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Biological Catalysis of Mutarotation of Glucose

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We have shown in previous papers (Keilin & Hartree, 1948*a*, 1952) that glucose oxidase, or notatin, is highly specific for β -glucose, the rate of oxidation of α -glucose being only about 0.64 % of that of β -glucose.

While determining the relative rates of oxidation of α - and β -glucose by two purified samples of notatin (*Y* and *Z*) we found that although their activities towards β -glucose, or equilibrium glucose, were virtually the same, their activities towards α -glucose differed very markedly, notatin *Z* being in this respect about eight times more active than notatin *Y*. This difference was ascribed by us to the presence in sample *Z* of an additional component capable of accelerating mutarotation.

In the present paper we shall examine the properties of this component and compare them with those of notatin itself. This will enable us to bring forward fresh evidence in support of the view that the mutarotation accelerating substance is independent of notatin, although until now the greatest concentration of this substance has been found in some highly purified samples of notatin. On the other hand, no attempt to isolate it in the pure form could be made without sacrificing much of the notatin which we could scarcely afford, considering the very limited availability of this enzyme and the great demand upon it for the study of kinetics of

reactions catalysed by different enzymes (Keilin & Hartree, 1948*b*).

Since the properties of the substance accelerating mutarotation are those of a highly active, thermolabile, non-dialysable catalyst we propose for it the name *mutarotase*. Considering that a variety of substances are capable of catalysing mutarotation, and that the intimate mechanism of such catalysis is still unknown, the name *mutarotase* given to the catalyst found in notatin preparations can only be regarded as a convenient provisional term, pending further evidence as to its enzymic nature. This would require more information on the properties of this catalyst based upon the study of preparations which are more active and more readily available than the highly purified samples of notatin used in the present study.

MATERIALS AND METHODS

Notatin. The two samples *Y* and *Z* described in the previous paper (Keilin & Hartree, 1952) were used in the present study.

Catalase, α - and β -glucose and phosphate buffer were the same materials as used in the previous paper.

α -Methyl glucoside had been prepared by the standard procedure from glucose and methanolic HCl.

β -Methyl glucoside was prepared by the method of Müller (1931). The melting point and mixed melting point with an authentic sample were 107–109° (uncorr.).

Fructose, lactose, maltose, xylose and sorbitol were pure bacteriological grades.

Anaerobic polarimetric measurements of mutarotation and manometric estimations of glucose oxidase activity were carried out as described in our previous paper.

RESULTS

It has already been established (Keilin & Hartree, 1952) that the addition under anaerobic conditions of 0.5 mg./ml. of notatin Z to a 4% solution of either α - or β -glucose in 0.2M-phosphate buffer pH 5.6, at 20°, approximately doubles the mutarotation constant (K).

Effect of enzyme concentration on mutarotation

In Table 1 and Fig. 1 are summarized the results of further polarimetric experiments carried out anaerobically, as previously described, on the effect of varying concentrations of notatin Z on the mutarotation of α - and β -glucose. These experiments

Table 1. Effect of different concentrations of notatin Z ('233-237') on the mutarotation of α - and β -glucose in presence of 0.2M-phosphate buffer, pH 5.6, at 20°

(s.d.: standard deviation. Data obtained by polarimetric measurements carried out anaerobically.)

Isomer	Glucose		Notatin concentration (mg./ml.)	$10^3 K$ (min. ⁻¹)	s.d. (%)
	Concentration (% w/v)				
α	4.0	1.0	185.9	1.24	
β	4.0	1.0	168.7	2.61	
α	4.0	0.5	131.4	1.00	
β	4.0	0.5	116.5	1.86	
α	4.0	0.3	101.0	1.58	
β	4.0	0.3	96.7	0.96	
α	4.0	0.2	90.7	1.40	
β	4.0	0.2	85.0	1.41	
α	4.0	0.1	78.6	1.44	
β	4.0	0.1	75.6	1.25	
α	4.0	0.0	65.8*	0.84	
β	4.0	0.0	66.4*	1.11	

* Values in absence of notatin taken from the previous paper (Keilin & Hartree, 1952).

show that the increment of the mutarotation constant above the value for buffer is proportional to notatin concentration and therefore to the concentration of mutarotase. Thus if the mutarotation constants are plotted against notatin concentrations a straight line is obtained which cuts the K axis at 0.0664, the mutarotation constant in 0.2M-phosphate buffer, pH 5.6. These experiments also show that K is significantly higher for α - than for β -glucose. Moreover, the values of K calculated from successive readings of optical rotation slightly drifted with time: upward for β -glucose and down-

ward for α -glucose. The constant thus tended towards a common mean as the mutarotation proceeded. However, since the equilibrium values of rotation were not influenced by the enzyme preparation the accelerating effect was a true catalysis.

Fig. 1. The influence of the concentration of notatin Z on the mutarotation constant for α - and β -glucose (4% w/v) in presence of 0.2M-phosphate buffer, pH 5.6, at 20°. ● = Mutarotation constant in buffer.

The effect of glucose concentration on the enzyme-catalysed mutarotation

The experiments carried out with different concentrations of α - and β -glucose, which are summarized in Table 2 and Fig. 2, show that the mutarotation constant falls with increasing glucose concentration and approaches the value of K for buffer alone (which is of course independent of glucose concentration). This unexpected relationship between K and substrate concentration will be discussed later.

Effect of other sugars on the catalysis of mutarotation and on the oxidation of glucose

The effect of various sugars on the mutarotation of α -glucose in presence of notatin Z was tested under the usual anaerobic conditions except that the notatin (0.27 ml. of 2% aqueous solution) was kept separate from the solution of the second sugar until all oxygen had been removed from the apparatus. This was achieved by placing the notatin solution in a small side bulb (Keilin & Hartree, 1952, Fig. 1, H). α -Glucose (400 ± 1 mg.) was weighed into the apparatus while the second sugar (4 mol. proportions) was dissolved in phosphate buffer (18 ml.) and allowed to stand overnight to reach rotational equilibrium before being placed

in the apparatus. In every case a control experiment in absence of notatin was carried out. From the results of these experiments, which are summarized in Table 3, it may be seen that the greatest inhibition is obtained with sugars which have a close

Table 2. *Effect of different concentrations of glucose on mutarotation of α - and β -glucose in presence of notatin Z and 0.2M-phosphate buffer, pH 5.6 at 20°*

(Data obtained polarimetrically under anaerobic conditions.)

Isomer	Glucose		Notatin concentration (mg./ml.)	10 ³ K (min. ⁻¹)	S.D. (%)
	Concentration (% w/v)				
α	1.33	0.2	125.5	1.68	
β	1.33	0.2	117.5	2.81	
α	2.66	0.2	98.3	1.47	
β	2.66	0.2	94.0	1.03	
α	4.00	0.2	90.7	1.40	
β	4.00	0.2	85.0	1.41	
α	5.65	0.2	82.2	0.94	
β	5.65	0.2	80.1	0.65	
α	4.00	0.0	65.8*	0.84	
β	4.00	0.0	66.4*	1.11	

* Values in absence of notatin taken from Keilin & Hartree (1952).

structural resemblance to glucose and, in particular, to β -glucose; in fact xylose and β -methyl glucoside are at least as effective as an excess of equilibrium glucose. Since the relative proportions of the α - and β -forms of the different sugars will vary, no strictly quantitative significance can be ascribed to the values expressing the relative degrees of inhibition of mutarotation produced by these sugars (Table 3). Owing to the very limited amount of notatin available no attempt was made to determine whether these inhibitions were competitive.

Table 3. *Effects of a second sugar (a) on the mutarotation under anaerobic conditions of α -glucose in presence and in absence of notatin Z; and (b) on the oxidation of equilibrium glucose by notatin Z*

((a) 400 mg. α -glucose + 4 equiv. of second sugar in 18 ml. 0.2M-phosphate buffer, pH 5.6; notatin 0.3 mg./ml.; 20°. (b) Oxidation of glucose determined manometrically: 30 mg. equilibrium glucose and 10 equiv. of second sugar; 30 μ g. notatin; 0.04 ml. catalase; 20°.)

Second sugar	10 ³ K (mutarotation)		Inhibition by second sugar in presence of notatin (%)	
	Without notatin	With notatin	Mutarotation of α -glucose	Oxidation of equilibrium glucose
None	66.7	129.2	—	—
β -Methyl glucoside	66.1	75.0	86	3
Xylose	63.7	73.9	83	8
Equilibrium glucose	64.6	77.8	78	0
Maltose	66.9	88.8	65	15
α -Methyl glucoside	67.5	90.5	63.5	0
Sorbitol	62.6	96.0	46.5	0
Lactose	64.6	105.3	35.0	12
Fructose	62.2	106.1	29.8	0

The effects of the same sugars on the rate of oxidation of glucose by glucose oxidase were also examined. In these experiments, which were carried out manometrically, the right-hand flasks received 30 μ g. notatin, catalase, 0.3 ml. 10% equilibrium glucose (in dangling tubes) and ten equivalents of the second sugar. The left-hand flasks received everything except the equilibrium glucose so as to compensate for any slow oxidation of the second sugar. The very slight inhibition shown in a few instances (Table 3) has no bearing upon the structural relationship between the substrate and the inhibitor.

Fig. 2. The influence of the concentration of α - and β -glucose on the mutarotation constants in a solution of notatin Z (0.2 mg./ml.) in 0.2M-phosphate buffer, pH 5.6, at 20°.

Effect of heating notatin Z on the catalysis of mutarotation

For the study of thermolability of mutarotase a solution of notatin Z containing 0.5 mg./ml. phosphate buffer was heated, in 20 ml. portions, under the varying conditions shown in Table 4. The different samples were then tested for their effect on mutarotation. For these tests, to 18 ml. of the

heated and filtered notatin solution, α -glucose was added anaerobically to give a concentration of 4%. The results of these experiments, summarized in Table 4, show that heating at 65° for only 10 min. abolishes the mutarotating activity of the enzyme preparation by 86%.

Table 4. *Irreversible inactivation of mutarotase after heating notatin to different temperatures*

(Mutarotation experiments carried out under the usual anaerobic conditions with 4% α -glucose and notatin Z (0.5 mg./ml.) at 20°.)

Treatment of notatin	10 ³ K (min. ⁻¹)	Inactivation (%)
Untreated	131.4	—
60° for 10 min.	118.4	20
65° for 10 min.	75.2	86
80° for 10 min.	72.6	90
100° for 30 min.	67.2	99
Control (no notatin)	66.4	—

Table 5. *The effect of 20 min. treatment at pH 9.1 followed by neutralization to pH 5.6 on mutarotation and on rate of glucose oxidation by notatin Z*

(For mutarotation, 720 mg. α -glucose was dissolved anaerobically in 18 ml. phosphate-borate buffer without and with notatin (final concentration 0.5 mg./ml.). Oxidase activity was determined as in Table 3 using 30 mg. equilibrium glucose and 0.1 ml. of the same phosphate-borate-notatin solution; temperature 20°. Standard deviations are absolute values and not percentages as given elsewhere.)

Solutions used for tests	10 ³ K (min. ⁻¹)	O ₂ uptake (μ l./hr.) (based on initial rate)
(I) Notatin incubated at pH 9.1 and neutralized to pH 5.6	105.4 $\pm$ 6.1*	174
(II) Notatin buffered to pH 5.6	110.0 $\pm$ 1.3	1380
(III) Phosphate-borate buffer as used for I and II	53.6 $\pm$ 0.73	—

* The high standard deviation was due to difficulties in taking readings with a slightly cloudy solution.

The effect of prior incubation at pH 9.1 on the mutarotase and glucose oxidase activities of notatin Z

For these experiments the following solutions were prepared: (a) notatin Z, 5 mg./ml. in water; (b) 0.05M-borax; (c) phosphoric acid, approximately 1M; (d) 0.2M-phosphate buffer, pH 5.6; (e) a mixture of 20 ml. borax and 2.0 ml. phosphoric acid (pH 5.6). From these solutions were prepared three mixtures as follows:

(I) 2 ml. *a* was added to 8 ml. *b*. After 20 min. at 20° the solution was neutralized to pH 5.6 by the addition of 9.2 ml. *d* and 0.8 ml. *c*.

(II) 2 ml. *a* was added to a mixture of 9.2 ml. *d* and 8.8 ml. *c*.

(III) As (II), but the notatin solution (*a*) was replaced by water.

In (I) the notatin had been at pH 9.1 for 20 min. while in (II) the pH had been maintained at 5.6. The inorganic constituents were identical in all three mixtures. To estimate glucose oxidase activity 0.1 ml. each of (I) and (II) were tested manometrically with catalase and 30 mg. equilibrium glucose under the standard conditions. For the determination of mutarotase activity, 18 ml. of (I), (II) and (III) were examined under anaerobic conditions in the polarimeter with 720 mg. α -glucose.

From the results of these experiments (Table 5) it is seen that while the high pH has inactivated the glucose oxidase by 87%, the mutarotase activity is not significantly affected.

Fractionation of notatin Z

Further confirmation of the view that mutarotation and oxidation of glucose by notatin Z are catalysed by two distinct enzymes, mutarotase and glucose oxidase, was obtained by a simple fractionation procedure.

For this purpose a 0.2% (w/v) aqueous solution of notatin Z was prepared and the insoluble residue filtered off. Of this solution (130 ml.), now containing 1.84 mg./ml., 5 ml. was set aside as a control. To the remaining 125 ml. was added 56.9 g. (NH₄)₂SO₄ to give 0.70 saturation. The resulting precipitate was centrifuged off, washed with 0.70 saturated (NH₄)₂SO₄, dissolved in a little water and dialysed against distilled water until salt free (fraction A). Two further fractions (B and C) were similarly obtained by raising the (NH₄)₂SO₄ concentration successively to 0.80 and 0.90 saturation respectively.

To 16 ml. A was added 1 ml. tricalcium phosphate gel (35.4 mg. dry weight). The mixture, which had a pH of 6.8, was centrifuged to give a clear pale-yellow solution and a yellow residue. The former was dialysed as before to give fraction A_i. The yellow residue was washed with 5 ml. distilled water, the water was discarded and the residue resuspended in 2 ml. water. The adsorbed material was removed from the calcium phosphate by addition of 0.5 ml. saturated potassium hydrogen oxalate which brought the pH to 7.4. The mixture was centrifuged and the clear bright-yellow eluate poured off. The almost white residue was again extracted with 3 ml. water and the extract added to the yellow eluate. This solution was dialysed to give fraction A_{ii}.

The volumes, dry weights and activities of the various fractions are given in Table 6. The activities are expressed as the initial rates of O₂ uptake obtained under the standard conditions but with 3.0 mg. equilibrium glucose. The volumes of enzyme taken for the activity determination were adjusted so that the rate of uptake did not exceed 10 μ l./min. since under these conditions the rate is constant for at least 5 min. and is directly proportional to the enzyme concentration. From the activity figures the volume of each fraction containing the same quantity of glucose oxidase as 0.2 ml. of the control could easily be calculated (Table 6, column 6).

In Fig. 3 are shown the apparent rates of oxidation by equivalent amounts of the various fractions,

Table 6. *Activities of glucose oxidase solutions obtained by fractionating notatin Z*

(Glucose oxidase activities expressed as $\mu\text{l. O}_2$ uptake/min./0.01 ml. enzyme solution in presence of 0.3 ml. 1% equilibrium glucose and 0.04 ml. catalase in 0.2M-phosphate buffer pH 5.6 at 20°.)

Fractions	Colour	Vol. (ml.)	Dry wt. (mg./ml.)	Activity	Volumes containing equivalent quantities of enzyme (ml.)
Notatin Z	Pale yellow	—	1.84	7.10	[0.2]
A	Yellow	26.5	2.15	14.67	0.0965
B	Yellow	13.5	3.95	19.22	0.0734
C	Yellow-brown	3.0	1.37	6.63	0.214
Ai	Yellow	16.0	2.60	13.62	0.0938
Aii	Pale yellow	7.3	0.78	4.52	0.314

of 3 mg. α -glucose. The results are plotted logarithmically as in the preceding paper (Keilin & Hartree, 1952). The dotted line in this figure represents the theoretical course of the $\alpha \rightarrow \beta$ change in

Fig. 3. Apparent rates of oxidation of 3 mg. α -glucose (plotted logarithmically) catalysed by various fractions obtained from notatin Z (see Table 6). Experiments carried out in presence of 0.04 ml. catalase, 0.2M-phosphate buffer, pH 5.6, at 20°; enzyme quantities as shown in Table 6 (column 6); - - - - -, theoretical course (if α -glucose would not be oxidized directly) depending upon mutarotation due to phosphate buffer; — — —, expected course if α -glucose is oxidized directly in absence of mutarotase.

phosphate buffer, if the back reaction were abolished, and hence the rate of oxidation if α -glucose were not directly oxidized by notatin. However, a slow direct oxidation of α -glucose by notatin does take

place and it can be calculated from our previous results (Keilin & Hartree, 1952, fig. 7) that with the glucose oxidase present in 0.2 ml. of control solution, the reaction would proceed as shown by the pecked line.

The increase in slopes of the full lines in Fig. 3, as compared with the pecked line, is thus a measure of the mutarotase/glucose oxidase ratios of the various fractions.

Since the oxidase activity of all fractions is the same, they must differ markedly in their content of mutarotase, which in fractions B and C was much higher, and in A and Aii much lower, than in the original preparation. Fraction Ai from notatin Z was completely devoid of mutarotase, and thus resembled in this respect the notatin Y examined in the previous paper (Keilin & Hartree, 1952).

Detection of mutarotase in culture medium of Penicillium notatum

The study of mutarotase in culture medium was carried out manometrically. The strain of *P. notatum* used by Coulthard *et al.* (1945) for the preparation of notatin was grown at 24° on the medium A of these workers. Cultures were kindly supplied by Mr C. E. Coulthard of the Boots Pure Drug Company.

After 1 week the medium was collected and filtered, 100 ml. lots were frozen at -15° in thin layers and dried, in the frozen state, in a vacuum desiccator (0.1 mm. Hg) over H_2SO_4 and KOH. The resulting light-brown powder was dissolved in about 10 ml. water and dialysed for 24 hr. against distilled water at 5°. The solution (15 ml.) was again dried in the frozen state and the residue dissolved in 5 ml. water (concentrate C). The ratio of the rates of oxidation of α - and β -glucose was obtained as follows: under the standard conditions for manometric experiments described in the previous paper (Keilin & Hartree, 1952), the complete oxidations of 3.0 mg. α -glucose by (1) 300 $\mu\text{g.}$ notatin Y (which is devoid of mutarotase) and (2) 300 $\mu\text{g.}$ Y plus 0.5 ml. C, were followed (Fig. 4, a and b). An experiment identical with (2) except that heated C was used showed that no thermostable catalysts of mutarotation, such as salts, were present in C (c). Two further experiments

(d and e) compared the rates of oxidation of 3.0 mg. α - and β -glucose by 0.5 ml. *C*.

The first-order reaction constants ($-2.30 \times \text{slope}$), as given in Fig. 4, were defined in the previous paper: K'_α (measured constant for α -glucose) = $K'_\alpha + k_\alpha$, where K'_α = sum of the constants for the direct oxidation of α -glucose and for the enzyme-catalysed mutarotation $\alpha \rightarrow \beta$ and k_α = constant for $\alpha \rightarrow \beta$ change in the phosphate buffer.

Fig. 4. Oxidation of 3.0 mg. β -glucose and apparent oxidation of 3.0 mg. α -glucose (plotted logarithmically) by a freeze-dried concentrate *C* (0.5 ml.) of a 7-day culture filtrate of *P. notatum*. Conditions as for Fig. 3. *a*, *C* + 300 μ g. notatin *Y* + α -glucose; *b*, notatin *Y* + α -glucose; *c*, boiled *C* + notatin *Y* + α -glucose; *d*, *C* + α -glucose; *e*, *C* + β -glucose. ---, theoretical course (if α -glucose would not be oxidized directly) depending upon mutarotation due to phosphate buffer.

The value of K'_α for 0.5 ml. *C*, i.e. the difference between the constants obtained from experiments *a* and *b*, is 0.090. In these two experiments the presence of excess notatin *Y* ensures that β -glucose, as it is formed, is oxidized virtually instantaneously and that, in consequence, the difference is a true measure of the sum of the oxidation of α -glucose by *C* and any catalysis of mutarotation additional to the buffer contribution. If *C* contains sufficient glucose oxidase to oxidize the β -glucose as rapidly as it is formed from the α -form, then a true value of K'_α could also be obtained from the difference between the constant for *d* (oxidation of α by *C*) and the constant for the $\alpha \rightarrow \beta$ change (pecked line in Fig. 4). Since the value of K'_α obtained in this way is very close to that obtained from *a* and *b* it follows that 0.5 ml. *C* does in fact contain almost sufficient glucose oxidase.

Experiment *e* gives directly the value of K'_β (0.461). In the preceding paper (Keilin & Hartree, 1952; Table 3) the activity of notatin towards β -

glucose was defined as $A_\beta = \frac{K'_\beta}{\text{g. enzyme}}$ and a value

of 3250 was obtained for an enzyme preparation which was about 90 % pure. From these figures it can be calculated that 0.5 ml. *C* contains about 130 μ g. of glucose oxidase. Since the dry weight of 0.5 ml. *C* is 5.0 mg., the preparation is about 2.6 % pure with respect to glucose oxidase. The change of slope in line *e* itself indicates a high mutarotase activity. In the preceding paper (Keilin & Hartree, 1952; fig. 3), this inflexion was observed when using 20 or 40 μ g. notatin in absence of mutarotase and it appeared to be due to the $\beta \rightarrow \alpha$ change during the first part of the experiment. The β -glucose was thus exhausted before the oxygen equivalent to 3.0 mg. glucose had been taken up and the latter part of the oxidation was limited by the rate of the $\alpha \rightarrow \beta$ back reaction in the phosphate buffer. With 80 μ g. notatin ($\approx$ about 72 μ g. pure enzyme) the oxidation was complete before an appreciable degree of mutarotation could take place. In the present experiments, however, the increased rate of mutarotation can affect the course of the oxidation of β -glucose by as much as 130 μ g. glucose oxidase.

For *C* the rate of oxidation of α -glucose, as percentage of the rate for β -glucose is thus

$$0.090/0.461 \times 100,$$

i.e. ~ 20 %. The comparable figures for mutarotase-free glucose oxidase is 0.64 % and for notatin *Z* ~ 5 % (Keilin & Hartree, 1952).

A second set of experiments similar to the above was carried out with a culture medium concentrate prepared in a slightly different manner: the filtered medium (100 ml.) was dialysed against distilled water until salt-free (24 hr.), and then freeze-dried and redissolved in 5 ml. water. Using this material it was found that both K'_α for α -glucose + concentrate, and K'_β (corresponding to *d* and *e* in Fig. 4) were reduced to about 0.049. Since the direct oxidation of β -glucose is a measure of glucose oxidase activity it follows that this enzyme had been approximately 90 % destroyed by the second method of concentration. However, K'_α for α -glucose + concentrate + notatin *Y* (cf. *a*) remained unchanged, indicating that the activity of mutarotase was the same in both concentrates.

Mutarotase in mycelia of *P. notatum*

Two 7-day mycelia (15 cm. diam.), grown as described in the previous section, were well washed with water, dried between filter paper, and disintegrated under 250 ml. acetone in a Waring blender. The mixture was filtered on a Büchner funnel and the powder allowed to dry in the air.

The powder (2.4 g.) was extracted for 30 min. with 72 ml. 0.2M-phosphate buffer pH 5.6 and centrifuged. The pale-yellow supernatant fluid was clarified by filtration through a Whatman no. 3 paper.

Manometric studies of mutarotation in this extract were not possible owing to the presence of an inhibitor of glucose oxidase. Tests for mutarotase were therefore carried out by the anaerobic polarimetric method described in the previous paper (Keilin & Hartree, 1952). The mutarotation constant K was determined using 4% (w/v) α -glucose in (a) 0.2M-phosphate buffer, pH 5.6, and (b) the buffer extract of the acetone powder. K was found to be 28% higher with b than with a .

DISCUSSION

We have previously shown that some samples of purified notatin, in addition to their highly specific catalytic activity in the oxidation of β -glucose, also have the property of accelerating the mutarotation of glucose. This property was found to be due to the presence in some samples of a mutarotation catalyst which, being thermolabile and not dialysable, is described here under the name of *mutarotase*.

Mutarotase and glucose oxidase. The evidence that mutarotase is not identical with glucose oxidase itself can briefly be summarized as follows:

(1) The two catalysts are not necessarily present in the same samples of notatin; thus sample Y contains only glucose oxidase, whereas sample Z contains both.

(2) By treatment of notatin Z with ammonium sulphate and calcium phosphate several fractions were obtained, one of which, like notatin Y , was completely devoid of mutarotase, whereas others showed mutarotase/oxidase ratios more than double that of notatin Z .

(3) A concentrated culture medium filtrate of *P. notatum* showed a mutarotase/oxidase ratio four times higher than that of notatin Z . A modification in the method of concentration reduced the glucose oxidase activity by about 90% without affecting the mutarotase. Such a preparation oxidized α - and β -glucose at essentially the same rate.

(4) The two catalysts show very marked differences in their resistance to alkali. Thus, treatment for 20 min. at pH 9.1 irreversibly destroys the glucose oxidase activity of notatin by nearly 90% but mutarotase activity is scarcely affected.

(5) They show very different susceptibilities to the action of other sugars; whereas glucose oxidase activity, on addition of ten equivalents of other sugars, is either not affected at all or is inhibited by not more than 15%, mutarotase activity, in presence of four equivalents of these sugars, is inhibited from 30 to 90%.

(6) They show an entirely different behaviour in relation to substrate concentration; whereas the

activity of glucose oxidase is proportional to glucose concentration until the enzyme approaches saturation with its substrate, the activity of mutarotase is approximately proportional to the reciprocal of the glucose concentration.

That the mutarotation catalyst is not an artifact, or a derivative of glucose oxidase produced by the treatment involved in the purification of the enzyme, is clearly shown by the fact that mutarotase can be detected in the culture medium and can be extracted, together with glucose oxidase, from the acetone-dried mycelium of *P. notatum*.

Reaction kinetics of mutarotase. In the experiments summarized in Tables 1 and 2 the calculated first-order reaction constants were in fact constant during the run except for the very slight upward and downward drifts with time in the case of β - and α -glucose respectively. This means that the mutarotation catalysed by mutarotase, apart from the buffer contribution, is in fact a first-order reaction in respect to glucose. The enzyme is thus not saturated with its substrate within the experimental range of glucose concentration and, if the enzyme concentration is fixed, K should be independent of glucose concentration within this range. The experiments recorded in Table 2 and Fig. 2, however, clearly show an inverse relationship between the mutarotation constant and glucose concentration, the rate of mutarotation falling off with increase of glucose concentration and gradually approaching that occurring in the phosphate buffer alone. Although the inhibition of enzyme activity by excessive concentrations of substrate has often been recorded, no explanation of this phenomenon has yet been given, which would apply equally well to all instances. In the case of mutarotation the inhibition by extra molecules of glucose is very similar to that obtained with structurally similar sugars or glucosides. Moreover, since β -methyl glucoside is more effective than α -methyl glucoside it is reasonable to assume that, as an inhibitor, β -glucose is more effective than the α -form. This would explain the fact that α -glucose invariably yielded higher values of K than did β -glucose (Tables 1 and 2).

Activity of mutarotase. The fact that much higher concentrations of notatin Z are necessary for demonstration of mutarotase activity than for oxidation of glucose does not imply that mutarotase is not highly active. In the first place the molar concentration of notatin in the mutarotation experiments was very much lower than that of the inorganic catalysts of mutarotation (water and ions), and in the second place not more than a small fraction of notatin Z consists of mutarotase. This latter point is a reasonable deduction from the diagrams of the sedimenting boundaries of notatin Z given by Cecil & Ogston (1948). From these diagrams it is clear

that the major impurity could not account for much more than 10% of the total sedimenting material and that as the sedimentation velocity of this impurity is about half that of glucose oxidase, its molecular weight will be approximately half that of notatin, i.e. about 75,000.

In presence of 4% glucose the value of K in the 0.2M-phosphate buffer is 6.6×10^{-2} , while for a solution of notatin Z alone (1 mg./ml.) it is 11.1×10^{-2} ; hence the 'catalytic coefficient' of mutarotase, i.e. the velocity constant for a molar solution, would be $11.1m/x$, where x is the percentage mutarotase in notatin Z (in terms of the dry weight). On the other hand, with 1.33% glucose the catalytic coefficient would be $26m/x$ (Table 2), while still higher values would presumably be attainable at lower glucose concentrations.

If it is assumed that the major impurity in notatin Z is in fact mutarotase then the above figures, 75,000 and 10, can be substituted for m and x to give an approximate estimate of the catalytic coefficient of mutarotase. The value so obtained is of the order of $2 \times 10^5 \text{ min.}^{-1}$ which is significantly higher than the figure for the OH^- ion (8.8×10^3), the most active catalyst for mutarotation so far recorded (Smith, 1936; Smith & Smith, 1937). On the other hand, should mutarotase be one of the minor impurities, then the activity would be markedly higher than 2×10^6 .

SUMMARY

1. Both culture filtrates of *Penicillium notatum* and extracts of mycelia, as well as the highly purified notatin (glucose oxidase) isolated from the

former, in addition to their property of catalysing the oxidation of glucose, also have the property of accelerating its mutarotation.

2. The acceleration of mutarotation of glucose by these materials is due to the presence of a thermolabile and non-dialysable catalyst for which the name *mutarotase* is proposed.

3. That mutarotase is a catalyst distinct from glucose oxidase is supported by the following evidence. (a) They do not always coexist in the same highly purified notatin sample: while all samples (Y and Z) catalyse the oxidation of glucose only some of them (Z) promote mutarotation. (b) Sample Z , which contains both catalysts, can be fractionated to give preparations with a mutarotase/glucose oxidase ratio varying from zero to about three times that of Z . (c) Mutarotase shows a much greater stability at higher pH than glucose oxidase. (d) Mutarotase, unlike glucose oxidase, is strongly inhibited by excess of glucose and by carbohydrates structurally similar to glucose. (e) The mutarotase/oxidase ratio in some *Penicillium* culture filtrates is higher than that shown by notatin Z .

4. Under the experimental conditions adopted the catalytic coefficient of mutarotase (the calculated first-order mutarotation constant for glucose in a molar solution of the catalyst) is estimated to be at least $2 \times 10^5 \text{ min.}^{-1}$ in presence of 4% glucose and would be greater at lower concentrations of glucose. This value is considerably higher than that for the OH^- ion ($8.8 \times 10^3 \text{ min.}^{-1}$) which is the most active catalyst of mutarotation hitherto known.

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