INTERACTION OF LINEAR ANALOGUES OF D-GLUCOSE WITH THE β-GLUCOSIDASE OF *STACHYBOTRYS ATRA*

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[*Manuscript received December* 3, 1968]

Summary

Certain linear analogues of D-glucose appear capable of interacting simultaneously with both the donor and acceptor centres of the β-glucosidase of *S. atra*. In particular, it has been shown directly that *N*-butyl-D-gluconamide can act as an acceptor for glucosyl transfer and indirectly, by extrapolation from the behaviour of related compounds, that it is a competitive inhibitor of the enzyme. For the generalized formula, D-*gluco*-$CH_2OH(CHOH)_4R$, highly polar R groups lead to effective interaction with the donor centre, manifested as competitive inhibition, and less polar R groups to effective interaction with the acceptor centre.

I. INTRODUCTION

I have previously described two extremes in the interaction between linear 6-carbon polyhydroxylic molecules with the configuration of D-glucose and the β-glucosidase of *Stachybotrys atra*. For D-gluconate the interaction takes the form of a simple competitive inhibition (Jermyn 1955). Sorbitol (D-glucitol) on the other hand is an acceptor of transferred glucosyl residues from aryl β-D-glucopyranosides, transfer taking place principally to the terminal primary hydroxyls (Jermyn 1966*b*).

The occurrence of transfer may be both observed directly by the inspection of chromatograms of enzymic digests and deduced from the non-equivalence of liberated glucose and phenol (Jermyn 1962). Nevertheless, from kinetic data it is impossible to argue in the reverse direction that transfer necessarily occurs or that, if such a transfer is known to take place, it is the sole type of interaction. The presence of some degree of competitive inhibition, i.e. of interaction with the binding centre for the donor substrate, cannot be excluded for such acceptors as sorbitol; the complementary uncertainty — whether or not some degree of transfer takes place at high concentrations of competitive inhibitors such as D-gluconate — is similarly enhanced by uncertainty about the significance to be attached to anomalies in kinetics. Consideration of the known specificities of donor and acceptor centres suggests that many molecules must have approximately equal affinities for both centres.

This paper records an attempt to find clear-cut evidence for mixed interactions using molecules intermediate in type between D-gluconate and D-glucitol. Linear molecules have been preferred since there is a body of evidence that many cyclic molecules of the D-glucopyranose type block the acceptor centre (Jermyn 1968). Such molecules are bound at the acceptor centre but their comparatively rigid

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structure does not permit a hydroxyl group to approach the active site in the correct orientation for transfer to take place. On the other hand, the correct (D-glucose) configurational relationship of H and OH groups on carbon atoms 2–5 without completion of a ring structure makes possible, as with D-gluconate, a binding strictly analogous to that of a donor substrate.

Two requirements for compounds to be tested are that cyclization of their molecules should be impossible and that they should be water-soluble. The importance of the first of these may be illustrated by noting that small effects with D-gluconate at high concentration and low pH would be impossible to interpret, since the highly effective competitive inhibitor D-glucono-1,5-lactone will always be present (Jermyn 1960). Low solubility in water precludes the higher D-glucose dialkyl dithioacetals, members of an otherwise interesting homologous series, from consideration.

The most satisfactory group combining the two requirements appeared to be compounds related to D-gluconamide, which are in general highly crystalline and readily purified. Even in this group certain limitations are imposed by the fact that disubstituted gluconamides (derivatives of secondary amines) are non-crystalline. This might have been deduced from the lack of information in the literature; attempts in this laboratory to prepare such compounds from a variety of secondary amines, both long-chain and cyclic, have invariably led to syrups of doubtful purity. The compounds studied here are therefore largely of the type D-*gluco*-$CH_2OH(CHOH)_4CONHR$, although certain other compounds (two dithioacetals, a benzimidazole, 1-nitro-1-deoxysorbitol, D-gluconate, and D-glucitol) that met the requirements have been included.

II. Materials and Methods

(*a*) *Preparation of Analogues*

Commercial sorbitol (D-glucitol) was recrystallized from aqueous ethanol until the ability to reduce alkaline copper (Somogyi) reagent could no longer be detected.

With the exception below, all compounds were laboratory preparations of compounds already described in the literature. The sample of D-gluconate used gave a negative reaction for lactones by the hydroxamate test under conditions where one part in 10^6 could have been detected.

Tris(hydroxymethyl)aminomethane (0·01 mole) and D-glucono-1,5-lactone (0·01 mole) were refluxed 15 min in methanol (20 ml). The resultant solution was set aside at −20°C until crystallization began; subsequent preparations were seeded from the first. Three recrystallizations from methanol gave white microneedles, m.p. 135°C, $[\alpha]_D^{20}+27{\cdot}4$ (*c*, 10 in H_2O) of *N-tris(hydroxymethyl)methyl-*D*-gluconamide*. Found: C, 39·8; H, 7·2; N, 4·4%. Calc. for $C_{10}H_{21}NO_9$: C, 40·1; H, 7·1; N, 4·7%.

(*b*) *Enzymology*

The basic enzyme sample was one from *S. atra* CMI 32542, prepared by Mr. A. C. Clark and to be described by him elsewhere, with a polysaccharide–protein ratio of 3:1 and no significant amounts of non-enzyme protein. Such a complex, with a reproducible particle weight of some 5×10^5 dalton, represents the ultimate stable preparation that can be achieved by non-violent physical means. The way in which protein–polysaccharide interaction stabilizes the enzyme, and its possible influence on the mechanism of enzyme action, remain to be established. It seems unlikely that such eventual elucidation will qualitatively affect the interpretations put forward here and elsewhere.

The substrate used throughout the kinetic work was *p*-nitrophenyl β-D-glucopyranoside, the temperature 28°C, and the buffer, unless otherwise noted, McIlvaine (citric acid–sodium

phosphate) buffer of pH 5·0. The higher concentrations of analogues were in general attained by adding weighed amounts to buffer–water mixtures; any initial heating and cooling needed to bring them into solution was completed before any additions of solutions of the temperature-sensitive substrate. Solutions of D-*gluco*-2-(1′,2′,3′,4′,5′-pentahydroxypentyl)benzimidazole above 10^{-2}M were supersaturated, but initiation of crystallization from solutions of this substance is long delayed in the absence of seeding. For the studies on D-gluconate, the pH of the buffer–potassium gluconate mixture was readjusted to the correct pH by adding solid citric acid before proceeding to the determination of enzyme activity.

The techniques used for measuring enzyme activity and preparing Lineweaver–Burk plots have been set out previously (Jermyn 1955) as have the techniques for comparing phenol and glucose liberation in the presence of added acceptors using phenyl β-D-glucopyranoside as the enzymic substrate. The latter substrate was also used in the preparation of enzyme digests for paper chromatography, which employed ascending chromatography on Whatman No. 1 paper and n-butanol–dimethylformamide–water (2:1:1) as the solvent. This mixture readily separates glucose from the glucosides of polyhydroxylic aglycones (Jermyn 1966). The alkaline silver nitrate and periodate–benzidine dips of Smith (1962) were employed to visualize glucose, glucosides, and gluconamides on the developed chromatogram.

III. Results

(*a*) *Competitive Inhibitors*

Three criteria for establishing simple competitive inhibition are (1) straight-line Lineweaver–Burk plots (the reciprocal plot of reaction velocity against substrate concentration has been used throughout) for the enzyme–substrate reaction, leading to a consistent K_m value; (2) similar straight lines for the enzymic reaction in the presence of inhibitor, all lines, whether for the inhibited or non-inhibited reaction, cutting the velocity axis at a common point; (3) a constant value of K_i when its value is calculated from the slopes of the Lineweaver–Burk lines for a range of inhibitor concentrations.

The experimental conditions have been deliberately chosen to conform with the first of these criteria throughout the kinetic studies on the β-glucosidase of *S. atra*. Although it is known that for concentrations of aryl D-glucopyranosides greater than $\simeq 10^{-2}$M Michaelis–Menten kinetics fail to hold, and increasing substrate concentrations actually decrease enzymic activity, no departures greater than experimental error can be found at concentrations less than 10^{-3}M and the reaction can be taken, for all practical purposes, as proceeding through a 1:1 enzyme–substrate complex.

The second and third criteria define a competitive inhibitor for the purpose in hand. In terms of the kinetic analysis, the following analogues were purely competitive: D-gluconate, D-gluconohydroxamate, D-gluconamide, *N*-methyl-, *N*-ethyl-, and *N*-phenyl-D-gluconamides, D-gluconhydrazide, *N′*-phenyl-D-gluconhydrazide, and D-glucose diethyldithioacetal. The relevant inhibitor constants are to be found in Table 2. Figure 1 demonstrates for the inhibitors of high and lowest affinity for the enzyme how closely their behaviour follows the classic competitive pattern.

(*b*) D-*Gluconate as Competitive Inhibitor*

It is impossible to obtain a consistent value of K_i for D-gluconate at any original pH unless allowance is made for the powerful buffering effect of this anion

and the enzymic digest is readjusted to the correct pH. The direction of the deviations suggested that K_i must fall sharply with increasing pH. Table 1 shows that this is so and that the trend of K_i values with pH is in the opposite direction to and much steeper than that for a competitive inhibitor (D-gluconhydrazide)

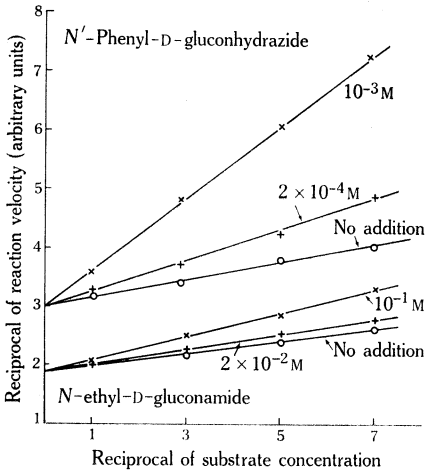

Fig. 1.—Simple competitive inhibitor behaviour by two linear D-glucose analogues. Unit substrate concentration = 10^{-3}M *p*-nitrophenyl β-D-glucopyranoside.

which would not be expected to be ionized over the pH range studied. No attempt was made to work below pH 4 since in this range the contribution of lactones to the observed inhibition (K_i for D-glucono-1,5-lactone is 5×10^{-6}M) would have to be taken into account.

TABLE 1

EFFECT OF pH ON THE APPARENT VALUES OF K_i FOR D-GLUCONATE AND D-GLUCONHYDRAZIDE AS COMPETITIVE INHIBITORS FOR THE ENZYMATIC HYDROLYSIS OF *p*-NITROPHENYL β-D-GLUCOPYRANOSIDE

An inhibitor of mixed type, *N*-2-hydroxyethyl-D-gluconamide, has been included for comparison

pH	$10^4 \times K_i$(M)		Ratio of K_i Values	Undissociated Gluconic Acid Present in 1M Gluconate (%)	Ratio of Previous Two Columns	$10^2 \times K_i$(M) for Mixed-type Inhibitor
	D-Gluconate	D-Glucon-hydrazide				
4·0	1·0	6·5	6·5	24	3·7	5·5
5·0	4·8	3·1	0·64	3·1	4·8	3·1
6·0	19	2·8	0·15	0·32	2·1	2·9
7·0	86	1·2	0·014	0·032	2·3	0·79

The obvious rationalization for the data of Table 1 is that the effective competitive inhibitor is undissociated gluconic acid, with the enzyme having little or no affinity for the gluconate anion. In the later columns of the table the affinity of the enzyme for gluconate is first "normalized" by making the assumption that the pH dependence of the hydrazide interaction is a measure of the effect of pH on the

binding site of the enzyme. This value is then compared with the proportion of undissociated gluconic acid calculated to be present in the solution, assuming a pK_a of 3·5 [Cannan and Kibrick (1938) give $pK_a = 3{\cdot}56$ for gluconic acid]. The agreement is as good as could be expected in the circumstances; the K_i for undissociated gluconic acid is $2\text{–}3 \times 10^{-5}$M.

An inhibitor of "mixed" type, *N*-2-hydroxyethyl-D-gluconamide, has been included in Table 1 for comparison with D-gluconhydrazide. The values for K_i show exactly the same trend in the same direction as for the hydrazide. This observation strengthens both the assumption that the K_i values for the hydrazide measure an overall trend in enzymic behaviour with pH and the hypothesis that K_i values for mixed inhibitors genuinely measure the effectiveness of a competitive inhibition.

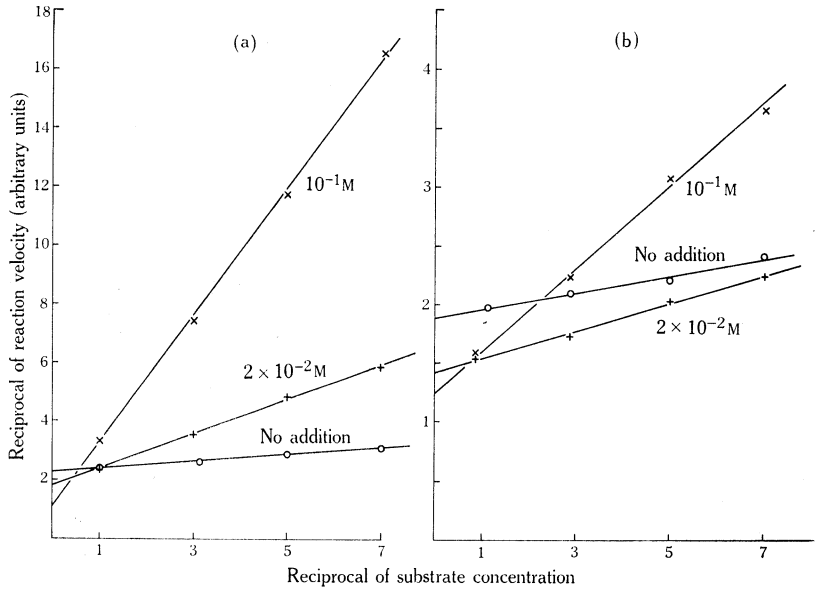

Fig. 2.—"Mixed" inhibitor behaviour by two linear D-glucose analogues. (*a*) *N*-Tris(hydroxymethyl)methyl-D-gluconamide; (*b*) *N*-cyclohexyl-D-gluconamide. Unit substrate concentration = 10^{-3}M *p*-nitrophenyl β-D-glucopyranoside.

(*c*) *"Mixed" Inhibition*

Most of the analogues tested satisfied the third criterion for competitive inhibition but not the second. In point of fact the maximum velocity of the enzyme reaction increases steadily with increasing analogue concentration in the manner already familiar for effective acceptors (Jermyn 1966*a*). Two representative instances are illustrated in Figure 2; Table 2 gives a full set of values of K_i.

The constancy of K_i and the shift in V with increasing analogue concentration are experimental observations, subject to the usual statistical uncertainties. The matter was pursued further using D-gluconamide and *N*-butyl-D-gluconamide at concentrations from 10^{-3} to 10^{-1}M. Statistical analysis using five analogue concen-

trations, and five substrate concentrations at each analogue concentration, showed no significant variation ($P > 0{\cdot}05$) between the various calculated values of V and K_i for D-gluconamide and of K_i for N-butyl-D-gluconamide, but a highly significant variation ($P < 0{\cdot}001$) for V for the N-butyl-D-gluconamide. The values of the last constant in fact formed a monotonically increasing series with increasing analogue concentration.

In view of the evidence to be presented that some degree of acceptor activity is shown by N-butyl-D-gluconamide, it is difficult to believe that there is not some influence of analogue concentration on apparent K_i. However, it is apparent that it is less than can be detected by the relatively simple experiment cited above.

TABLE 2

EFFECTS OF LINEAR D-GLUCOSE ANALOGUES ON THE DECOMPOSITION OF 10^{-3}M p-NITROPHENYL β-D-GLUCOPYRANOSIDE BY *S. ATRA* β-GLUCOSIDASE AT pH 5 AND 28°C

Results for K_i and V are given for two analogue concentrations giving conveniently measurable degrees of inhibition

Analogue	Set 1			Set 2		
	Concn. (M)	10^3K_i (M)	V/V_0	Concn. (M)	10^3K_i (M)	V/V_0
D-Gluconamide	0·002	2·9	1·00	0·01	3·1	1·00
N-Methyl-	0·02	42	1·00	0·1	4·7	1·00
N-Ethyl-	0·02	130	1·00	0·1	130	1·00
N-Propyl-	0·02	51	1·12	0·1	50	1·21
N-Butyl-	0·02	36	1·19	0·1	36	1·31
N-Cyclohexyl-	0·02	31	1·31	0·1	37	1·50
N-Benzyl-	0·02	13	1·51	0·1	1	1·60
N-Phenyl-	0·002	2·5	1·00	0·01	2·1	1·00
N-2-Hydroxyethyl-	0·02	28	1·40	0·1	31	1·51
N-Tris(hydroxymethyl)-methyl-	0·02	5·0	1·34	0·1	5·8	2·11
D-Gluconhydrazide	0·005	0·31	1·00	0·02	0·31	1·00
N'-Phenylhydrazide	0·0002	0·24	1·00	0·001	0·26	1·00
D-Glucohydroxamic acid	0·002	0·22	1·00	0·01	0·25	1·00
D-Glucose diethyldithioacetal	0·02	6·2	1·00	0·1	6·3	1·00
D-Glucose ethylenedithioacetal	0·02	24	1·07	0·1	24	1·15
D-*gluco*-2-(Pentahydroxy-pentyl)benzimidazole	0·02	17	0·87	0·1	37	0·77
D-Glucitol	0·02	12	1·46	0·1	37	2·10
1-Nitro-1-deoxysorbitol	0·02	33	0·84	0·1	69	0·65

(*d*) *Acceptor Activity of Gluconamides*

Considering the series of N-alkyl-D-gluconamides only, it is apparent that there is a break in properties between N-ethyl- and N-propyl-D-gluconamide. A reasonable hypothesis is that it is only for the latter compound that the relative affinity for the acceptor centre is sufficient to modify the dominant effect of the competitive inhibition on the observed kinetics. This hypothesis involves taking the observed

increase in V at its face value, i.e. as being due to the presence of an acceptor more efficient than water. This last supposition is amenable to direct experimental test.

The terminal members of the series, D-gluconamide and *N*-butyl-D-gluconamide were incorporated at concentrations up to 0·5M in digests containing the β-glucosidase (1500 units/ml) and phenyl β-D-glucopyranoside (up to 0·4M, the approximate solubility limit at 20°C) and samples withdrawn at intervals for chromatography until glucoside breakdown was complete. With D-gluconamide no spot was ever seen other than those corresponding to glucose, glucoside, and amide. With *N*-butyl-D-gluconamide on the other hand one further spot, intermediate in R_F between the amide and glucose and best visualized with the periodate–benzidine reagents, appeared under a variety of conditions. Since the amide possesses only one primary hydroxyl group the single transfer product involving it should be preponderant (Jermyn 1966*b*).

This implied conclusion was checked by studying the glucose–phenol liberation ratio in digests involving 4×10^{-3}M phenyl β-D-glucopyranoside and dilute enzyme. At pH 5, the ratio was 0·82 in the presence of 0·1M *N*-butyl-D-gluconamide, 0·68 for 0·3M. The implied value of T_{50} (Jermyn 1966*a*) was $\simeq$0·6M, where T_{50} is the concentration of added acceptor at which the glucosyl residue is transferred in equal amounts to acceptor and water. *N*-Butyl-D-gluconamide is thus an acceptor, more efficient than water certainly, but not of high affinity for the enzyme compared with highly efficient acceptors. The ratio did not depart significantly from unity for D-gluconamide.

(*e*) *Effect at High Substrate Concentrations*

The existence of a sudden break in the mechanism of interaction with the enzyme in the D-gluconamide homologous series between the *N*-ethyl and *N*-propyl amides appears *a priori* improbable. If the observed break is in fact due to the changing ratio in the affinity for the two centres bringing certain effects to a detectable level only at a certain point in the series, it should be possible to arrange conditions such that the interaction with one centre can be studied in isolation. The obvious course is to block the donor centre completely by working at very high substrate concentrations. Unfortunately the secondary affinity of the substrate for the acceptor centre becomes marked at this point, and the three-way competition of donor, analogue, and water for the acceptor centre leads to kinetics that cannot by analysed in any simple way. However, it remains possible to draw certain qualitative conclusions.

The concentrations used in the experiments of Table 3, 0·08M in *p*-nitrophenyl β-D-glucopyranoside (the solubility limit at 28°C) and 0·5M in the amides, and the known values of K_m for the glucoside (3×10^{-5}M) and of K_i for the amides ($>10^{-3}$M) lead to a calculated value of virtually 100% occupancy of the donor centre by glucoside molecules. On the other hand the depression of the reaction velocity in the absence of analogues (60% of that at 10^{-3}M substrate) and the expected values of T_{50} ($>$0·6M, the value for the *N*-butyl amide) suggests roughly comparable values for the degree of occupancy of the acceptor centre by glucoside, *N*-butyl amide, and water molecules. Under these conditions, any propensity of an amide to displace water or glucose from the acceptor centre and act as an acceptor more effective than water, even though to a lesser degree than manifested by the *N*-butyl amide, should lead to increase in reaction velocity.

Table 3 shows that this supposition is justified. There is a steady increase in rate in proceeding from the ineffective D-gluconamide to its *N*-butyl derivative. Since the latter has been shown to be an acceptor, the whole series may reasonably be attributed to increasingly effective acceptor action. The apparent break between the *N*-ethyl and *N*-propyl amides is thus apparent rather than real and due to the limitations of an experimental method.

TABLE 3

EFFECT OF D-GLUCONAMIDES (0·5M) ON THE ENZYMATIC BREAKDOWN OF *p*-NITROPHENYL β-D-GLUCOPYRANOSIDE (0·08M)

N-substituent in D-gluconamide	None	Methyl	Ethyl	n-Propyl	n-Butyl
Relative optical density of released *p*-nitrophenol*	1·02	1·14	1·23	1·45	1·53

* Control = 1·00. Since the optical density of the liberated *p*-nitrophenol had to be measured at 425 mμ to eliminate the unworkably high blank due to the optical density of the concentrated glucoside at 400 mμ, Beer's law no longer applies strictly and the relative optical density underestimates the higher enzyme activities.

(*f*) *Effects of* D-*Galactonhydrazide*

D-Gluconhydrazide is one of the most effective competitive inhibitors of the β-glucosidase known, and certainly the most effective acyclic one. The effect of reversing the configuration around C-4 to give D-galactonhydrazide are quite dramatic [Fig. 3(*a*)]. Detectable competitive inhibition is abolished and a small but definite "blocking" effect appears in its place. Since the concentrations of D-galacton-

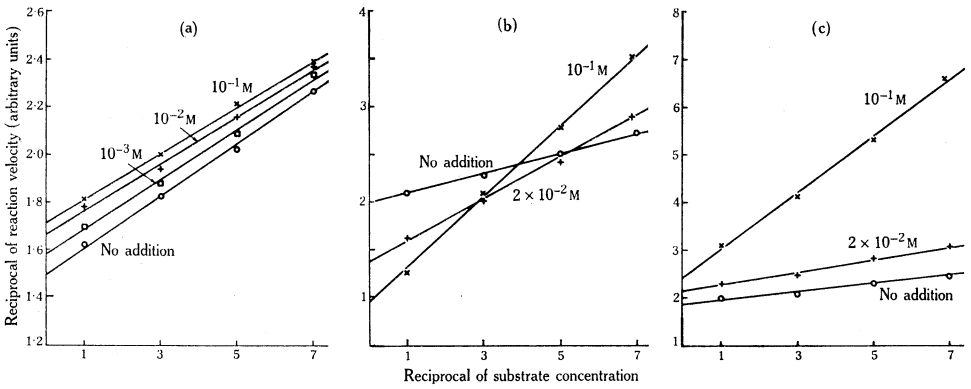

Fig. 3.—Interaction between β-glucosidase and D-galactonhydrazide (*a*), D-glucitol (*b*), and D-*gluco*-2-(1′,2′,3′,4′,5′-pentahydroxypentyl)benzimidazole (*c*). Unit substrate concentration = 10^{-3}M *p*-nitrophenyl β-D-glucopyranoside.

hydrazide used in producing the data of Figure 3(*a*) would, for D-gluconhydrazide, have led to the suppression of detectable enzyme activity through competitive inhibitor action, blocking or "stimulation" may theoretically exist for D-gluconhydrazide without being detectable in practice. Indeed, the results with D-galactonhydrazide

may be looked on as a roundabout method of establishing the presence of some affinity between D-gluconhydrazide and the acceptor site by abolishing its interaction with the donor site in a parallel, but much neater, way to the blocking achieved in the previous section by the use of excess substrate.

Since extrapolation suggests that enzyme activity does not tend to zero with increasing hydrazide concentration, it appears that this analogue is a less effective acceptor than water and not a true blocking agent, although the Lineweaver–Burk plots for the two groups of agents are of the same general type [cf. Fig. 3(*c*)].

(*g*) *Special Cases*

Three analogues interact with the enzyme in ways different from those described in the first two sections.

D-Glucitol (sorbitol) is a known effective acceptor. Its behaviour [Fig. 3(*b*); Table 2] cannot be equated in any way to competitive inhibition, neither V nor K_i being constant. Interaction with the acceptor centre obviously far outweighs that with the donor centre. Indeed, whether the latter interaction occurs to a degree comparable in any way with the former cannot be determined from the data.

D-*gluco*-2-(1′,2′,3′,4′,5′-Pentahydroxypentyl)benzimidazole [Fig. 3(*c*)] and 1-nitro-1-deoxysorbitol [an essentially similar plot to that of Fig. 3(*c*)] represent the group of substances that appear to block the acceptor centre without acting as effective acceptors. The figure shows the characteristic feature of their kinetics, a steady decrease in V with increasing analogue concentration. The calculated value of the affinity constant for the benzimidazole (the concentration at which half the acceptor centres are occupied) is 0·5M. This may be compared with the value for T_{50} of 0·6M deduced for *N*-butyl-D-gluconamide. For acceptors, T_{50} and the affinity constant are not identical but are of similar magnitude (Jermyn 1966*a*). For these blocking agents, unlike the stimulating acceptors exemplified in Figure 2, "K_i" is not found to have a constant value (Table 2). But they may nevertheless be readily distinguished from true non-competitive inhibitors to which they otherwise approximate. In addition, the extended Lineweaver–Burk lines fail to meet on the abscissa (substrate axis) in the characteristic test for non-competitive inhibition.

IV. Discussion

The primary assumption that has been made here, and earlier elsewhere, that Lineweaver–Burk plots of the type of those in Figure 2 represent a mixture of interaction with the donor and acceptor centres appears to require some justification. Attempts to develop a mathematical treatment lead to complex expressions with many constants that require drastic simplification before they can be manipulated and it is probably sufficient for the present purpose to treat the matter intuitively.

The value of K_i for competitive inhibition is deduced from the expression $R = 1+(K_i/[I])$, where R is the ratio of the slopes of the Lineweaver–Burk lines for the inhibited and non-inhibited reaction. For simple competitive inhibition one of the two quantities that determine R, the slope corresponding to the non-inhibited reaction, is fixed, and constancy of K_i can thus be demonstrated by a linear relationship between the slope corresponding to the inhibited reaction and the

reciprocal of [I]. If the inhibitor also acts as an acceptor, constancy of K_i with changing [I] thus implies that the slope of the non-inhibited reaction with water alone is identical with that of the experimentally non-accessible theoretical reaction with a mixture of water and inhibitor exerting its acceptor function only. Conditions, however, are always such that the two functions are being exerted simultaneously, and in any case there is no theoretical reason to suppose that the slope should remain constant. However, Figure 3(*a*) and earlier reports (Jermyn 1966*a*) show that the hypothesis is often not far from the truth. Minor variations in the slope will be confounded with the much greater experimental errors in determining the relatively low rate of the inhibited reaction and therefore produce no statistically significant change in K_i.

Theory suggests that by assigning suitable values to the rate constants the relationship between the slope of the Lineweaver–Burk lines and the concentration of an added acceptor can take almost any form we please. The finding in a given case that there is an approximate constancy of slope with changing acceptor concentration is thus to be treated as an experimental one rather than as a theoretical deduction. The interpretation of the data of the present kind is therefore perhaps best put in a form reversed from that of the initial approach. Where a molecule is known to be an acceptor and there is a reasonable assumption from its structure that it is a competitive inhibitor, then constancy of K_i argues a near constancy of slope on passing from the reaction with water alone to that with both water and acceptor. For D-glucitol, however, it seems that the assumption that the slope of the "non-inhibited" Lineweaver–Burk line does not depart too far from that of the base (water reaction) Lineweaver–Burk line can no longer be made, and no deductions can therefore be made from the values of "K_i".

The interaction of the β-glucosidase with inhibitors and acceptors has already been exhaustively discussed (Jermyn 1966*a*, 1968). However, earlier investigations have always used an eclectic choice of reagents, e.g. β-glucopyranosides as competitive inhibitors, primary alcohols as acceptors, sugars and their derivatives such as turanose and benzyl β-D-fructopyranoside as blocking agents for the acceptor centre. This paper represents an attempt to understand the requirements for such interaction in terms of variations in a single type of molecular structure.

The results suggest that for compounds of the type $CH_2OH(CHOH)_4R$ to react with the donor centre, the molecule must have the D-*gluco* configuration overall, and that the group R must be strongly polar; however, there is at present no basis for correlating the nature of the R group and the strength of the interaction. The observed order –COOH > –CONHOH, $-CONHNH_2$, $-CONHNHC_6H_5$ > $-CONH_2$, $-CONHC_6H_5$ > $-CONHCH_3$, –CONHalkyl generally > $-CONHC_2H_5$, with about an order of magnitude difference in K_i between succeeding groups appears significant but, in the absence of detailed knowledge of the active site, the series could not have been predicted.

There is nothing in the data to contradict previous conclusions that both the interaction of multiple hydroxyl groups and bulky non-polar groups facilitate interaction with the acceptor centre. Indeed, there is a striking parallel between the interactions with this centre of the homologous series of *N*-alkyl-D-gluconamides and the series of simple n-alkanols. Perhaps the most novel observation is the

inhibitory effect of a strongly polar group on this interaction; the requirements for effective interaction with donor and acceptor centres appear to show a trend in opposite directions. The details of correlation between structure and affinity for the acceptor centre remain empirical observations, however, even though they are capable of some prediction on this basis. Thus the observed large shift in V in the presence of N-(2-hydroxyethyl)-D-gluconamide led to the synthesis of N-tris(hydroxymethyl)-methyl-D-gluconamide and the observation of the largest shift in V in the series. The mechanisms underlying this result remain obscure; since tris(hydroxymethyl)-aminomethane is itself a potent acceptor (Jermyn 1966*a*), transfer may well be to this moiety of the molecule rather than the gluconyl moiety.

Once molecules other than those directly related to D-gluconamide are considered, effects become even more difficult to rationalize and must for the moment be taken as matters of observation only. Thus no explanation can be offered for the sharply contrasting behaviour of D-glucose diethyldithioacetal and D-glucose ethylenedithioacetal; *a priori* it might have been expected that it was the first and least polar of the pair that had the highest affinity for the donor centre and the least for the acceptor centre. However, steric effects may have to be taken into consideration in many cases; thus it appears that the bulk of the benzimidazole group plays some part in the action of the pentahydroxypentylbenzimidazole as a blocking agent for the acceptor centre. The affinity of this analogue for the donor centre is, however, similar to that of the alkyl gluconamides.

V. Acknowledgment

I thank Mr. A. C. Clark for the sample of purified enzyme used in this study.

VI. References

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