1958)

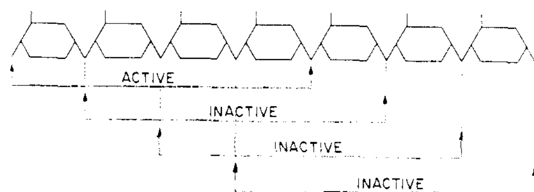


Fig. 1.—Schematic representation of active and inactive complexes of a heptaose with beta-amylase in which four consecutive groups are necessary for binding and in which a free terminal C4 group is necessary for enzyme action after binding has occurred.

these classical equations since the concentration of inhibitor is directly proportional to the concentration of substrate. Thus, for any one substrate, variation of inhibitor concentration cannot be performed independently of variation of substrate concentration. In Fig. 1, possible active and inactive complexes of beta-amylase with maltoheptaose are depicted. Generalization of this relationship shows that for any polymer of n units complexed by an exo-ase there will be one active and n-m inactive complexes, where m represents the number of monomers over which the binding site is extended and n is the number of monomers in the polymer. Thus the concentration of inhibitor is given by equation 6. Substitution of this expression in equation 5 will

$$(\mathrm{I}) = (n - m)(\mathrm{S}) \qquad (6)$$

give equation 7. Equation 7 can be simplified

$$\frac{1}{v} = \frac{1}{V_\mathrm{m}}\left(1 + \frac{K_\mathrm{m}}{(\mathrm{S})} + (n - m)\frac{K_\mathrm{m}}{K_\mathrm{i}}\right) \qquad (7)$$

into the classical Michaelis–Menten formulation of equation 8 in which, however, V_m' and K_m' are

$$\frac{1}{v} = \frac{1}{V_\mathrm{m}'}\left(1 + \frac{K_\mathrm{m}'}{(\mathrm{S})}\right) \qquad (8)$$

complex functions defined by equations 9 and 10. Thus a typical Lineweaver–Burk plot will produce

$$V_\mathrm{m}' = V_\mathrm{m}\left(\frac{1}{1 + (n - m)(K_\mathrm{m}/K_\mathrm{i})}\right) \qquad (9)$$

$$K_\mathrm{m}' = K_\mathrm{m}\left(\frac{1}{1 + (n - m)(K_\mathrm{m}/K_\mathrm{i})}\right) \qquad (10)$$

a straight line but the inhibition constant K_i will be concealed in the observed Michaelis constant, K_m', and the observed maximum velocity, V_m'. However, both V_m' and K_m' are functions of n-m and determination of these constants as a function of n-m can test the theory. If inhibition by internal segments occurs, plots of $1/k_\mathrm{m}'$ *vs.* n-m and $1/V_\mathrm{m}'$ *vs.* n-m will be straight lines. In the former case, the slope will be $1/K_\mathrm{i}$ and the intercept $1/K_\mathrm{m}$. In the latter case, the slope will be $K_\mathrm{m}/K_\mathrm{i}$ and the intercept $1/V_\mathrm{m}$.

Inhibition by the Cyclohexa- and Cycloheptaamylose.—The most conventional method for determining the competitive inhibitor constant is by a Lineweaver–Burk[5] plot in the presence and absence of an inhibitor. This procedure is technically impossible with starch because its effective molecular weight is unknown and thus the calculation of substrate concentration is precluded. However, a method which circumvents this difficulty has been described by Dixon,[6] who suggested that a plot of $1/v$ against (I) at two different substrate concentrations be made. If the two lines intersect above the (I) axis, the inhibitor is competitive and projection of this point on the (I) axis is $-K_\mathrm{i}$. If the lines intersect on the (I) axis, the inhibitor is non-competitive.

(5) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

Experimental

Materials.—The sweet potato beta-amylase used was the twice recrystallized material prepared by the procedure of Balls, *et al.*,[7] and purchased from the Worthington Biochemical Company.

Crystalline methyl-α-maltotetraoside was kindly furnished by Dr. John Pazur.

Cyclohexa- and cycloheptaamylose were donated by Dr. Robert Baldwin and their per cent purity determined polarimetrically.[8]

The preparation of amylodextrin has been described elsewhere.[9] The preparation used in these studies had an average chain length of 24 units determined by Nelson's[10] procedure with the modified heating time suggested by Whelan.[11] The per cent. moisture was measured by determining the total carbohydrate content of an accurately weighed sample by the anthrone procedure.[12]

Preparation of Enzyme Solutions.—Solutions of beta-amylase were prepared by centrifuging several mg. of the Worthington suspension in a micro test tube (5 mm. glass tube sealed at one end), discarding the supernatant liquid and dissolving the crystals in 0.16 to 0.2 M acetate buffer at pH 4.8.

Determination of K_m'.—In a typical run, 1 ml. of enzyme and 1 ml. of a series of substrate solution (prepared by accurate dilution of exactly weighed amounts), at reaction temperature, were rapidly mixed and incubated for 10 min. Hydrolysis was terminated by the addition of 1 ml. of Nelson's copper reagent and the extent of the reaction was determined by measuring the color in a Beckman DU spectrophotometer at 660 mμ. In all cases appropriate substrate blanks were determined.

Measurement of Inhibition Constants of Cycloamyloses.—In a typical run, 1 ml. of beta-amylase was added to a series of tubes containing 1 ml. of a starch–cycloamylose solution. The concentration of starch was held constant but the concentration of the cycloamylose was systematically varied. After incubation at constant temperature for 10 min., the hydrolysis was arrested by addition of 1 ml. of Nelson's copper reagent and the maltose liberated, measured as above.

In all cases, the measured velocities are initial velocities, determined when the extent of hydrolysis was 10% or less.

Results

To test the possibility of competitive inhibition by the cycloamyloses, the method of kinetic analysis proposed by Dixon[6] was used and the results are shown in Fig. 2. It is seen that both the cyclohexa- and the cycloheptaamylose curves intersect above the abscissa indicating competitive inhibition. By projecting the point of intersection onto the abscissa the inhibitor constants (K_i) are obtained and found to be 0.50×10^{-3} for cyclohexaamylose and 0.48×10^{-3} for the cycloheptaamylose. The results, therefore, established that within experimental error cyclohexa- and cycloheptaamylose are equally tightly bound to the enzyme.

(6) M. Dixon, *Biochem. J.*, **55**, 170 (1953).

(7) A. K. Balls, M. K. Walden and R. R. Thompson, *J. Biol. Chem.*, **173**, 9 (1948).

(8) D. French, in "Advances in Carbohydrate Chemistry," Vol. XII, M. L. Wolfrom, editor, Academic Press, Inc., New York, N. Y., 1957, p. 189.

(9) J. A. Thoma, H. B. Wright and D. French, *Arch. Biochem. Biophys.*, **85**, 452 (1959).

(10) N. Nelson, *J. Biol. Chem.*, **153**, 375 (1944).

(11) W. J. Whelan, J. M. Bailey and P. J. P. Roberts, *J. Chem. Soc.*, 1293 (1953).

(12) R. J. Dimler, W. C. Schaefer, C. S. Wise and C. E. Rist, *Anal. Chem.*, **24**, 1411 (1952).

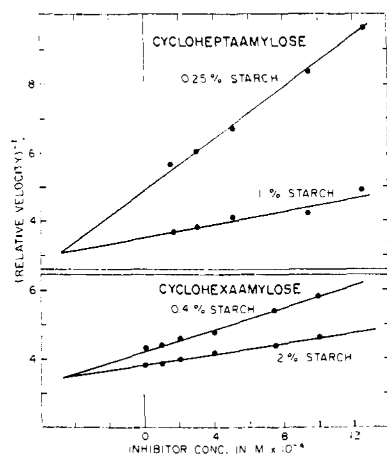


Fig. 2.—Evaluation of inhibitor constants for cyclohexa-amylose and cycloheptaamylose. Conditions: 0.1 M sodium acetate–acetic acid buffer, pH 4.8, 25.0 ± 0.1°.

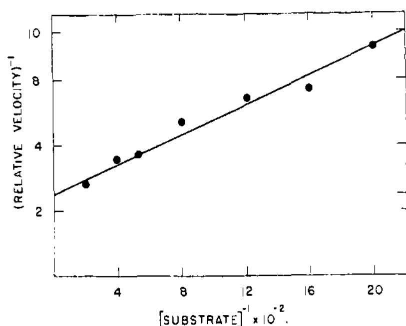


Fig. 3.—Kinetics of hydrolysis of methyl-α-maltotetra-oside by beta-amylase. Conditions: 0.08 M sodium acetate–acetic acid buffer at pH 4.8, 25.0 ± 0.1°.

Figure 3 is a typical Lineweaver–Burk plot for the hydrolysis of methyl-α-maltotetraoside. A reasonable fit to a straight line is obtained from which a Michaelis constant of 0.89 ± 0.05 × 10^{-3} M is calculated. In Fig. 4 an equivalent plot for the 24-unit amylodextrin is shown. From the straight line at 25°, a K_m' of 1.23 ± 0.03 × 10^{-4} is obtained. The K_m' for a 44-unit amylodextrin has been measured by Bailey and French[13] at 35°. To correct this value to 25° the K_m' of the 24-unit amylodextrin was measured at 35° as well as 25°. The K_m' at 35° for the 24-unit polysaccharide is 1.72 ± 0.06 × 10^{-4} and, using the appropriate ratio, this gives a K_m' for a 44-unit amylodextrin at 25° of 0.53 ± 0.03 × 10^{-4}.

The equations derived in the theoretical section show that if binding by internal segments of the polysaccharide chain occurs, the reciprocal of the observed Michaelis constants should be a linear function of n-m. For this particular system, a value of m = 4 was chosen because beta-amylase hydrolyzes maltotetraose at approximately the same rate as maltopentaose and maltohexaose but several orders of magnitude faster than maltotriose.[11,14] It is seen in Fig. 5 that a plot of the reciprocal of K_m' *versus* n-4 gives a straight line as predicted by this theory.

(13) J. M. Bailey and D. French, *J. Biol. Chem.*, **226**, 1 (1957).
(14) J. M. Bailey and W. J. Whelan, *Biochem. J.*, **67**, 540 (1957).

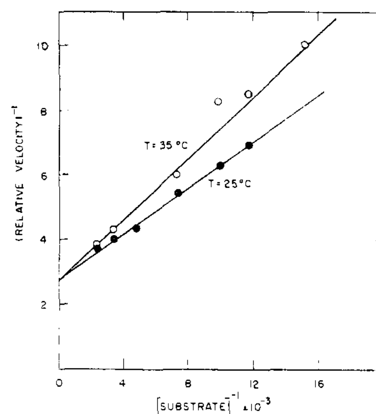


Fig. 4.—Kinetics of hydrolysis of an amylodextrin having an average chain length of 24 glucose units. Conditions: 0.1 M sodium acetate–acetic acid buffer at pH 4.8, 25.0 ± 0.1°. (No particular significance should be attached to the common intercept because different enzyme concentrations were used.)

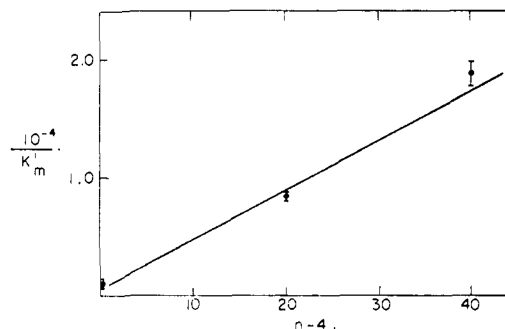


Fig. 5.—Effect of chain length (n) on the observed Michaelis constant (K_m') for substrates of beta-amylase assuming an active site spanning 4 monomer units (m = 4).

It is possible to evaluate from Fig. 4 and equation 7 both the K_m of the "true" substrate, *i.e.*, the terminal end of the polysaccharide, and the K_i for the interior segments. These values and the corresponding K_i values for the cyclohexa- and cycloheptaamylose are summarized in Table I.

TABLE I

MICHAELIS CONSTANT AND INHIBITOR CONSTANTS FOR INTERACTION OF POLYGLUCOSE COMPOUNDS WITH BETA-AMYLASE

Compound	K_m or apparent K_i in $M \times 10^3$	Effective K_m or K_i in $M \times 10^3$
Nonreducing end of starch chain	0.89 ± 0.05 (K_m)	0.89 ± 0.05
Internal segment of starch chain	2.35 ± .15 (K_i)	2.35 ± .15
Cyclohexaamylose	0.50 ± .04 (K_i)	3.00 ± .24[a]
Cycloheptaamylose	0.48 ± .03 (K_i)	3.36 ± .21[a]

[a] Observed K_i is multiplied by 6 for cyclohexaamylose and by 7 for cycloheptaamylose to correct for statistical probability of binding.

It is to be noted that the K_m for the nonreducing end of the starch chain is close to the value of K_i. Since the K_m is always equal to or larger than the true dissociation constant, k_{-1}/k_1, the actual binding of the nonreducing end of the starch chain is apparently even stronger than either of the cyclic

polymers or the internal segments. The similarities of the values suggest that the binding constant for the nonreducing end is equal or almost equal to the observed K_m value.

The measured value of K_m is a function of the value chosen for m. The slope of the line is not very steep, hence changes in the value of K_m would not be very great for small changes in m. Thus, if the actual number of binding groups is five instead of four, a value of K_m of 7×10^{-4} would have been obtained.

Discussion

The internal maltose units of the starch molecule and the cycloamyloses are not hydrolyzed by beta-amylase. Since the only difference between the terminal and internal maltose units is the free unsubstituted hydroxyl at the C4 position, this group must play a key role in the specificity. On the basis of the template theory, this role could be explained either by assuming that this hydroxyl provides a key hydrogen bond without which no net attraction occurs or by assuming that the hydroxyl fits into a small cavity which is unable to accommodate glucosyl or polyglucosyl groups. Both of these explanations lead to the prediction that these analogs will not be bound to the active site of the enzyme. However, both of these analogs were shown to be competitive inhibitors and hence some other explanation is needed.

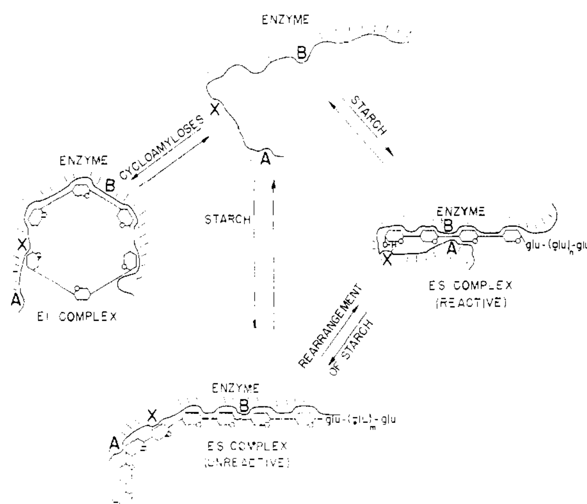


Fig. 6.—Schematic mechanism for beta-amylase specificity.

The induced-fit theory postulates that a compound while forming a complex with the enzyme may induce a change in protein conformation. If the compound is a substrate, this change brings the appropriate catalytic groups into alignment with each other and the bond to be broken. A compound, however, may have some of the structural features necessary for binding without producing the alignment necessary for enzyme action. In the case of beta-amylase, the binding of the internal segments and the cycloamyloses would not lead to reaction if the C4 hydroxyl is important in this alignment. A schematic illustration is shown in Fig. 6. When the terminal end of the starch chain is at the active site, the catalytic group A is induced into proper alignment with group B and the bond to be broken. The C4 hydroxyl may play either an active role by forming a hydrogen bond with the group X or a passive role by being small enough to allow the desired conformation (or a combination of the two). The cycloamyloses and internal segments, however, cannot induce such a folding of the protein and hence no reaction ensues even though the glucose units are bound at the active site. Figure 6 shows a gross folding of a portion of the protein but far more subtle changes in geometry are probably sufficient to promote enzyme action. The binding patterns presented in this work, therefore indicate that beta-amylase follows an induced-fit specificity pattern. It adds further support for the generality of the induced-fit theory since the substrate analog is larger than the substrate in this case whereas it was smaller or equal in size in previous examples.[4,15]

The quantitative values of K_i for the cyclic glucose polymers are further indications for flexibility of the enzyme. The seven-membered glucose polymer has an appreciably larger radius that the six-membered polymer and these molecules are rather rigid because of the steric restraints.[8] Hence, the close similarity of the binding constants of the six-membered and seven-membered rings indicate that the enzyme conforms to the substrate rather than *vice versa*. Moreover, the similarity in the corrected binding constants (see Table I) of the cycloamyloses and internal segments support the argument that the latter are acting as competitive inhibitors.

The discovery that a polymeric substrate can act as its own competitive inhibitor naturally could apply to any other exo-ase. Indeed, it has implications to enzymes in general, since there are many conceivable cases in which the substrate can be adsorbed on the active site in a number of different ways. Attachment in any except the "correct" manner would then lead to an inactive enzyme substrate complex and the substrate would act as a competitive inhibitor. The phenomenon of substrate competitive inhibition described here is distinctly different from the type in which two or more molecules[16] are adsorbed on the active site. In the latter instance, deviations from Michaelis–Menten kinetics are observed at high substrate concentrations. However, if only *one* substrate molecule is absorbed at the active site in such a manner that an unreactive complex is formed no change in the gross Michaelis–Menten kinetics will be observed. It has been shown in the theory section that this phenomenon results because the inhibitor concentration cannot be varied independently of the substrate concentration. In such systems, the observed K_m and V_m will be complex functions involving the "true" K_m, the number of ways in which inactive com-

(15) D. E. Koshland, Jr., in "The Enzymes," Revised ed., P. Boyer, H. Lardy and K. Myrback, editors, Academic Press, Inc., New York, N. Y., 1959. p. 305; D. E. Koshland, Jr., *J. Cell. Comp. Physiol.*, **54**, Supplement 1, 245 (1959).

(16) K. J. Laidler and J. P. Hoare, This Journal, **71**, 2699 (1949); J. B. S. Haldane, "Enzymes," Longmans, Green and Company, Ltd., London, 1930.

plexes can be formed and the inhibitor constants for these inactive complexes. Since V_m is the turnover number which will ultimately be of importance in evaluating the mechanism of enzymic catalysis, the existence of this type of inhibition should be kept in mind if a substrate might be attracted to the enzyme in more than one possible arrangement.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA]

The Absolute Configuration of the *myo*-Inositol 1-Phosphates and a Confirmation of the Bornesitol Configurations[1]

BY CLINTON E. BALLOU AND LEWIS I. PIZER[2]

RECEIVED NOVEMBER 30, 1959

An optically active *myo*-inositol 1-phosphate and *myo*-inositol 1-O-methyl ether have been synthesized from galactinol, a galactoside of *myo*-inositol in which the absolute configuration of the 1-position of the *myo*-inositol involved in the linkage is known. Thus, the absolute configurations of the two products are known. The *myo*-inositol 1-phosphate is the enantiomorph of the product obtained by base hydrolysis of soybean phosphoinositide, while the methyl ether is identical with (+)-bornesitol, a naturally occurring 1-o-methyl *myo*-inositol. (−)-Bornesitol has been synthesized from quebrachitol (1-O-methyl-(−)-inositol) by Anderson and Post. Thus, both enantiomorphs of bornesitol have been obtained by synthesis.

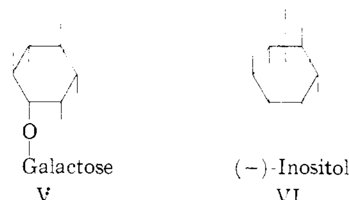
myo-Inositol occurs in nature in several conjugated forms. Being a *meso*-compound, it may, like glycerol, be substituted in such a way as to form optically active conjugates. Thus, three optically active monomethyl ethers have been described[2]; the *myo*-inositol part of galactinol[3] has been shown to be asymmetrically substituted with D-galactose[4]; and, it has recently been demonstrated that the *myo*-inositol moiety in the phosphoinositide from soybean[5] and from beef liver[6] is asymmetrically linked to glycerol phosphate.

The cyclic form of the inositols has led to some difficulty in nomenclature, since there is no direct way in which positions in the inositol rings can be related stereochemically to a straight chain reference such as glyceraldehyde. However, the generally accepted convention[7] for numbering the positions in the *myo*-inositol ring leads to the two mirror images below (I and II).

These differ only in the direction in which the ring is numbered, and for a substituted *myo*-inositol, that direction of numbering is followed which gives the substituent the lowest number.

While positions 2 and 5 have planes of symmetry, positions 1,3 and 4,6 form enantiomorphic pairs. Indeed, two of the monomethyl ethers of *myo*-inositol found in nature[8] correspond to the 1,3-pair of isomers, III and IV.

One is called (+)-bornesitol (III) and the other (−)-bornesitol (IV), the absolute configurations of the two having been established by the synthesis of the latter from quebrachitol (1-O-methyl-(−)-inositol).[9] The absolute configuration of the substituted *myo*-inositol in galactinol has been found to be (V).



The D-galactose is attached to that position which on inversion leads to (−)-inositol (VI). Cleavage of the (−)-inositol ring along the dotted line would give a straight chain polyol with the L-mannitol configuration.[10]

Recent work published from this Laboratory has established that the base hydrolysis of soybean phosphoinositide yields an optically active *myo*-inositol phosphate in which the phosphate group was shown to be in one of the enantiomorphic 1-positions.[5] To establish the optical purity of this isolated material as well as its absolute configuration, we have carried out the synthesis of an asymmetric *myo*-inositol 1-phosphate. The starting material for this work was galactinol, which has the structure and absolute configuration shown in V. Complete benzylation was carried out in dimethylformamide with benzyl bromide and silver oxide according to the method of Kuhn, *et al.*[11] The

(1) Reported in part in a previous publication, C. E. Ballou and L. I. Pizer, THIS JOURNAL, **81**, 4745 (1959).

(2) S. J. Angyal, P. T. Gilham and C. G. MacDonald, *J. Chem. Soc.*, 1417 (1957).

(3) R. J. Brown and R. F. Serro, THIS JOURNAL, **75**, 1040 (1953).

(4) E. A. Kabat, D. L. MacDonald, C. E. Ballou and H. O. L. Fischer, *ibid.*, **75**, 4507 (1953).

(5) F. L. Pizer and C. E. Ballou, *ibid.*, **81**, 915 (1959).

(6) H. Brockerhoff and D. J. Hanahan, *ibid.*, **81**, 2591 (1959).

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

(8) A. Girard, *Compt. rend.*, **73**, 425 (1871); V. Plouvier, *ibid.*, **241**, 983 (1955).

(9) L. Anderson and G. G. Post, Abstracts 134th Meeting of the American Chemical Society, Chicago, 1958, 13-D.

(10) C. E. Ballou and H. O. L. Fischer, THIS JOURNAL, **75**, 3673 (1953).

(11) R. Kuhn, I. Löw and H. Trischmann, *Ber.*, **90**, 203 (1957).