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The Use of Deoxyfluoro-D-glucopyranoses and Related Compounds in a Study of Yeast Hexokinase Specificity

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1. 2-Deoxy-2-fluoro-D-glucose, 2-deoxy-2-fluoro-D-mannose and 2-deoxy-2,2-difluoro-D-*arabino*-hexose are good substrates for yeast hexokinase. 2. 3-Deoxy-3-fluoro-D-glucose and 4-deoxy-4-fluoro-D-glucose are poor substrates and have very similar K_m values (8×10^{-2}M). 3. Neither α- nor β-D-glucopyranosyl fluoride is a substrate or inhibitor. 4. Studies with 2-chloro-2-deoxy- and 2-*O*-methyl derivatives of D-glucose and D-mannose have revealed that little chemical modification is possible at position 2 without substantial loss in substrate binding. 5. The variation in the value of K_m for the D-hexose derivatives was associated with a corresponding change in the value of K_m for $MgATP^{2-}$ showing that the binding of $MgATP^{2-}$ is modified by the binding of the sugar.

Three hexokinase isoenzymes (I–III) are present in mammalian tissues (Katzen & Schimke, 1965) and an additional isoenzyme (IV), glucokinase, is present in liver (Vinuela *et al.*, 1963). It has been shown that there are quantitative differences between the hexokinase isoenzyme profile of normal liver and that of Morris minimum deviation hepatomas (Shatton *et al.*, 1969; Sato *et al.*, 1969). It has also been shown that the total amount of hexokinase/g of tissue is greater in a range of rat carcinomas, including mammary carcinoma and Morris hepatomas, than in either normal or regenerating liver (Knox *et al.*, 1970). As hexokinase is one of the rate-determining enzymes in the glycolytic sequence (Weber, 1968), we have been prompted to investigate the possibility of designing selective inhibitors of the hexokinase isoenzymes to evaluate their potential as anti-tumour agents.

Before any attempt to design active-site directed irreversible inhibitors (Baker, 1967) is likely to be successful, a more precise definition of the binding requirements of each of the isoenzymes I–IV is required. As highly purified preparations of yeast hexokinase are readily available, this enzyme has been used as a model in preparation for studies on mammalian hexokinases. Literature data reviewed by Walker (1966) suggests that the substrate specificities of yeast and mammalian hexokinases are comparable.

The substrate specificity studies described by Sols *et al.* (1958) showed that, of the epimers of D-glucose, only the 2-epimer (D-mannose) was a good substrate for yeast hexokinase (ATP–D-hexose 6-phosphotransferase, EC 2.7.1.1). These results, which support previous findings (Gottschalk, 1950), imply that the hydroxyl groups at positions 1, 3, 4 and 6 of the D-glucose molecule play an essential role in the formation of the enzyme–substrate complex. Although the precise nature of this role for each hydroxyl group is unknown, several possibilities can be envisaged. For example, a hydroxyl group can function as a proton donor and/or a proton acceptor in the formation of a hydrogen bond. Chemical reactions are also a possibility, for example, in the case of the anomeric centre, acetal formation (involving an *O*-, *N*- or *S*-glucoside) and esterification of enzyme carboxyl groups. Moreover, there is still doubt concerning the structure of the D-glucose molecule (cyclic or open chain form) that binds to hexokinase and the sequence of events when yeast hexokinase phosphorylates D-glucose. Thus, both an ordered sequential mechanism with glucose binding before $MgATP^{2-}$ (Hammes & Kochavi, 1962; Noat *et al.*, 1968; DelaFuente & Sols, 1970) and a random bisubstrate mechanism (Rudolph & Fromm, 1970; Boyer & Silverstein, 1963) have been proposed.

As a result of an extensive synthetic programme (Bessell *et al.*, 1971, and references cited therein) the complete series of deoxyfluoro derivatives of D-glucopyranose and numerous related fluoro sugars have become available. This type of compound is of potential value in substrate specificity studies of enzymes that utilize carbohydrates. When a hydroxyl group in D-glucose is replaced by a fluorine substituent the number of possibilities for interactions at the active site of hexokinase is decreased. Thus a fluorine substituent can function only as a hydrogen-bond acceptor, and, if present at the anomeric centre (glycosyl fluoride), it will not only block acetal formation, but also prevent equilibration of the sugar between the furanose, pyranose, septanose and open chain forms. Additionally, the marked inductive

effect of a fluorine substituent will increase the acidity of a vicinal hydroxyl group [i.e. in the grouping CF–C(OH)] and thereby influence the interaction of the latter group at the active centre of the enzyme. We now report on the behaviour of the deoxyfluoro-D-glucopyranoses and some related compounds as substrates for yeast hexokinase.

Experimental

Materials

Yeast hexokinase C-301 (200–300 units/mg) was obtained from Sigma (London) Chemical Co., London S.W.6, U.K.

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49, type I purity from yeast, 350 units/mg), ATP, and $NADP^+$ were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Yeast hexokinase has been shown to be a mixture of four isoenzymes with similar kinetic properties (Ramel *et al.*, 1971). All the D-hexose derivatives used in this study were synthesized in these laboratories and were analytically pure and homogeneous as shown by paper and thin-layer chromatography. The synthesis of the following compounds has been described elsewhere: 2-deoxy-2-fluoro-D-glucose, 2-deoxy-2-fluoro-D-mannose (Adamson *et al.*, 1970), 2-deoxy-2,2-difluoro-D-*arabino*-hexose (Adamson *et al.*, 1971), 2-chloro-2-deoxy-D-glucose, 2,2-dichloro-2-deoxy-D-*arabino*-hexose (Adamson & Foster, 1969), 2-deoxy-2-fluoro-D-galactose (Adamson & Marcus, 1970), 2-*O*-methyl-D-glucose (Oldham & Rutherford, 1932), 2-*O*-methyl-D-mannose (Deferrari *et al.*, 1967), 3-deoxy-3-fluoro-D-glucose (Foster *et al.*, 1967), 4-deoxy-4-fluoro-D-glucose (Barford *et al.*, 1970), 6-deoxy-6-fluoro-D-glucose (Bessell *et al.*, 1971), α-D-glucopyranosyl fluoride (Helferich *et al.*, 1926) and β-D-glucopyranosyl fluoride (Helferich & Gootz, 1929).

Synthesis of 2-chloro-2-deoxy-D-mannose

3,4,6-Tri-*O*-acetyl-2-chloro-2-deoxy-β-D-mannopyranosyl chloride (Igarashi *et al.*, 1968) (630mg) was suspended in 0.05M-H_2SO_4 (10ml) and was heated at 90°C for 1.5h. The resulting solution was neutralized with $BaCO_3$ (500mg), filtered through a Hyflo pad, and concentrated under reduced pressure. The product was eluted from a column (25cm × 2.5cm) of Kieselgel (Merck, 7734) with ethyl acetate–methanol (5:1, v/v). Concentration of the appropriate fractions gave 2-chloro-2-deoxy-D-mannose (184mg, 50% yield) as a chromatographically homogeneous, amorphous solid, $[\alpha]_D^{30}$ −6° (*c* 1 in water, equilibrium value).

The structure of 2-chloro-2-deoxy-D-mannose was confirmed by the following reaction sequence. Treatment of 2-chloro-2-deoxy-D-mannose with sodium acetate and acetic anhydride in the usual way (Adamson *et al.*, 1970) gave 1,3,4,6-tetra-*O*-acetyl-αβ-D-mannopyranose as a syrup. The proton magnetic resonance (p.m.r.) spectrum (60MHz, 25% solution in C^2HCl_3, internal standard Me_4Si) contained signals at τ 3.7 (doublet, $J_{1,2}$ 1Hz, *H*-1, α-anomer), 4.0 (doublet, $J_{1,2}$ 1Hz, *H*-1, β-anomer), 7.80, 7.85, 7.90, 7.95 (singlets, 4-OAc). The magnitude of the coupling constants ($J_{1,2}$) for *H*-1 indicates a *gauche* relationship with *H*-2 in each anomer and therefore confirms the *manno* configuration.

3,4,6-Tri-*O*-acetyl-2-chloro-2-deoxy-α-D-mannopyranosyl bromide was synthesized from the foregoing tetra-acetate by conventional treatment with hydrogen bromide in acetic acid and was converted into methyl 3,4,6-tri-*O*-acetyl-2-chloro-2-deoxy-β-D-mannopyranoside by a Koenigs–Knorr reaction with methanol–silver carbonate. The product had m.p. 116°C, unchanged by admixture with the authentic sample kindly supplied by Dr. K. Igarashi and $[\alpha]_D^{30}$ −85° (*c* 0.4 in chloroform); Igarashi *et al.* (1968) recorded m.p. 120.5–121°C, $[\alpha]_D^{22.5}$ −86.9° (in chloroform). The two compounds had the same R_F value [0.40, on t.l.c. with ether–light petroleum (b.p. 60–80°C), (3:1, v/v)] and gave identical i.r. spectra.

Initial-rate measurements

The assay method employed was basically that described by Darrow & Colowick (1962) and Lazarus *et al.* (1966) with glycyl glycine (0.01M), Cresol Red, and 0.015M-$MgCl_2$. The decrease in E_{560} of Cresol Red is a quantitative measure of the acid produced in the reaction. The assay system was standardized by the addition of 1μmol of HCl, which produced a ΔE_{560} of approx. 0.040. All rates were measured at pH8.6 with a Pye Unicam (Cambridge, U.K.) SP.500 monochromator with a Gilford model 220 absorbance indicator (Gilford Instrument Laboratories, Ohio, U.S.A.), the output of which was connected to a Honeywell strip chart recorder (Honeywell Ltd., Brentford, Middx., U.K.). The cuvette compartment was kept at 30°C. The full-scale deflexion of the recorder was set to correspond to an extinction of 0.1 unit and the chart speed was adjusted to give recorded traces with an angle of inclination of between 20° and 60°. Silica cells (1cm light-path) were filled with all the components of the reaction mixture except the enzyme, in a total volume of 2.9ml. The reaction was initiated by the addition of 0.1ml of a suitably diluted solution of enzyme.

All initial rates were determined in triplicate and the mean value was used for the determination of K_m and $V_{max.}$.

K_i values were determined by using the double-reciprocal plot of Lineweaver & Burk (1934). When α-D-glucopyranosyl fluoride and β-D-glucopyranosyl

fluoride were tested as inhibitors, hydrolysis, which released HF, markedly interfered with the determination of the rate of phosphorylation. The rate of phosphorylation was therefore measured by observing the change in E_{340} of a reaction mixture also containing 1×10^{-4}M-NADP and an excess of glucose 6-phosphate dehydrogenase at pH7.5 (0.1M-tris–HCl buffer); α-D-glucopyranosyl fluoride and β-D-glucopyranosyl fluoride are moderately stable between pH5 and 8 (Barnett, 1969).

Data processing

Values of K_m and $V_{max.}$ in eqn. (1) were obtained by the method of Wilkinson (1961); the Fortran program was written by Dr. L. I. Hart and run on the London University CDC 6600 computer.

$$v=(V_{max.}[S])/(K_m+[S]) \quad (1)$$

The program provided estimates of K_m and $V_{max.}$ with standard errors, coefficients of variation and confidence intervals.

Values of K_a, K_b, K_{ia} and V in eqn. (2) were obtained by an analogous method with a computer program written by Cleland (1963). The program was slightly modified by Dr. L. I. Hart to run on the CDC 6600 computer. This program provided estimates of the constants with standard errors.

$$v=\frac{V[A][B]}{K_{ia}K_b+K_a[B]+K_b[A]+[A][B]} \quad (2)$$

Eqn. (3) was used for a mixture of two substrates and an enzyme and was derived by Thorn (1949) by an extension of the Michaelis–Menten theory.

$$v_t=\frac{Vx/K+V'x'/K'}{x/K+x'/K'+1} \quad (3)$$

When $x=x'=s$ and $V_{max.}$ is the maximum initial velocity for an equimolar mixture of two substrates (Whittaker & Adams, 1949) eqn. (4) can be derived.

$$K_m/K_m'=\frac{V-V_{max.}}{V_{max.}-V'} \quad (4)$$

Determination of the ratio K_m/K_m' enabled a check to be made on the K_m values for the sugars.

Results

The experimentally determined kinetic constants are given in Table 1. The K_m and $V_{max.}$ values were determined, in most cases, by using eqn. (1). The concentration of the other substrate was at least 10 times its K_m value except in the case of 3-deoxy-3-fluoro-D-glucose and 4-deoxy-4-fluoro-D-glucose when this was not possible because of the relatively high K_m values. Eqn. (2) was used to calculate the K_a and K_b values when 2-chloro-2-deoxy-D-glucose and ATP were substrates. Compounds that were found not to be substrates for yeast hexokinase showed no initial velocity at a concentration of

Table 1. *Experimentally determined kinetic constants*

For experimental details see the text. Compounds that are not substrates: α-D-glucopyranosyl fluoride, β-D-glucopyranosyl fluoride, 2-*O*-methyl-D-glucose, 2,2-dichloro-2-deoxy-D-*arabino*-hexose, 2-deoxy-2-fluoro-D-galactose, 2,3-anhydro-D-mannose (kindly provided by Professor J. G. Buchanan), 2-chloro-2-deoxy-D-mannose, 2-*O*-methyl-D-mannose. Compounds that are not inhibitors: α-D-glucopyranosyl fluoride, β-D-glucopyranosyl fluoride, 2-deoxy-2-fluoro-D-galactose, 2-deoxy-2,2-dichloro-D-*arabino*-hexose, 2,3-anhydro-D-mannose. Compounds that are inhibitors: 2-*O*-methyl-D-glucose (K_i 60mM), 2-*O*-methyl-D-mannose (K_i 7mM). Values are means±S.E.M.

Compound	K_m (mM)	Relative $V_{max.}$	K_m $MgATP^{2-}$ (mM)	Calculated K_m'/K_m (glucose)	Experimental K_m'/K_m (glucose)
D-Glucose	0.17	1.00	0.20	1	1
2-Deoxy-D-*arabino*-hexose	0.59± 0.11	0.85	0.36±0.11	—	—
2-Deoxy-2-fluoro-D-glucose	0.19± 0.03	0.50	0.26±0.05	1.14	1.36± 0.37
2-Deoxy-2-fluoro-D-mannose	0.41± 0.05	0.85	0.66±0.25	2.46	0.86± 0.75
2-Deoxy-2,2-difluoro-D-*arabino*-hexose	0.13± 0.02	0.53	0.21±0.02	0.78	0.91± 0.47
3-Deoxy-3-fluoro-D-glucose	70 ±30*	0.10	2.3 ±0.3†	—	—
4-Deoxy-4-fluoro-D-glucose	84 ±30*	0.10	1.9 ±0.1‡	—	—
2-Chloro-2-deoxy-D-glucose	2.1 ± 0.6	0.54	0.97±0.33	12.6	9 ±10

* Concn. of ATP, 4.1mM.
† Concn. of 3-deoxy-3-fluoro-D-glucose, 10mM.
‡ Concn. of 4-deoxy-4-fluoro-D-glucose, 10mM.

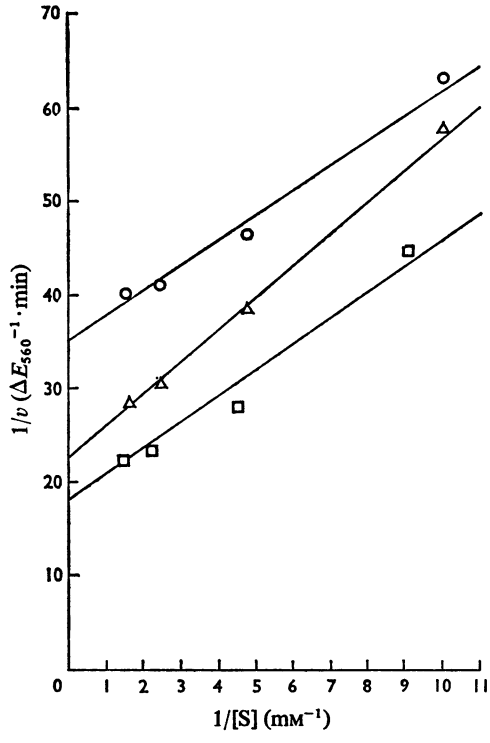

Fig. 1. *Reciprocal plots for the effect of* 2-*deoxy*-2-*fluoro*-D-*glucose on the phosphorylation rate of* D-*glucose*

For experimental details see the text. □, D-Glucose; △, 2-deoxy-2-fluoro-D-glucose+D-glucose in equimolar concentrations; ○, 2-deoxy-2-fluoro-D-glucose.

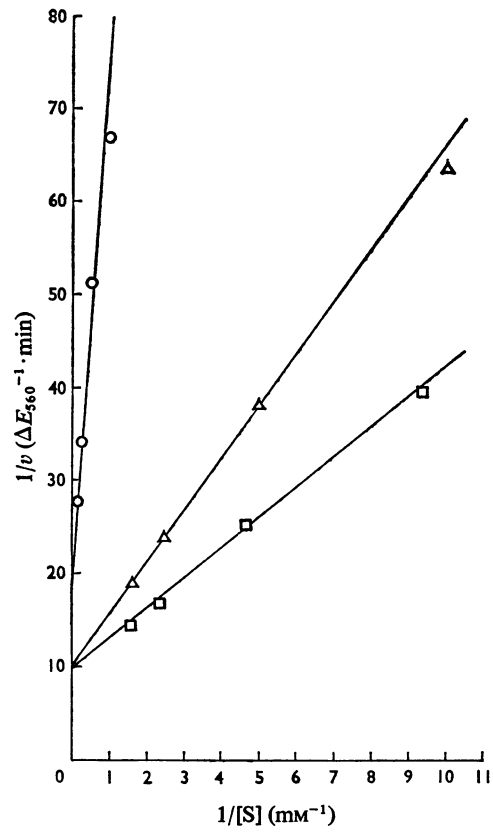

Fig. 2. *Reciprocal plots for the effect of* 2-*chloro*-2-*deoxy*-D-*glucose on the phosphorylation rate of* D-*glucose*

For experimental details see the text. □, D-glucose; △, 2-chloro-2-deoxy-D-glucose+D-glucose in equimolar concentrations; ○, 2-chloro-2-deoxy-D-glucose.

$\geqslant$10mM with an enzyme concentration of at least 5 units/ml.

The calculated value of K_m'/K_m, where K_m' and K_m are the Michaelis constants of the halogenated D-glucose derivative and D-glucose respectively, was obtained by dividing the experimentally determined K_m' values by the published value of K_m, 1.7×10^{-4}M (Fromm & Zewe, 1962). The experimentally determined value of K_m'/K_m was obtained by using eqn. (4) where V, $V_{max.}$ and V' are the maximum velocities for D-glucose, for the two substrates in equimolar concentrations, and for the halogenated D-hexose, respectively. The velocity constants were calculated by using eqn. (1). The standard error for the experimentally determined quotient K_m'/K_m was calculated from the property of additivity of the squares of the coefficients of variation. The inaccurate value of K_m'/K_m obtained experimentally for 2-deoxy-2-fluoro-D-mannose was due to the similarity of the values of V, $V_{max.}$ and V'.

The effect of the equimolar concentration of 2-deoxy-2-fluoro-D-glucose on the rate of phosphorylation of D-glucose is shown by a double-reciprocal plot in Fig. 1. The effect of 2-chloro-2-deoxy-D-glucose is shown in Fig. 2.

Those deoxyhalogenohexoses that were substrates for yeast hexokinase gave 6-phosphates. Evidence supporting the assigned structures has been obtained by H. D. Beckey, A. B. Foster, M. Jarman, H.-R. Schulten & J. H. Westwood (unpublished results).

Discussion

The commercial yeast hexokinase preparation used in this work for the determination of kinetic parameters was a mixture of isoenzymes. The K_m values reported by Ramel *et al.* (1971) for the individual

isoenzymes differ by up to twofold in magnitude. Thus, the results reported here should be regarded as no more than semi-quantitative in nature as there is no basis at present for assuming that the hexose derivatives used behave similarly with the various hexokinase isoenzymes. Nevertheless, the halogeno derivatives can be divided into four groups: (1) good substrates, e.g. 2-deoxy-2-fluoro-D-glucose, 2-deoxy-2,2-difluoro-D-*arabino*-hexose, 2-deoxy-2-fluoro-D-mannose and 2-deoxy-D-*arabino*-hexose; (2) moderate substrates, e.g. 2-chloro-2-deoxy-D-glucose; (3) poor substrates, e.g. 3-deoxy-3-fluoro-D-glucose and 4-deoxy-4-fluoro-D-glucose; (4) non-substrates, e.g. α-D-glucopyranosyl fluoride and β-D-glucopyranosyl fluoride.

It is possible to relate the semi-quantitative K_m value of a derivative to its binding to the enzyme, because yeast hexokinase appears to operate by a steady-state random mechanism with all the steps in the reaction sequence in rapid equilibrium relative to the interconversion of the ternary complex (Rudolph & Fromm, 1971).

The results obtained in the present work are in agreement with those of previous studies on the substrate specificity of yeast hexokinase that modification only at C-2 of the D-glucose molecule can be made without loss of binding to the enzyme. The replacement of HO-6 in D-glucose by a fluorine substituent substantially decreases binding to yeast hexokinase. 6-Deoxy-6-fluoro-D-glucose, which cannot be phosphorylated by the enzyme, is a competitive inhibitor (K_i 12mM). The several replacements of HO-3 and HO-4 by fluorine substituents also decrease binding to the enzyme. 3-Deoxy-3-fluoro-D-glucose and 4-deoxy-4-fluoro-D-glucose are both substrates with similar $V_{max.}$ values and K_m values of 70 and 84mM respectively. These data suggest that HO-3 and HO-4 could have similar roles in substrate–enzyme binding and that the hydroxyl groups at position 3, 4, and 6 do not function solely as hydrogen-bond acceptors when located at the receptor site.

The replacement of HO-2 in D-glucose by a fluorine substituent gives 2-deoxy-2-fluoro-D-glucose which is a good substrate with a K_m value similar to that of D-glucose. The corresponding replacement in D-mannose gives 2-deoxy-2-fluoro-D-mannose, which is a substrate with a K_m value similar to that of D-mannose and with a comparable $V_{max.}$. Of particular interest is the geminal difluoro compound, 2-deoxy-2,2-difluoro-D-*arabino*-hexose, which is a very good substrate for yeast hexokinase and is a good inhibitor of D-glucose metabolism *in vitro*; 2-deoxy-2,2-difluoro-D-*arabino*-hexose 6-phosphate is not a substrate for glucose 6-phosphate dehydrogenase (E. M. Bessell & P. Thomas, unpublished work).

2-Chloro-2-deoxy-D-glucose is a substrate (K_m 2mM), but the 2-epimer, 2-chloro-2-deoxy-D-mannose, is not a substrate, showing that there is more scope for chemical modification at position 2 in the D-*gluco* than in the D-*manno* series. As might be expected, 2,2-dichloro-2-deoxy-D-*arabino*-hexose is neither a substrate nor a competitive inhibitor.

Although chemical modification of the D-glucose molecule is restricted as far as substrate specificity is concerned, this is not so with respect to competitive inhibitor specificity. It has been shown (Sols *et al.*, 1958) that 2-acetamido-2-deoxy-D-glucose and 2-*C*-hydroxymethyl-D-glucose are competitive inhibitors (K_i values 1mM) as are 2-*O*-methyl-D-glucose and 2-*O*-methyl-D-mannose, but the latter compounds have higher K_i values. These results suggest that poor binding may not be due so much to steric hindrance associated with a bulky group at C-2 in the D-*gluco* series but to the lack of a group able to donate a hydrogen bond. In fact, 2-(3,5-dinitrobenzamido)-2-deoxy-D-glucose is a better competitive inhibitor of yeast hexokinase than is 2-acetamido-2-deoxy-D-glucose (Maley & Lardy, 1955). The *N*-acetyl and *C*-hydroxymethyl groups are each capable of donating a hydrogen bond whereas the *O*-methyl group is not. This factor may be important in the design of active-site-directed irreversible inhibitors of hexokinase.

The possible importance of a hydrogen-bond-donating group at C-2 is not easily reconciled with the fact that the 2-fluoro derivatives 2-deoxy-2-fluoro-D-glucose, 2-deoxy-2-fluoro-D-mannose and 2-deoxy-2,2-difluoro-D-*arabino*-hexose are good substrates for yeast hexokinase. One explanation of this situation would follow if HO-2 displaced a water molecule on the receptor site of the enzyme but compensated by providing a hydrogen bond. Replacement of HO-2 by a fluorine or hydrogen substituent might result in the water molecule at the receptor site not being displaced because of the smaller size of the substituent. An additional factor may be operative in the binding of 2-deoxy-2,2-difluoro-D-*arabino*-hexose, which has a K_m value similar to that of D-glucose. The electron-withdrawing effect of the CF_2 group will increase the acidity of HO-3 and thereby increase its proton donating capability in hydrogen-bond formation.

Replacement of the hydroxyl group at position 2, 3, 4 or 6 by a fluorine substituent does not result in complete loss of binding to the enzyme. However, replacement of HO-1 in α- or β-D-glucopyranose by a fluorine substituent has this effect. The effect can be explained by postulating either that HO-1 plays a vital role in the binding, or that D-glucose does not bind in its pyranose form.

The variation in the K_m values of ATP with different sugar substrates shows that the binding of ATP is modified. These results are in accordance with the 'induced fit' postulated by DelaFuente *et al.* (1970) to explain the modification of adenosine triphosphatase activity in the presence of D-xylose and D-lyxose.

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