A Rotographic Study of Mutarotase

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Glucose oxidase is a specific enzyme which catalyses the oxidation (dehydrogenation) of glucose. It was studied in detail during the past fifty years and was proved to be identical with penicillin A, penicillin B and Notatin (see Theorell, 1951). It has been prepared in a highly purified form by Coulthard *et al.* (1945), and its characteristics have been firmly established by Keilin & Hartree (1948*a*). Its specificity is truly remarkable, as shown by these authors (Keilin & Hartree, 1952*a*), in that essentially only β -D-glucose is oxidized by the enzyme. In the course of these studies, they noted certain irregularities and they concluded that some glucose oxidase preparations contained another agent, which they called mutarotase. This was in accordance with an earlier observation by Bentley & Neuberger (1949). Keilin & Hartree (1952*b*) indicated, in their succeeding paper, that this agent is a different entity from glucose oxidase and it is probably an enzyme which catalyses the mutarotation of glucose. Based on the molecular weight determination of Cecil & Ogston (1948), they estimate that mutarotase has the remarkably high catalytic coefficient of $2 \times 10^8 \text{ min.}^{-1}$, as compared to the best inorganic catalyst, namely the hydroxyl ion, with a coefficient of $8.8 \times 10^3 \text{ min.}^{-1}$. For this reason it seemed desirable to study this enzyme in more detail.

An additional impetus to this work was our concern with automatic-recording polarimetry, which is a method of choice in such investigations. Automatic-recording polarimetry—'rotography', in short—is based on our instrument which we have described previously (Levy, Schwed & Fergus, 1950). By its use difficult kinetic studies can be performed with ease and this has been demonstrated with the penicillin—penicillinase system (Levy, 1951). However, owing to the nature of that problem, reactions of essentially only zero order were considered (Levy, 1950), and therefore the problem of mutarotase seemed a suitable test for the application of rotography to first-order kinetics.

EXPERIMENTAL

General technique

The automatic-recording polarimeter, or rotograph, was used in the present work as described previously (Levy *et al.* 1950). It was necessary, however, to construct an anaerobic

handling system because the mutarotase used is present as an impurity of glucose oxidase and the activity of the latter had to be eliminated by the exclusion of oxygen. This was accomplished by the use of a polarimeter flow cell of 4 dm. length equipped with plane-parallel windows. This cell was constructed and generously supplied by O. C. Rudolph & Sons, Caldwell, New Jersey, U.S.A. To the inlet of this tube was connected a sintered glass filter and separating funnel,

Fig. 1. The mutarotation of α -D-xylose. The left-hand curve shows the changes in optical rotation of 0.1M xylose solution containing 0.06% glucose oxidase in 0.005M phthalate buffer, pH 4.7 at 25°. The right-hand curve shows the changes occurring in the absence of enzyme. During the initial period of 3–5 min. the curves are irregular owing to the time required for mixing the solutions and the attainment of balance of the instrument. Each subsequent cycle represents 0.5 angular degree. The results plotted in Fig. 2 represent the changes in optical rotation, expressed in scale divisions, taking place between times subsequent to the attainment of balance and t_{∞} .

and water-saturated N_2 was fed under pressure to the top of the funnel and the outlet of the tube through stopcocks. The substrate was first placed into the funnel and, by letting N_2 into the outlet of the tube, the system and the solution were cleared of air. After about 15 min. the enzyme preparation was added. The gas flow was maintained and so rapid mixing occurred. By reversing the gas flow the sample was filtered through the sintered glass filter and pumped under N_2 into the polarimeter tube. A rotogram was then prepared. The appearance of such a record is shown in Fig. 1, which represents the mutarotation of xylose with and without mutarotase. By plotting the corresponding values of the abscissa versus time on a semi-logarithmic paper the first-order rate constant can be evaluated graphically or according to the equation:

$$k = \frac{2.303}{t_2 - t_1} \log_{10} \frac{\text{divisions } (t_\infty) - \text{divisions } (t_2)}{\text{divisions } (t_\infty) - \text{divisions } (t_1)}$$

Such a plot, using the data of Fig. 1, is shown in Fig. 2 with the points used for its construction indicated. These points are chosen arbitrarily and are only shown to indicate the precision usually obtained. If a large scatter is encountered, any number of additional points can be used and thus fuller advantage taken of the continuous record. Using this general procedure a large number of mutarotation experiments were performed. To assess the catalytic activity, the rate constant of mutarotation of a control (k_c) and that of the accelerated reaction (k_a) was used to express the acceleration (A), where $A = \frac{k_a - k_c}{k_c}$.

The reproducibility of the rate constant depends on various factors. Usually the instrumental precision is not limiting. However, in very slow reactions the long-range

Fig. 2. The mutarotation of xylose. Upper curve without, lower curve with mutarotase (glucose oxidase). Data plotted from Fig. 1.

stability is of importance. Conversely, in very rapid reactions the evaluation of the record is difficult. Turbid or coloured solutions may limit the signal and precision, whereas temperature fluctuation alters the rate without affecting the precision. Because of this complex situation an arbitrary decision to consider accelerations of 0.05 as the limit of reproducibility was made. When comparison is only between two runs, differences are not considered significant unless the acceleration exceeds 0.1. This limit is rather conservative, but it was found not to be confining, as will be evident.

Materials

Enzymes. Several lots of commercial glucose oxidase were tested for mutarotase activity but no activity was found by the rotographic technique. These were samples of 'Deoxygenase' produced by Takamine Laboratory, Inc., Clifton, New Jersey, U.S.A., and supplied through the courtesy of Dr E. G. Snyder. This finding was further verified qualitatively by measuring the oxidative power of these preparations using equilibrium glucose and α -glucose. Since much slower rates resulted with the latter, the presence of a sizeable quantity of mutarotase was excluded. It was concluded that mutarotase was either lacking due to the manufacturing process or because *Aspergillus niger*, from which the oxidase had been prepared, does not produce mutarotase. The work of Keilin & Hartree (1952a, b) was done with a highly purified product obtained from *Penicillium notatum* fermentation by Boots Pure Drug Co. Ltd. Such a preparation was generously supplied to us by Dr W. F. Short and the work reported here was carried out with this specimen. It was marked 274-56, 5/9/42, and with glucose as a substrate it exhibited $Q_{O_2} = 50000 \mu\text{l./mg./hr.}$ The preparation 233-237, used by Keilin & Hartree, under essentially identical conditions was reported to show $Q_{O_2} = 40000 \mu\text{l./mg./hr.}$ The mutarotase activities were also very similar, and it was concluded that these two lots are essentially identical.

The α -amylase used in one experiment was a highly purified preparation of mould amylase which is marketed for analytical purposes by General Biochemicals, Inc. The pepsin, used in another experiment, was of the same manufacture and was a twice-crystallized preparation.

Sugars. These were analytical grade chemicals. α -D-Glucose was prepared from such grade D-glucose (Mallinckrodt Chemical Works, New York, U.S.A.) in accordance with standard procedure (Hudson & Dale, 1917). It was dried *in vacuo* to constant weight.

D-Fructose (Eastman no. 679) was dried *in vacuo*. It consisted almost entirely of the β -form.

α -D-Mannose was obtained from Nutritional Biochemicals Corp. Because of the significance of the results obtained with this sugar, as shown below, these experiments were repeated with another preparation from Difco Laboratories.

β -Lactose was prepared from an Eastman preparation by recrystallization and drying *in vacuo*. The resulting preparation was tested for glucose content by a method essentially as developed by Keilin & Hartree (1948b). It showed a glucose content of less than 0.1%.

β -Maltose (Eastman no. 154) was also tested by this procedure. Its glucose content was found to be less than 0.5% and it was used without purification.

α -D-Galactose was tested in a like manner and was found to contain 3.7% of glucose. The high glucose content of a

preparation labelled 'Free from glucose' (Pfaunstiehl Chemical Co.) is not surprising, since Keilin & Hartree (1948*a*) reported 4% glucose in 'pure' commercial galactose. This preparation could be used, nevertheless, because glucose and galactose were found by us to exhibit similar mutarotation, and the small amount of glucose did not cause any detectable error.

α -D-Xylose (Matheson Co. no. 2717) contained no detectable amount of glucose.

All other chemicals were analytical grades and merit no discussion.

RESULTS

Effect of mutarotase on glucose

Keilin & Hartree (1952*b*) have reported in their basic work on mutarotase that at pH 5.6 in 0.2M phosphate buffer at 20° the rate constant of the mutarotation of α -glucose is 0.0658 min.⁻¹. They also determined the acceleration of this mutarotation at various concentrations of their mutarotase preparation. At 0.06% the value was about 0.139 min.⁻¹ (by interpolation of their results) representing an acceleration of about 1.1. Since the rotograph was operated at a higher temperature (about 24° in these preliminary experiments) the effect of temperature needs consideration. In general, mutarotation of carbohydrates shows a temperature coefficient for 10° of about 2.5, and Isbell & Pigman (1937) give the figure of 2.6 for α -D-glucose around room temperature. Using this figure, the data of Keilin & Hartree (1952*b*) correspond to about 0.11 min.⁻¹ for α -glucose and 0.23 min.⁻¹ for the accelerated run at this higher temperature. Using the same buffer, the rate constant for glucose was found by us to be 0.11 min.⁻¹ and for the mutarotase-accelerated reaction, 0.22 min.⁻¹ (acceleration, 1.0). Thus, not only were their findings verified but it was evident that the two preparations are virtually identical with respect to mutarotase activity (cf. Experimental section).

Effect of metal ions

Before proceeding further with the studies of mutarotase, certain aspects of the mutarotation of glucose were considered. Isbell & Pigman (1937) state that 'the effect of traces of heavy metals on the rates of mutarotation is pointed out and attention is directed to errors caused by the use of nickel-plated brass tubes in mutarotation measurements'. It seemed important to verify this statement. In these experiments 0.005M phthalate buffer of pH 4.6 was used as was done by Isbell & Pigman. The mutarotation constant for α -glucose at 20° is given by them as 0.0146 min.⁻¹, which can be estimated to correspond to about 0.026 min.⁻¹ at 25°. This is within experimental error of our values, shown in Table 1. The salts of heavy metals used were cupric acetate, zinc acetate, nickel chloride and mercuric chloride. Also listed is an experiment with ethylenediaminetetraacetic acid (Versene). Gluconic acid, as its sodium salt, was also included in this set of experiments because of its possible presence if, owing to faulty operation, some oxidation of glucose occurs.

Inspection of the first section of the table shows excellent reproducibility, which may, however, be fortuitous. The second section shows that the four heavy metals, even in relatively high trace concentrations (0.001M) do not alter the mutarotation rate.

It must be appreciated that the addition of a sizeable amount of any ion or salt will cause significant acceleration, e.g. from the data cited in Tables 1 and 3, it can be calculated that the rate of mutarotation of glucose in 0.2M phosphate buffer is more than 4 times that in 0.005M phthalate buffers. The nature of the salt or ion (as it affects the dissociation constant) is of importance, as was pointed out by many workers, but within the limit of significance as defined, and in the practical ranges as used, such anions as phosphate, phthalate and gluconate show

Table 1. *The effect of salts on the mutarotation of 0.1M glucose at 25°*

Molarity of phthalate buffer ($\times 10^{-3}$)	Addition*	pH	<i>k</i> (min. ⁻¹)	Acceleration
5	—	4.6	{ 0.0247 0.0245	— —
7	—	4.7	0.0246	—
5	Zn(C ₂ H ₃ O ₂) ₂	4.7	0.024	-0.04
5	Cu(C ₂ H ₃ O ₂) ₂	4.7	0.023	-0.09
5	NiCl ₂	4.5	0.025	0
5	HgCl ₂	4.4	0.027	+0.08
5	Ethylenediaminetetraacetic acid	4.8	0.026	+0.04
5	Sodium gluconate†	3.7	0.0235	-0.06
2.5	Sodium gluconate‡	3.6	0.025	0

* All 0.001M except gluconate (see below).

† Anomalous result owing to mutarotation of the gluconate (0.002M).

‡ Equilibrium gluconate (0.0025M).

negligible variations. However, if the gluconate itself is undergoing mutarotation anomalous rotograms may be obtained (see Table 1).

pH optimum of mutarotase

With this background information, the problem of mutarotase was attacked. A point of importance was the pH optimum of this agent. Keilin & Hartree (1952*b*) used pH 5.6 in their work because the optimum activity of glucose oxidase is at that point. Isbell & Pigman (1938) recommended pH 4.2 or 4.6 for mutarotation studies in general, because of the minimal rates in that range. For the same reason they employed extremely dilute phthalate buffers. It was realized that a generally valid pH optimum for mutarotase activity would not be established while working with a preparation of glucose oxidase which contains but a small portion of mutarotase. However, for the purpose of this study it was deemed necessary to obtain a practical approximation, at least in the neutral-to-acid range, where the lowest natural mutarotation rates occur.

In these experiments a stock solution of glucose oxidase was used in 0.01M phthalate buffer of varying pH. The solutions were 0.1M with respect to glucose. The results are shown in Table 2. The agreement with the published pH dependence of the mutarotation of glucose (Isbell & Pigman, 1938) is good. It is also indicated that the pH optimum of mutarotase may be somewhat higher than that of glucose oxidase and possibly somewhat flatter, although in view of the impurity of the preparation (with respect to mutarotase) these conclusions must be considered tentative. It was concluded, however, that further experiments should be carried out at the plateau of maximum activity where the pH dependence is at a minimum. The advantage of using dilute buffer to magnify the accelerating effect of mutarotase is also clear.

Table 2. *The effect of pH on the activity of mutarotase*

0.1M Glucose in 0.01M phthalate buffer at 25°, glucose oxidase, 0.0294%.

pH	<i>k</i> (min. ⁻¹)		Acceleration
	Glucose	Glucose + enzyme	
6.0	0.0262	—	—
5.8	—	0.0937	2.60*
4.7	0.0245	0.0833	2.40
4.0	0.0245	0.0741	2.02
3.2	0.0258	0.0495	0.92

* Obtained by graphical interpolation of the results for the uncatalysed mutarotation.

Nature of mutarotase

The chemical nature of mutarotase may also merit some discussion. Keilin & Hartree (1952*b*) do not make any statement, but their choice of name implies a suggestion of its enzymic nature. Moreover, the tremendous activity (on a molar basis) almost precludes any known inorganic catalyst. They also showed that mutarotase is heat labile (although not to the same extent as glucose oxidase) and is not diffusible. This proof was extended in the present work. To 2 ml. of the glucose oxidase stock solution (0.0294%)* and 50 ml. of buffer, pH 3.2, crystalline pepsin (15 mg.) was added and the mixture digested at 25° for 45 min. Subsequently, a rotogram with 0.1M glucose was prepared. The rate constant of mutarotation was found to be 0.0301 min.⁻¹. Since the catalysed pH 3.2 run (Table 2) represents a control, it was calculated that owing to this treatment over 81 % of the mutarotase activity was lost. This is further proof of the protein nature of mutarotase.

In this connexion a recent paper by Blom & Schwarz (1952) is of interest. In discussing the actions of α - and β -amylases on starch, they propose 1:5-glucofuranosidic linkages in starch, together with the accepted 1:4- and 1:6-linkages. In discussing this hypothesis and the possible degradation mechanisms, Hopkins (1953) postulates 'mutarotase' activity for α -amylase but doubt about this activity has been expressed in a later (personal) communication from Prof. Hopkins. Considering the fungal source of mutarotase, it would not seem impossible that actually α -amylase is involved, although it is unlikely that this would have escaped the attention of previous investigators. To test this possibility the following experiment was carried out. To 50 ml. of 0.1M α -D-glucose in 0.01M phthalate buffer, pH 6, was added 1 ml. of a solution containing 30.1 mg. of amylase. The rate constant was found to be 0.0267 min.⁻¹, which is well within analytical error of the control (see Table 2) of 0.0262 min.⁻¹. In view of the fact that half the weight of glucose oxidase, which contains but a small portion of mutarotase, showed an acceleration of 2.6 under similar conditions (which is more than 100 times that caused by amylase if the figure 0.0267 is taken at face value), one cannot consider α -amylase as 'mutarotase'. This does not mean that α -amylase preparations may not accelerate mutarotations, as do practically all ionized compounds, nor is any criticism of this new theory of starch structure implied. It seems, however, that mutarotase is a unique entity and most probably an enzyme which accelerates the mutarotation of glucose to an extent not encountered heretofore.

* Final concentration.

Specificity of mutarotase

As a proof that glucose oxidase and mutarotase are different entities, Keilin & Hartree (1952b) showed that a number of equilibrium sugars inhibit mutarotase action while scarcely affecting that of glucose oxidase. This pointed to the possibility that the sugars may be substrates of mutarotase. To explore this, lactose, maltose and xylose were tested under conditions similar to those employed by Keilin & Hartree, as shown in Table 3.

These results indicated that there is indeed an accelerating action on the mutarotation of sugars other than glucose. However, the effect was close to the limit of discrimination by the technique. Therefore more favourable conditions were chosen for subsequent experiments, as discussed above. The concentration and pH of the buffer were decreased and 0.005M phthalate buffer of pH 4.7 was used throughout. The concentration of sugars (0.1M) was maintained and also the concentration of glucose oxidase (0.06%). The selection of the sugars was planned in such a fashion as to give most information on specificity. The individual runs were evaluated as shown above (cf. Figs. 1 and 2) and the results are summarized in Table 4.

Table 3. *The action of mutarotase on various aldoses*

0.1M Sugar solutions in 0.2M phosphate buffer, pH 5.6, with 0.06% glucose oxidase.

Substrate	k (min. ⁻¹)		Acceleration
	Enzyme absent	Enzyme present	
α -D-Glucose	0.11	0.22	1.0
β -Lactose	0.084	0.11	0.31
β -Maltose	0.0098	0.011	0.12
α -D-Xylose	0.37	0.42	0.14

Table 4. *The action of mutarotase on sugars*

0.1M Sugar solutions in 0.005M phthalate buffer, pH 4.7, with 0.06% glucose oxidase.

Sugar	k (min. ⁻¹)		Acceleration
	Enzyme absent	Enzyme present	
α -D-Glucose	0.025	0.13	4.20
β -D-Fructose	0.17	0.18	0.05
α -D-Xylose	0.079	0.11	0.39
α -D-Mannose	{ 0.064	{ 0.064	0
	{ 0.066	{ 0.066	
Glucosamine	0.028	0.028	0
α -D-Galactose	0.028	0.166	3.37
β -Maltose	0.021	0.027	0.29
β -Lactose	0.018	0.042	1.33

DISCUSSION

Mutarotation is a rather general term not confined to the chemistry of sugars and which indicates merely that there is a spontaneous change in optical activity. Even in the case of the carbohydrates there are several different reactions which can cause mutarotation. In this branch the most common cause of mutarotation is the change between α - and β -configuration of sugars. However, mutarotation does also occur with changes in ring structure, i.e. pyranose and furanose structures. It seemed unlikely that mutarotase should catalyse such diverse phenomena and the results with fructose indicate that the pyranose-furanose transformation is not accelerated. Conversely, the acceleration of the $\alpha \rightleftharpoons \beta$ type mutarotation appears to be quite general. Obviously α -D-glucose is the preferred substrate. However, α -D-galactose is almost equally acceptable (Table 4), presumably due to the close structural similarity. Apparently the reverse symmetry on the fourth carbon atom is of small importance. It is noted, however, that the linking of a second sugar at that point reduces the effectiveness of mutarotase and, surprisingly, in this case the symmetry appears to have a pronounced effect (cf. lactose and maltose). A large depression of activity is also caused by lack of the sixth carbon, as shown with xylose which is otherwise identical in configuration with glucose. However, all of these aldoses are acted upon by mutarotase to a large extent. The deciding locus of action appears to be the second carbon atom. Reverse symmetry to glucose at that atom abolishes mutarotase action completely (mannose) and the substitution of the hydroxyl group, at that point, by an amino group equally nullifies action (glucosamine).

The outlines of specificity are clear and other sugars are expected to fall into this pattern. The relative lack of specificity of mutarotase is distinct from that of glucose oxidase and this leaves no doubt that they are two distinct enzymes. This lack of specificity seems to justify also the term 'mutarotase', which would seem too broad if only glucose were acted upon.

It was noted that in order to maintain comparable conditions in these studies of specificity, the concentrations of the enzyme and the sugars were kept constant, which resulted in a tenfold range of reaction rates (0.018 min.⁻¹ to 0.18 min.⁻¹). The fastest changes were thus of the order of 0.25 angular degree/min.⁻¹ which could not have been followed visually with any degree of precision. The slowest changes necessitated many hours of continuous recording, which would have represented a formidable task by conventional visual polarimetry. Therefore it appears that automatic operation, or rotography, is of value in this type of

work and it is recommended for all applications where continuous changes of optical activity are involved.

SUMMARY

1. No evidence was found that heavy metal ions (Cu^{2+} , Zn^{2+} , Ni^{2+} and Hg^{2+}) catalyse the mutarotation of glucose.

2. The strong catalytic action of mutarotase was verified. That pepsin largely destroys this activity may be taken as additional proof of the protein nature of mutarotase.

3. α -Amylase was found to have no mutarotase activity.

4. The specificity of mutarotase was investigated in greater detail. It appears that the enzyme catalyses only some $\alpha \rightleftharpoons \beta$ transformations. Of the aldoses tested, glucose and galactose seem to be the best substrates, indicating that the configuration at the fourth carbon atom is not decisive. However, the mutarotation of the disaccharides lactose and maltose is accelerated to a lesser degree. The lack of a hydroxymethyl group on the fifth carbon atom (xylose) also reduces the activity. The configuration at the second carbon atom of the sugar molecule is decisive, since neither mannose nor glucosamine serves as a substrate. These studies indicate the range of specificity of mutarotase.

5. An incidental outcome of this work was that it demonstrated the general utility of automatic-recording polarimetry or rotography.

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The Effect of Certain Barbiturates on the Porphyrin Metabolism of Rabbits

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Since the introduction of the barbiturates to clinical medicine in 1903, there have been conflicting reports on their possible relation to human acute porphyria. Dobrschansky (1906) described a typical case of acute porphyria, without paralysis, occurring in a patient after prolonged administration of diethylbarbituric acid. Haxthausen (1927) reported the development of skin photosensitivity and the presence of excessive amounts of porphyrins in the urine of an epileptic who had been taking ethylphenylbarbiturate for some time. Eliaser & Kondo (1942), Denny-Brown & Sciarra (1945), Prunty (1946) and Jørgensen & With (1947) have described deterioration in the clinical state of acute porphyria patients with the development of severe or even fatal paralysis, after the administration of certain barbiturates, among which were

ethylphenylbarbituric acid and ethyl(1-methylbutyl)barbituric acid. Waldenström (1939, 1940) was convinced that barbiturates may precipitate attacks in cases of latent porphyria and that they seriously affect the prognosis of the disease. On the other hand, Günther (1922), Turner (1938) and Discombe & D'Silva (1945) failed to note any relation between barbiturates and acute porphyria.

Information on the relation of barbiturates to normal human porphyrin metabolism has been obtained from observations on the urinary porphyrin excretion in cases of barbiturate poisoning. Rosendorff (1910), Sowden (1910) and MacLean (1912) found no abnormal porphyrinuria in patients who had ingested single large doses of diethylbarbituric acid, while Rommel (1912) observed dark-red urine in a patient who had taken 25 g. of