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STUDIES OF THE INHIBITION BY MALTO-OLIGOSACCHARIDES OF THE CYCLISATION REACTION CATALYSED BY THE CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Klebsiella pneumoniae* M 5 al WITH GLYCOGEN

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ABSTRACT

The substrate qualities of malto-oligosaccharides for the disproportionation reaction catalysed by the cyclodextrin glycosyltransferase {(1→4)-α-D-glucan:[(1→4)-α-D-glucopyranosyl]transferase (cyclising) EC 2.4.1.19} from *Klebsiella pneumoniae* M 5 al have been re-investigated. Maltose failed to be homologised with measurable velocity. The initial rates of disproportionation and the affinities of the enzyme increased with the chain lengths of the substrates. Maltopentaose was the smallest saccharide which, by disproportionation, yielded longer chains being cyclised initially. D-Glucose did not affect the initial cyclisation from glycogen, but served as acceptor for the "chain-shortening" reaction. Maltose inhibited the initial cyclisation reaction in a linearly competitive manner. Maltotriose and maltotetraose inhibited the cyclisation reaction competitively, the inhibition kinetics pointing to the binding of two effector-molecules to the enzyme. Competitive inhibition was also found with malto-pentaose, -hexaose, and -heptaose. The degrees of inhibition increased from maltose to maltotetraose, and decreased with the larger saccharides; maltotriose and maltotetraose were the most effective inhibitors of the initial cyclisation. Some possibilities for the subsite-mechanisms are discussed.

INTRODUCTION

The precise mechanism of the cyclisation reaction *1* [where G_n, G_m, and G_x are (1→4)-α-D-glucopyranosyl chains with n, m, and x units, respectively, and cG_x is a cyclomaltopolyose (cyclodextrin) containing x residues] catalysed by the cyclodextrin

$$G_n \underset{\text{coupling}}{\overset{\text{cyclisation}}{\rightleftharpoons}} G_{(n-G_x)} + cG_x \qquad 1$$

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$$G_n + G_m \overset{\text{disproportionation}}{\rightleftharpoons} G_{(n-G_x)} + G_{(m+G_x)} \qquad 2$$

glycosyltransferases {(1→4)-α-D-glucan:[(1→4)-α-D-glucopyranosyl]transferase (cyclising) EC 2.4.1.19, CGT} is unknown. The one-substrate reaction, proceeding via a binary EG_n-complex and obeying Michaelis–Menten kinetics, is thought to be a special type of disproportionation (two-substrate reaction *2*, proceeding via a ternary EG_nG_m-complex), where the part of a (1→4)-α-D-glucopyranosyl chain (substrate or donor) containing the non-reducing end is transferred via HO-1 to mainly HO-4 of a suitable acceptor. For cyclisation, the non-reducing end of one chain serves as acceptor, whereby the helical conformation of the substrate may be a prerequisite for the reaction[1]. As the CGTs catalyse the two-substrate reactions disproportionation and coupling, they have been supposed to possess two binding-sites for substrate and acceptor, respectively[1,2]. Cyclisation can occur only if the acceptor binding-site is empty, otherwise the disproportionation reaction takes place. Indeed, the presence of malto-oligosaccharides affects the yields of cyclodextrins from starch. Thus, a 34% starch solution hydrolysed to a glucose equivalent (g.e.) of 1, yielded 45% of cyclodextrins but only 17% from[3] a starch of g.e. 12.

Investigation of the reaction mechanisms requires a knowledge of the substrate qualities for both disproportionation and cyclisation. H.p.l.c. of long-term digests showed maltose to be a very poor substrate, whereas maltotriose and maltopentaose were homologised at markedly higher rates, yielding malto-oligosaccharides $>G_{11}$, and 7.6 and 31% of cyclodextrins, respectively[4]. Chains with an average d.p. of 16 were used[1,5] for assaying the CGT of *Klebsiella pneumoniae* M 5 al. The maximum rate of cyclisation was 6.2 kat.kg^{-1} of protein (K_m 1.03mM), indicating that chains of these lengths were suited for cyclisation. Evidently, the substrate qualities increase with increase in chain length, pointing to the necessity of occupying a series of subsites for maximum activity, as found for various amyloses[6–9].

As the presence of malto-oligosaccharides decreases the yields of cyclodextrins from starch, it is of interest to determine the precise nature of their effect on the initial rate of cyclisation. The investigation is complicated by the fact that cyclisation and disproportionation occur simultaneously with most of the substrates suited for cyclisation. Besides cyclisation and disproportionation (mechanisms of exo-attack), the CGTs catalyse a rapid endo-cleavage of the long amylose chains[10,11], and the long B-chains of the basic structure[12,13] of the amylopectin molecules[14]. It is questionable, at present, whether the chain-shortening is due[15,16] to hydrolytic activity of the CGTs, or whether it represents another type of disproportionation reaction. The marked enhancement of chain-shortening by malto-oligosaccharides[11] points to the second possibility. Of the substrates assayed so far, only glycogen underwent cyclisation exclusively in the initial phase of the transfer reactions. We now describe the effect of D-glucose and malto-oligosaccharides (up to maltoheptaose) on the initial velocity of cyclisation of the CGT from *Klebsiella pneumoniae* M 5 al with the glycogen of *Escherichia coli* K 12.

EXPERIMENTAL

Materials. — CGT from *Klebsiella pneumoniae* M 5 al was isolated from the culture filtrate of continuously grown bacteria, and purified to electrophoretic homogeneity[17,18]. Glycogen from *Escherichia coli* K 12, grown by continuous two-stage cultivation, was isolated and purified as described[19]. Maltotriose was prepared by digestion of pullulan with pullulanase [pullulan:(1→6)-glucanohydrolase, EC 3.2.1.41][20,21]. Maltose, malto-tetraose, -pentaose, -hexaose, and -heptaose were commercial materials. The malto-oligosaccharides were purified by preparative h.p.l.c.

Analytical methods. — Total carbohydrate was determined with the anthrone reagent[22,23], reducing sugars with the Nelson reagent[24], and protein by the biuret method[25]. H.p.l.c. was performed on Waters μ Bondapak-NH_2 columns (3.9 × 300 mm), using acetonitrile–water (65:35) at 1.5 mL/min (1,200 p.s.i., 30°) with refractometric detection and 20-μL samples. The carbohydrate contents of the peaks were calculated by planimetry, and calibrated with maltose–maltoheptaose[4].

For the determination of the initial rates of disproportionation, 1.5–20mM solutions (1 mL) of maltose–maltopentaose in 10mM Tris–HCl buffer (pH 7.2, 5mM $CaCl_2$) were incubated with CGT (3–30 μg/mL) at 30° for 2 min. The enzymic reaction was stopped by boiling, and the products were analysed by h.p.l.c. The unit of activity was defined as $mol.L^{-1}.s^{-1}$ of substrate consumption per kg of protein. The values for the maximum rate $V_{(dispr)}$ were calculated from the double reciprocal plots[26] $1/v_{o(dispr)}$ against 1/[S].

The initial rates of cyclisation were calculated using a photometric assay[5]. Solutions of glycogen (0.3–1.5 g/L) in 10mM Tris–HCl buffer (pH 7.2, 5mM $CaCl_2$) were brought to 0.05–0.2mM concentrations of iodine using 0.1M iodine in dimethyl sulfoxide, and pre-incubated (30°) for 2 h. Likewise, the initial rates of cyclisation with the malto-oligosaccharides were determined with 12.5–20mM solutions in 10mM Tris–HCl buffer (pH 7.2, 5mM $CaCl_2$), containing 0.05mM iodine. The concentrations of the effectors were 4–40 (D-glucose), and 1.9–20mM (maltose–maltoheptaose). The final concentrations were obtained by adding appropriate volumes to the assays from 0.6M stock solutions. The transfer reactions (30°) were started by the addition of CGT (final concentration, 0.34 mg/L). The enzymic activities were given as kat per kg of protein.

The classification of the inhibition type was performed by graphical analysis using the double reciprocal plot[26] ($1/v_i$ *versus* 1/[S]), the re-plot[27] of slopes 1/[S] and of K_m^{app} (apparent Michaelis constant in the presence of inhibitor) against [I] and $[I]^2$ (where I is the inhibitor), respectively, and the Dixon-plot[28] ($1/v_i$ against [I]). Steady-state treatment was performed. As maltotriose and maltotetraose could not be cyclised initially, the reaction kinetics were treated as for one-substrate reactions.

RESULTS AND DISCUSSION

The initial rate of cyclisation of the CGT with the glycogen from *Escherichia coli* K 12 was determined in the absence of effectors. From the double reciprocal plot ($1/v_o$ against 1/[S]), values of 2.74 kat.kg^{-1} of protein for $V_{(cycl)}$, and 0.286 g.L^{-1} for K_m were calculated; it is not possible to give the molar concentration of the glycogen of very high molecular weight. For initial cyclisation, only the outer chains (A- and exterior B-chains) are the effective substrates. As the degree of beta-amylolysis was 46%, 0.286 g of the glycogen contained 0.142 g of exterior chains (average d.p. 14), corresponding to a concentration of ~58μM.

The initial rates of disproportionation with various concentrations of maltose–maltopentaose were determined by quantitative h.p.l.c. Initial disproportionation activity could not be detected with maltose. With maltotriose–maltopentaose, both the rates and the affinities increased markedly with increase in chain length (Table I). As shown by h.p.l.c., maltose was the main product formed initially from the higher malto-oligosaccharides. D-Glucose was not formed, and only small amounts of maltotriose and larger disproportionation products could be detected (Fig. 1). The maltosyl residue of a chain is important for the binding of the substrate, and the (1→4)-α-D-glucosidic bond following the maltosyl residue is split preferentially.

As shown by the photometric assay, disproportionation of maltotetraose yielded no products which allowed initial cyclisation (Fig. 1A). Initial cyclisation, however, occurred with maltopentaose (Table I, Fig. 1B). If $2G_5 \rightarrow G_8 + G_2$ initially, the smallest malto-oligosaccharide for cyclisation would be malto-octaose. The initial disproportionation of malto-hexaose and -heptaose yielded products that were cyclised at markedly higher rates (Table I, Fig. 1C).

Effects of D-*glucose and maltose–maltoheptaose on the initial rate of cyclisation with glycogen as substrate.* — D-Glucose is a commonly used acceptor for the coupling reaction *1* and must have some affinity for the acceptor binding-site of the

TABLE I

INITIAL DISPROPORTIONATION AND CYCLISATION CATALYSED BY THE CGT FROM *Klebsiella pneumoniae* WITH VARIOUS MALTO-OLIGOSACCHARIDES[a]

Substrate[b]	$V_{(disp)}$ (*kat.kg^{-1} of protein*)	K_m[c] (*mM*)	$V_{o(cycl)}$[d] (*kat.kg^{-1} of protein*)
Maltose	—	—	—
Maltotriose	2.22	11.78	—
Maltotetraose	4.76	10.53	—
Maltopentaose	5.71	8.33	0.92
Maltohexaose	n.d.	n.d.	1.3
Maltoheptaose	n.d.	n d.	1.4

[a]See Experimental. [b]The initial concentrations were in the range of 1.5–20mM. [c]At 30° in 10mM Tris–HCl buffer (pH 7.2, 5mM $CaCl_2$). [d]The initial substrate concentration was 12.5mM.

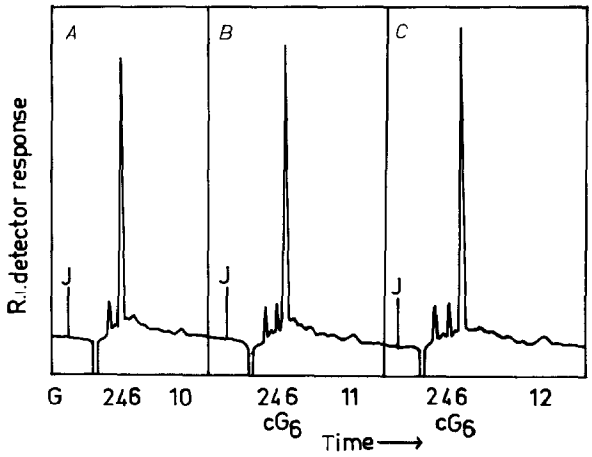

Fig. 1. H.p.l.c. of the initial disproportionation products (2 min, 30°) of *A*, maltotetraose (8 mg/L of CGT); *B*, maltopentaose (4 mg/L of CGT); and *C*, maltohexaose (3 mg/L of CGT) (see Experimental).

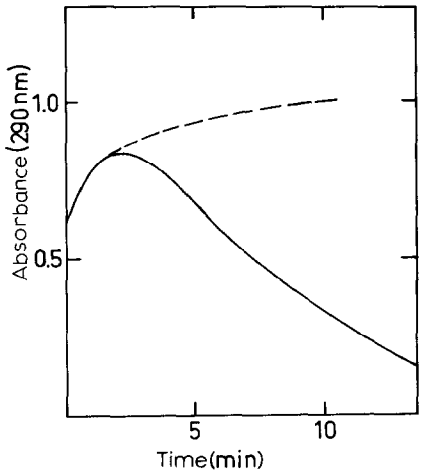

Fig. 2. Effect of D-glucose (40mM) on the initial rate of cyclisation at 30° with glycogen (1.5 g/L). The concentration of CGT was 0.34 mg/L; -----, increase in absorbancy in the absence of effector.

enzyme. Surprisingly, D-glucose up to 40mM did not affect the initial rate of cyclisation. With higher concentrations, the initial increase in absorbance (due to the formation of the cyclomaltohexaose–iodine complex) was followed by a marked decrease in absorbance caused by a loss of turbidity of the substrate solution (Fig. 2). Thus, D-glucose served as acceptor for disproportionation (*i.e.*, chain-shortening reaction) of the substrate, but it is not clear why D-glucose did not inhibit the initial cyclisation by interacting with the acceptor binding-site. This fact may be due to a rather low affinity for that site, or to an alteration of the enzyme affinity by bound cyclomaltohexaose allowing the binding for the coupling reaction.

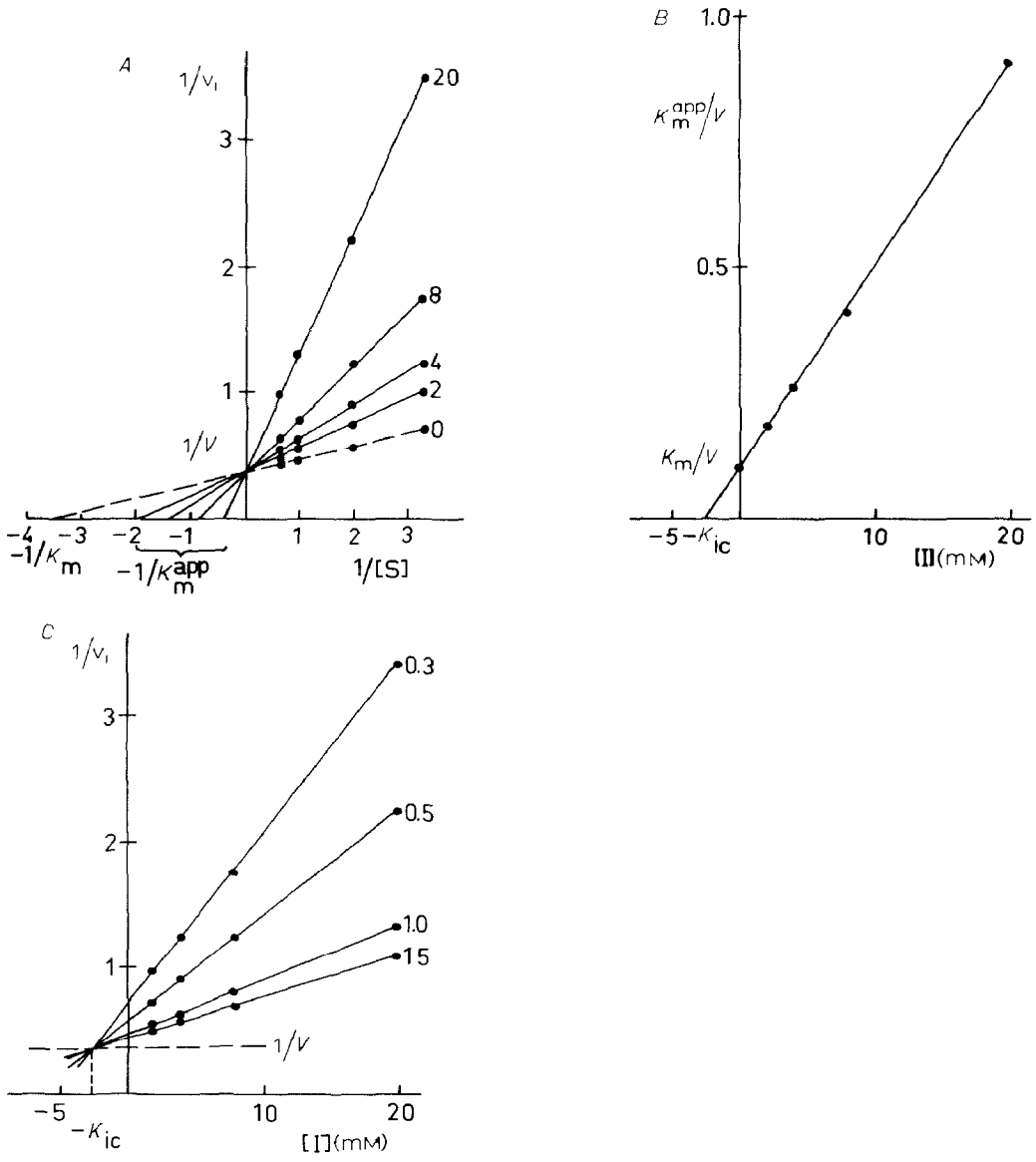

Fig. 3 Inhibition by maltose of the initial cyclisation reaction at 30° with glycogen: *A*, double reciprocal plot of $1/v_i$ *versus* 1/[S] ([S] = g/L; the numbers of the lines give the mM concentration of maltose); *B*, re-plot of the slopes 1/[S] against [I]; *C*, Dixon-plot $1/v_i$ against [I] (the numbers of the lines give the concentration of substrate in g/L) (see Experimental).

For maltose, the double reciprocal plots $1/v_i$ *versus* 1/[glycogen] for each concentration gave straight lines which intersected the $1/v_i$-axis at $1/V_{(cycl)}$ (Fig. 3A). As there was only a slope- and no intercept-effect (maltose affecting only K_m^{app}), the inhibition was competitive, given for one-substrate reactions by Eq. *3*. The re-plot

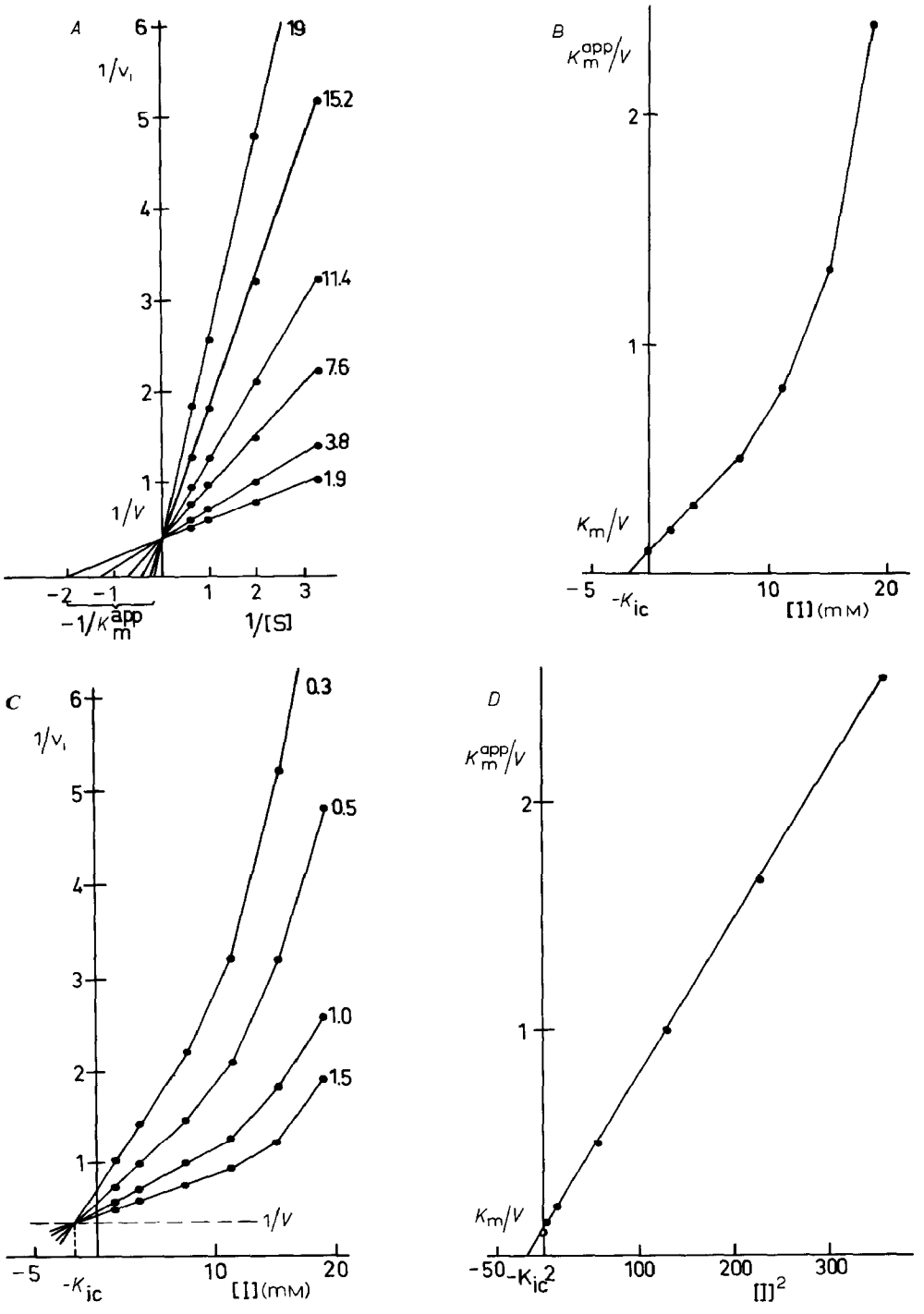

Fig. 4. Inhibition by maltotriose of the initial cyclisation reaction at 30° with glycogen: *A*, double reciprocal plot $1/v_i$ *versus* 1/[S] (the number of the lines give the mM concentration of maltotriose); *B*, re-plot of the slopes 1/[S] against [I]; *C*, Dixon-plot $1/v_i$ against [I] (the numbers of the lines give the concentration of substrate in g/L); *D*, re-plot of the slopes 1/[S] against $[I]^2$ (see Experimental).

$$v_i = V \frac{[S]}{[S] + K_m(1 + [I]/K_{ic})} \qquad 3$$

of the slopes $1/[S] = K_m/V\,(1 + [I]K_{ic})$ against [I] gave a straight line (slope = $K_m/V\,K_{ic}$) with an intercept on the ordinate at K_m/V, *i.e.*, control slope at [I] = 0. Accordingly, the inhibition was linearly competitive. The intercept on the [I] axis gave $-K_{ic}$ (inhibitor constant for competitive inhibition) = 2.6mM (Fig. 3B). The Dixon-plot (*4*) gave straight lines for each substrate concentration, which intercepted in

$$\frac{1}{v_i} = \frac{1}{V}\left[1 + \frac{K_m}{[S]}\right] + \frac{K_m[I]}{V[S]K_{ic}} \qquad 4$$

the second quadrant at the ordinate value of $1/V$. From the negative abcissa value of the intersection point, again a K_{ic} of 2.6mM was determined (Fig. 3C). Within the limits of error, the experimental data agreed well with those calculated using Eq. *3* ($V_{(cycl)}$ = 2.74 kat.kg^{-1} of protein, K_m = 0.286 g.L^{-1}, and K_{ic} = 2.6mM). From the inhibition kinetics, it was evident that one maltose molecule was bound per enzyme molecule.

For maltotriose, the double reciprocal plots yielded straight lines for each concentration, which intercepted on the $1/v_i$-axis at $1/V_{(cycl)}$, indicating the competitive nature of inhibition (Fig. 4A). The re-plot of slopes 1/[S] against [I] (Fig. 4B), and also the Dixon-plot (Fig. 4C), gave upward concave curves pointing to an enhanced inhibition by the higher concentrations of maltotriose. Both the re-plot of slopes 1/[S] and the Dixon-plot gave nearly straight lines for the lower [I], from which an apparent K_{ic} of 1.8mM was determined.

One explanation for curved lines obtained in the Dixon-plot is the binding of two molecules of inhibitor to the enzyme molecule. Indeed, plotting the slopes 1/[S] against $[I]^2$ yielded a straight line which intersected the ordinate at $\sim K_m/V$ (Fig. 4D). The intercept on the $[I]^2$ axis gave $-K_{ic}$ = 4.47mM. Within the limits of error, the experimental data agreed well with those calculated using Eq. *5*, where V = 2.74 kat.kg^{-1} of protein, K_m = 0.286 g.L^{-1}, and K_{ic}, K_i' = 4.47mM. The K_{ic} de-

$$v_i = \frac{V}{1 + K_m/[S][1 + [I]/K_{ic} + [I]^2/K_{ic}K_i']} \qquad 5$$

termined from the re-plot of slopes 1/[S] against [I] and the Dixon-plot was artifactual, and there was a binding of two inhibitor-molecules to the enzyme molecule independent of the inhibitor concentration.

For maltotetraose, the double reciprocal plot gave straight lines for each concentration, which intercepted the $1/v_i$-axis at $1/V_{(cycl)}$ (Fig. 5A). The re-plot of slopes 1/[S] against [I] (Fig. 5B), and also the Dixon-plot (Fig. 5C), gave upward concave curves, again indicating the binding of two effector molecules to the enzyme molecule. K_{ic} could not be determined from the re-plot of slopes 1/[S] and the

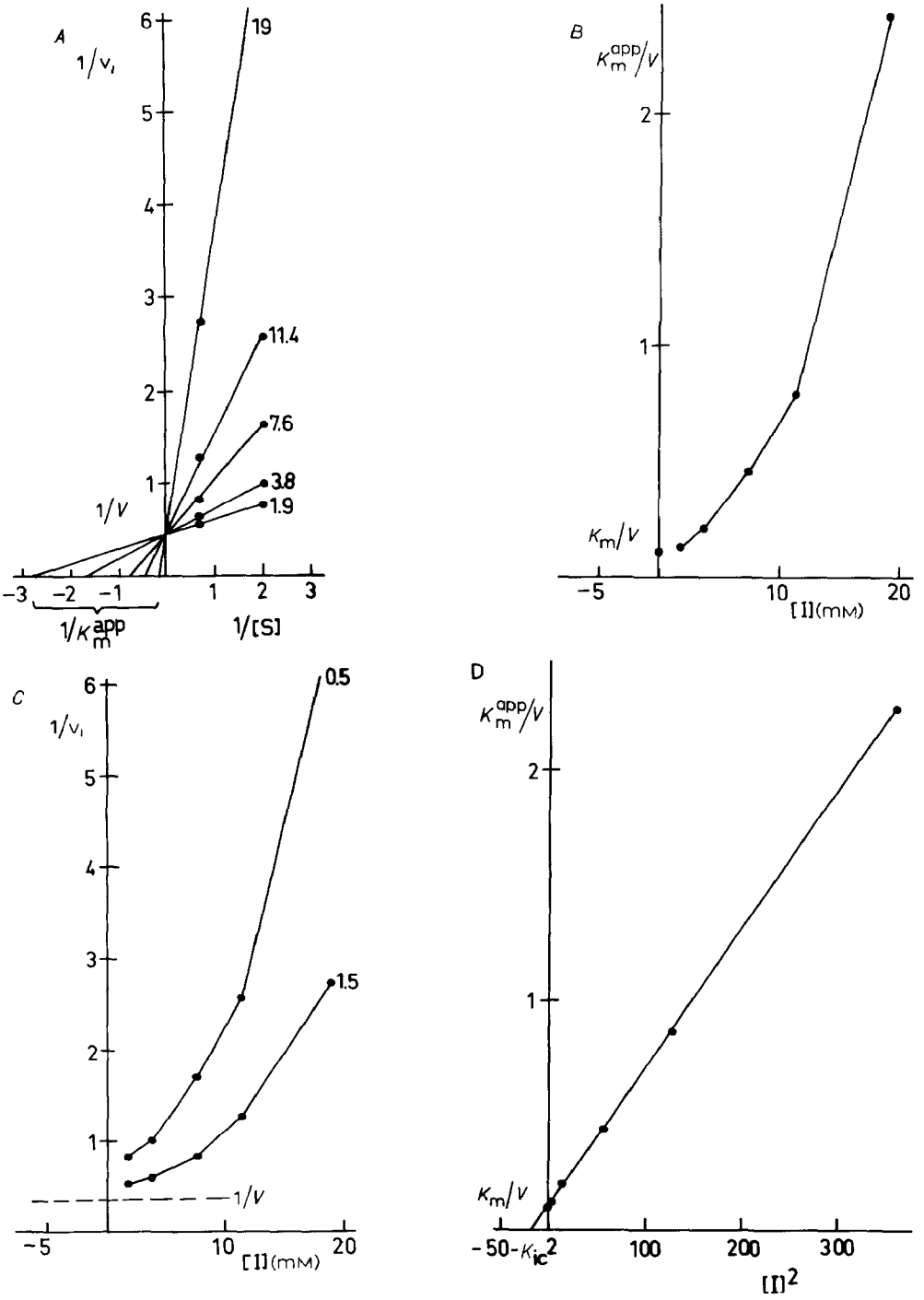

Fig. 5. Inhibition by maltotetraose of the initial cyclisation reaction at 30° with glycogen: *A*, double reciprocal plot $1/v_i$ *versus* 1/[S] (the numbers of the lines give the mM concentration of maltotetraose); *B*, re-plot of the slopes 1/[S] against [I]; *C*, Dixon-plot $1/v_i$ against [I] (the numbers of the lines give the concentration of the substrate in g/L); *D*, re-plot of the slopes 1/[S] against $[I]^2$ (see Experimental).

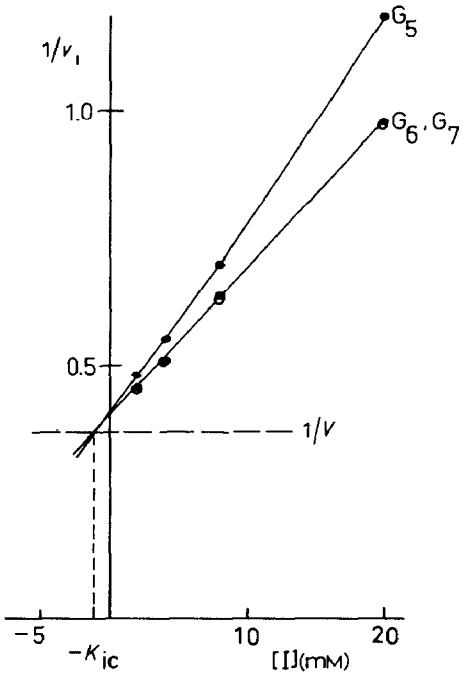

Fig. 6. Inhibition by maltopentaose–maltoheptaose of the initial cyclisation reaction at 30° with glycogen (1.5 g/L). Dixon-plot $1/v_i$ against [I] (see Experimental).

Dixon-plot. As with maltotriose, the re-plot of slopes 1/[S] against $[I]^2$ gave a straight line which intersected the ordinate at K_m/V, and the abcissa at $-K_{ic}^2$ = 4.24mM (Fig. 5D). The experimental data coincided with those calculated using Eq. 5 ($V_{(cycl)}$ = 2.74 kat.kg^{-1} of protein, K_m = 0.286 g.L^{-1}, and K_{ic},K_i' = 4.24mM).

Although maltopentaose–maltoheptaose, by disproportionation, were substrates for initial cyclisation, they also competitively inhibited the cyclisation from glycogen (Fig. 6). The values found for v_i at [I] = 19mM were lower than those found for the initial rates of cyclisation with the saccharides as the substrates. Obviously, glycogen and saccharides compete for the binding sites of the enzyme in such a way that none of them could be cyclised with optimum rate. From the Dixon-plot, values of <1mM for K_{ic} were determined.

The re-plot of the values for $K_m^{app} = K_m(1 + [I]/K_{ic})$ obtained by the double reciprocal plots against the effector concentration should be similar to that of the slopes 1/[S] against [I]. Indeed, the values of K_m^{app} increased linearly with maltose, but upward concave curves were obtained with increasing concentrations of maltotriose and maltotetraose, indicating that much more substrate was needed to prevent the effectors from combining with the active sites of the enzyme (Fig. 7).

The degrees of inhibition [$\varepsilon_i = (v_o - v_i)/v_o$] increased from maltose to maltotetraose. There was no linear correlation between the inhibitor and the substrate concentrations. Linear [I]-dependent decreases were found only for [S] up to 1

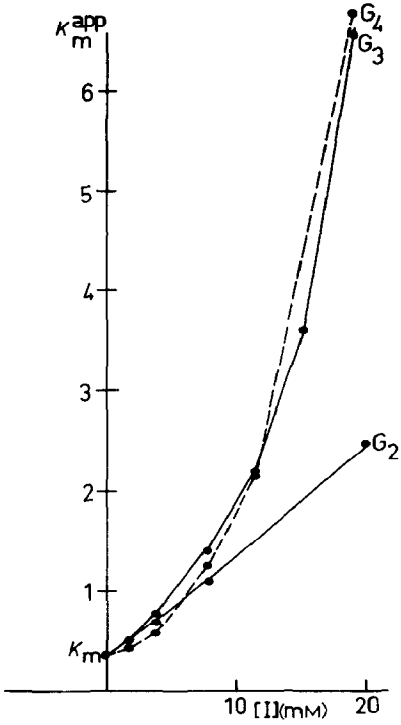

Fig. 7. Re-plot of K_m^{app} obtained by the double reciprocal plots for maltose–maltotetraose (G_2–G_4) against [I].

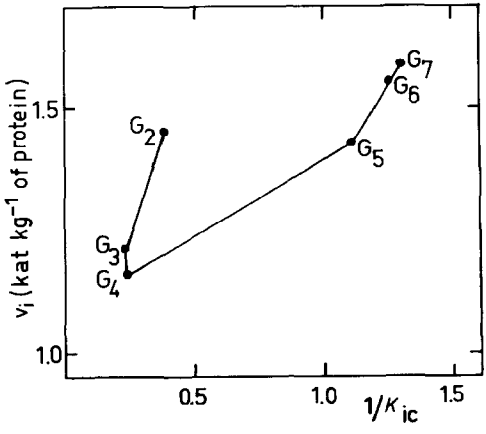

Fig. 8. Plot of the v_i values determined for maltose–maltoheptaose (G_2–G_7) at [S] = 1.5 g/L and [I] = 7.6mM against $1/K_{ic}$ (see text).

g.L^{-1}. One molecule of maltose and two molecules of maltotriose and maltotetraose were bound to the enzyme molecule. Accordingly, the reciprocal of the K_{ic} (maltose) indicates the affinity for one of the binding sites of the enzyme, whereas the reciprocals of the K_{ic} values for maltotriose and maltotetraose give the average affinities for the two binding-sites. The higher malto-oligosaccharides, by disproportionation, are substrates for initial cyclisation and, therefore, must have additional substrate qualities. The v_i values found for [S] = 1.5 g.L^{-1} and maltose–maltoheptaose concentrations of 7.6mM were plotted against $1/K_{ic}$ (Fig. 8), from which it is evident that the most effective inhibitors of the initial cyclisation reaction with glycogen are maltotriose and maltotetraose.

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