recent methods, that described by Kodicek & Pepper (1948*b*) had been in use in this laboratory, but the present medium with *L. casei* has completely replaced the former procedure and was found to give more satisfactory results.

In the assay of pteroylglutamic acid the conjugates, with the exception of Teropterin, showed an activity in general agreement with the findings of other workers.

SUMMARY

1. An improved medium was developed for the assay of pteroylglutamic acid with *Lactobacillus casei* and was tested for some other B vitamins and tryptophan.

2. The titrimetric dose-response curves for riboflavin, nicotinic acid, calcium pantothenate, biotin and tryptophan were satisfactory.

3. The medium has been used in particular for the microbiological assay of total nicotinic acid, and the results were in good agreement with the chemical values. The bound form of nicotinic acid present in cereals was found to be less active than the free vitamin.

4. A number of conjugates of pteroylglutamic acid and other pteridines were tested.

We wish to thank Dr A. Albert for the synthetic pteridines, Dr T. H. Jukes for pteroylgutamic acid and conjugates, Dr J. J. Pfiffner for the vitamin B_c conjugate, Dr L. D. Wright for the biocytin concentrate, Dr K. Folkers for biotin and rhizopterin, and the Flour Millers Association for the supply of the different brans.

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Specificity of Glucose Oxidase (Notatin)

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(*Received* 23 *May* 1951)

The antibiotic 'notatin' isolated in a very highly purified state from culture medium filtrates of *Penicillium notatum* by Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick (1942, 1945) was shown by these authors to be identical with the glucose oxidase discovered by Müller (1928, 1941) in cultures of different moulds (for a summary of the literature, see Keilin & Hartree, 1948*a*). The enzyme is a flavoprotein (Coulthard *et al.* 1945) and the prosthetic group has been identified as alloxazine-adenine-dinucleotide (Keilin & Hartree, 1946).

A study of the catalytic activities of this enzyme revealed its very high specificity for glucose (Keilin & Hartree, 1948*a*) which is quantitatively oxidized to gluconic acid. Previous workers had shown that the reaction proceeds according to the equation

$$C_6H_{12}O_6 + O_2 + H_2O = C_6H_{12}O_7 + H_2O_2.$$

In presence of catalase, which decomposes the peroxide to oxygen and water, the overall reaction is

$$C_6H_{12}O_6 + \tfrac{1}{2}O_2 = C_6H_{12}O_7.$$

Further experiments showed that notatin could be successfully used for manometric estimation of glucose in biological material in presence of other carbohydrates and proteins, and that the method could be extended to studies of the kinetics of different enzyme reactions which yield glucose (Keilin & Hartree, 1948*b*).

Of about fifty other sugars and their derivatives tested only mannose, xylose, 6-methyl glucose and 4:6-dimethyl glucose were oxidized, although the rates of oxidation were only 1–2% that of glucose, whereas the remainder were practically unoxidized. This investigation has now been extended to the complete range of aldohexoses with the result that notatin can be considered as strictly specific for glucose.

Early in 1949 it was suggested to us by Dr A. Gottschalk that it would be interesting to determine whether the specificity of notatin extends to the α- and β-forms of glucose. It would seem reasonable to assume that one form would be more actively oxidized since stereospecificity is a general property of enzymes. Such a specificity for the α-configuration has been shown by Cori & Cori (1940) and by Campbell & Creasy (1949) who found that the α-form of glucose is the specific inhibitor of the synthesis by phosphorylases of polysaccharides from glucose-1-phosphate which itself has the α-configuration. Even chemical oxidizing agents react at different rates with the two isomers. Thus bromine (Isbell & Pigman, 1933) and hypoiodous acid (Reeve, 1951) will, under certain conditions, oxidize β-glucose 35 and 25 times respectively more rapidly than the α-isomer.

Experiments with notatin have shown that β-glucose is the more rapidly oxidized isomer and that the rate of oxidation of α-glucose is very slow indeed when the necessary corrections for mutarotation have been applied. While our experiments were in progress we learned from Dr A. Neuberger that an independent study, the results of which have since been published (Bentley & Neuberger, 1949), also indicated that β-glucose was the more readily oxidized form.

EXPERIMENTAL

Materials

Notatin. Samples of notatin used in this investigation were supplied by the Boots Pure Drug Company through the courtesy of Dr W. F. Short. They were samples of two different batches labelled '410–417' and '233–237' which we shall denote as *Y* and *Z* respectively in this paper. The amount of water-insoluble material in these samples was *Y*, 37% and *Z*, 8%. However, the glucose oxidase activities of the two samples were equal in terms of the soluble fraction (Q_{O_2}, 40,000 μl./mg. dry wt./hr. in air at 20° and pH 5·6). The weights of notatin given in the text refer to the dry weight of the soluble material.

Catalase was obtained from horse-liver by a method previously described (Keilin & Hartree, 1945). All traces of ethanol had been removed by prolonged dialysis. The solution was 0·44 mM with respect to haematin.

Sugars. α- and β-Glucoses were prepared according to Hudson & Dale (1917). These sugars were normally dried in vacuum over P_2O_5 immediately after their preparation and so obtained anhydrous. One air-dried sample of α-glucose was found on analysis to be the monohydrate. (Found: C, 36·5; H, 7·34. Calc. for $C_6H_{12}O_6.H_2O$: C, 36·3; H, 7·16%. 302 mg. lost 26·3 mg. on heating at 105° for 24 hr. Calc. for $1H_2O$: 27·5 mg.) Whenever the monohydrate was used the weight recorded is the equivalent of anhydrous glucose. The specific mutarotations given in Table 1 agree closely with those of Hudson & Yanovsky (1917). For the study of equilibrium glucose, a 1% aqueous solution was brought to equilibrium by heating 10 min. at 100°.

Table 1. *Specific rotations of samples of α- and β-glucose used in the present investigation*

(4% (w/v) solutions of glucose in water at 20°; 22 cm. tube.)

Glucose isomer	Initial specific rotation (°)	Equilibrium specific rotation (°)
β-Glucose	17·8	52·5
α-Glucose	111·4	52·5
α-Glucose monohydrate	100·2	47·6
α-Glucose monohydrate after heating at 105° for 24 hr.	110·0	52·3

As to other sugars used in the present study, a sample of D-altrose was supplied by Prof. C. S. Hudson, D-allose, D-talose and D-idose (syrup) were supplied by Prof. T. Reichstein and samples of D-talose and D-gulose ($CaCl_2$ derivative) were supplied by Dr H. S. Isbell. 4:6-Benzylidene glucose was prepared according to Zerfas (1931).

Phosphate buffer in all experiments was a mixture of 920 ml. 0·2 M-KH_2PO_4 and 80 ml. 0·2 M-Na_2HPO_4. Its pH, measured with glass electrode at 20°, was 5·61 which is the optimum for notatin.

Manometric methods

The oxidation of glucose by notatin was followed in Barcroft differential manometers at 20°. Except where otherwise stated, the right-hand flasks received notatin and 0·04 ml. catalase dissolved in 3·3 ml. buffer, while the glucose was added from a dangling cup after temperature equilibration. The left-hand flasks received 3·3 ml. buffer.

When using more than 100 μg. notatin in a manometer flask the rate of O_2 uptake, especially with β-glucose, is very rapid and in order to reduce experimental error all measurements, with the exception of those recorded in Fig. 4, were carried out in quadruplicate with rapid shaking of manometers (150/min.). Little advantage can be gained by working at 0° since not only mutarotation but also the oxidation catalysed by notatin drops considerably.

The α- and β-glucoses were weighed into dangling cups in the dry form, and since the most convenient quantity for a Barcroft manometer is about 3 mg. it was scarcely practical as a routine measure to weigh this out to an accuracy (1–2%) which would be within the experimental

error. The sugars were therefore weighed out to within 10% of 3 mg., and the periodical readings of a set of four identically filled manometers were converted to percentage of final readings (corresponding to complete oxidation) before being averaged. In presence of catalase the oxidation of 3 mg. glucose requires 187 μl. oxygen. The results are expressed as plots of $\log_{10}$ % glucose unoxidized against time (see Kinetics).

Polarimetric methods

Mutarotation was studied in a 22 cm. jacketed tube in which the temperature was maintained at $20\pm0{\cdot}05°$. For measurements of rotation in absence of notatin the glucose was weighed into a 15 ml. volumetric flask and made up with water or buffer to the mark at zero time. Immediately after transfer of the solution to the polarimeter tube the readings of rotation were begun and a series of about a dozen was made within 30–40 min. (excluding the final reading) when buffer was used as solvent; with water the experiment was extended for a longer period. A sodium lamp was used as light source.

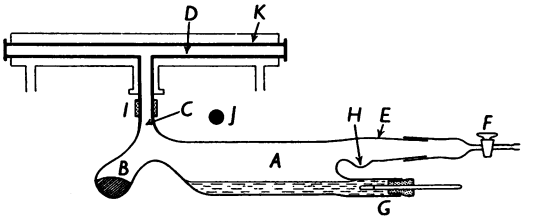

Fig. 1. Arrangement for filling a 22 cm. jacketed polarimeter tube with a solution of glucose and notatin in buffer under anaerobic conditions. For explanation of letters see text.

For the study of mutarotation in presence of notatin the experiments had to be carried out under strictly anaerobic conditions in order to avoid oxidation of the glucose. This was achieved by means of the apparatus shown in Fig. 1. The tube *A* of circular section was drawn out at one end to a bulb *B* and to a side arm *C* of the same diameter as the filling orifice of the polarimeter tube *D*. To the other end of *A* were fused two parallel narrower tubes: *E* which carried a standard ground joint and tap *F*, and *G* which held a thermometer in a rubber stopper. The small depression *H* was used in experiments to be described in a later paper (Keilin & Hartree, 1952).

By having *A* clamped vertically it was possible to introduce a known weight of glucose via *C* into *B*. The polarimeter tube, the end plates of which had been sealed on with 'picein' wax, was then attached by means of a rubber sleeve *I* and the entire apparatus clamped horizontally as shown in Fig. 1 to a retort-stand clamp which is shown in cross-section at *J*. The solution of notatin in buffer was introduced into *A* via the standard joint of the branch *E* the tap being then fixed in position with vacuum grease.

Air was removed from the system by threefold evacuation and filling with N_2 that had been purified over hot copper, the apparatus being gently rocked about *J* to assist the removal of dissolved O_2. The jacket *K* of the polarimeter tube *D* was connected to a constant-temperature circulating system by tubing flexible enough to allow the transfer of the liquid in *A* to the polarimeter tube by the following procedure: when the notatin solution in *A* had been brought to about 20° (the warmth of the hand sufficed) the apparatus was tilted to and fro about *J*, through 90° each way, until the glucose had dissolved (zero time). Then, by turning the apparatus through 180° so that *A* was horizontal and uppermost, and rocking it gently, the solution, free from air-bubbles, could be rapidly passed into the polarimeter tube. The clamp *J* was so placed that at this stage the polarimeter tube could be readily lowered into the polarimeter trough and a series of rotation readings begun within 1·5 min. of mixing the glucose with the notatin solution.

Notation. In order to avoid confusion which might arise in using α to denote one of the forms of glucose and also the optical rotation, the symbols α and $[\alpha]$ as normally used in polarimetry will be replaced in this paper by θ and $[\theta]$, together with suffixes α, β and eq. to denote the isomers and the equilibrium mixture.

Kinetics

Oxidation of glucose. The oxidations of α- and β-glucose catalysed by notatin under the standard manometric conditions can be expressed by the equation

$$K'=\frac{1}{t}\ln\frac{a}{a-x},$$

where K' is the velocity constant of a first order reaction (K' being used to avoid confusion with the mutarotation constant K). This equation can be rewritten

$$\log_{10}\frac{a-x}{a}=-\frac{K'}{2{\cdot}303}t.$$

Hence, by plotting log % sugar unoxidized against time, a straight line is obtained with slope $=-\frac{K'}{2{\cdot}303}$; the velocity constants for oxidation of isomers α and β will be denoted by K'_α and K'_β respectively.

Mutarotation of glucose. If k_α and k_β are the velocity constants in the equilibrium α-glucose $\underset{k_\beta}{\overset{k_\alpha}{\rightleftharpoons}}$ β-glucose and K is the first order mutarotation constant, it can be shown that

$$K=\frac{1}{t}\ln\frac{\theta_0-\theta_{eq.}}{\theta_t-\theta_{eq.}}=k_\alpha+k_\beta.$$

Since no measurement at zero time is possible the more general equation

$$K=\frac{2{\cdot}303}{t_2-t_1}\log_{10}\frac{\theta_1-\theta_{eq.}}{\theta_2-\theta_{eq.}}$$

has been used in which θ_1 represents the first reading and θ_2 the ten or more subsequent readings. For each experiment only the mean value of K and the standard deviation have been given. The calculation of $[\theta]_0$ for each glucose isomer, i.e. $[\theta]_\alpha$ and $[\theta]_\beta$ enables the values of k_α and k_β to be determined since $k_\alpha+k_\beta=K$ and $\frac{k_\alpha}{k_\beta}=\frac{[\theta]_\alpha-[\theta]_{eq.}}{[\theta]_{eq.}-[\theta]_\beta}$.

Effect of phosphate buffer on mutarotation of glucose

As shown in Table 2, mutarotation in presence of 0·2 M-phosphate buffer pH 5·6 is more than four times faster than in distilled water. The figures obtained for mutarotation constants in water agree

with those of Richards, Faulkner & Lowry (1927) whose average figure was 0·0146 and also with those of Isbell & Pigman (1937) when their results are converted to a natural logarithm basis. According to Hudson & Dale (1917) K is almost independent of glucose concentration provided the latter does not exceed 10%. In phosphate buffer

$$K = k_\alpha + k_\beta = 0{\cdot}0664 \text{ min.}^{-1}$$

and since we could find no significant differences between the specific rotations in buffer and in water the data of Table 1 have been substituted in the equation above to give a value of 1·70 for k_α/k_β. From these figures it can be calculated that $k_\alpha = 0{\cdot}0418$ min.$^{-1}$ and that 64% β-glucose is present at equilibrium in the phosphate buffer.

Table 2. *Mutarotation constants at 20° of α- and β-glucose in 0·2 M-phosphate buffer,* pH 5·6 *and in water*

(22 cm. tube. S.D. = standard deviation (%).)

Isomer	Concentration (%, w/v)	Solvent	$10^3 K$ (min.$^{-1}$)	S.D.
α	3·98	Buffer	65·8	0·84
α	2·03	Buffer	66·6	1·11
α	0·803	Buffer	66·6	5·4
β	4·01	Buffer	66·4	1·11
α	4·01	Water	14·51	0·94
β	4·00	Water	14·56	1·15

Mean $10^3 K$ in buffer: **66·4** min.$^{-1}$.

Oxidation of α- and β-glucose by notatin

The study of the oxidation of α- and β-glucose by notatin is complicated by mutarotation of both forms which is already more than four times faster in phosphate than in water and which may be further accelerated by a factor present in some samples of notatin.

The amount of notatin used in manometric experiments can be so adjusted that the enzyme is fully saturated by substrate and in this case the initial rate of O_2 uptake with either β- or equilibrium glucose will be proportional to the amount of enzyme present and from such an experiment the Q_{O_2} can be calculated. The conditions are satisfied when 10 μg. notatin is used in presence of 0·1 M-substrate.

For the comparative study of the behaviour of notatin towards α- and β-glucose, however, it becomes necessary to use much lower concentrations of glucose (~0·005 M) in order that the oxidation can be followed manometrically to completion and also to avoid a too rapid formation of the other isomer.

Oxidation of glucose catalysed by notatin Y ('410–417')

The comparative study of the oxidation of α-, β- and equilibrium glucose catalysed by notatin Y was carried out manometrically as described above (see Methods) in the presence of 80 μg. enzyme and 3 mg. of the corresponding forms of glucose.

The results of these experiments summarized in Fig. 2 clearly show that notatin catalyses the oxidation of β-glucose much more rapidly than that of α-glucose. In the case of equilibrium glucose

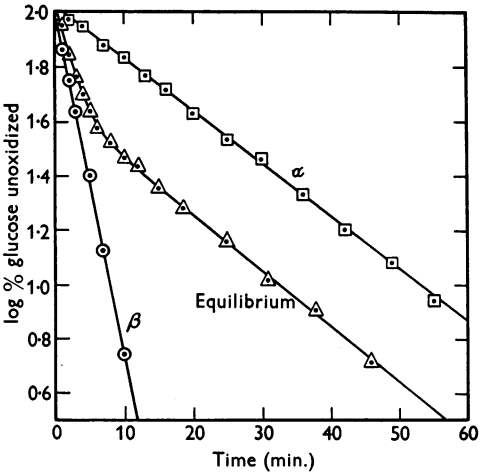

Fig. 2. The oxidation of α-, β- and equilibrium glucose by notatin Y. 80 μg. notatin, 0·04 ml. catalase, 3 mg. glucose as indicated (from dangling cup: α- and β-glucose in dry state, equilibrium glucose as 1% solution), 0·2 M-phosphate buffer (pH 5·6) to 3·3 ml. Experiments carried out in Barcroft differential manometers in quadruplicate and averaged after determination of % sugar unoxidized at each time interval. $T = 20°$. $\log \frac{(a-x)}{a} = -\frac{K'_\beta}{2{\cdot}303} t$; or log % glucose unoxidized $= 2 - \frac{K'_\beta}{2{\cdot}303} t$.

there is a break in the line corresponding to the change from β to α oxidation. This inflexion is reached within 5–7 min. during which time, since the β-form is being rapidly removed, the α to β change will predominate over the reverse reaction. The straight line obtained for α-glucose must represent the combined rates of mutarotation of α-glucose to the readily oxidizable β-glucose and any direct oxidation of α-glucose.

Oxidation of β-glucose by notatin Y. The oxidation of 3 mg. β-glucose by various amounts (10–300 μg.) of notatin Y is represented in Fig. 3. The change of slope of the logarithmic plot in experiments with 20 and 40 μg. notatin is presumably due to the fact that sufficient α-glucose is formed during the first part of the experiment to modify the rate of O_2 uptake at a later period when the mutarotation $\alpha \rightarrow \beta$ becomes the limiting factor in the reaction. With only 10 μg. notatin present, the straight line obtained indicates that the rate of oxidation of

β-glucose is not faster than that of mutarotation so that if an appreciable amount of α-glucose is formed the reverse reaction α- → β-glucose is if anything faster than the oxidation of β-glucose. In other words, with 10 μg. notatin at no time during the reaction does the mutarotation become a limiting factor.

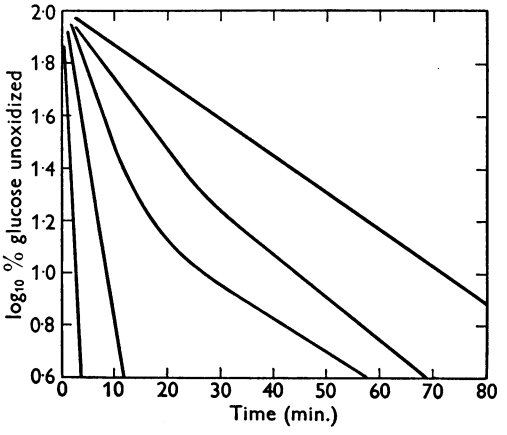

Fig. 3. The oxidation of β-glucose by different quantities of notatin *Y*. The five curves from right to left correspond to 10, 20, 40, 80 and 300 μg. notatin. 0·04 ml. catalase, 3 mg. β-glucose from dangling cup, notatin as indicated, 0·2 M-phosphate (pH 5·6) to 3·3 ml. $T = 20°$. For the rest see explanation of Fig. 2. See also Table 3.

In Table 3 are given the velocity constants K'_β derived from the initial slopes of the curves shown in Fig. 3. The last column of this table shows the activities A_β of this enzyme preparation expressed as the first-order velocity constant K'_β divided by grams dry weight of enzyme. The mean value is 3250.

Table 3. *Relationship between K', the velocity constant for oxidation of β-glucose by notatin Y and the weight of notatin taken*

(Data taken from Fig. 3. Activity of notatin, $A_\beta = K'_\beta$/g. enzyme.)

Notatin (μg./flask)	Initial slope $\left(-\frac{K'_\beta}{2{\cdot}303}\right)$	K'_β	A_β
10	−0·0139	0·0320	3200
20	−0·0268	0·0618	3090
40	−0·0565	0·130	3230
80	−0·120	0·276	3450
300	−0·393	0·905	3020
		Mean	**3250**

The oxidation of α-glucose by notatin Y. Since α-glucose is oxidized very slowly, the analysis of the kinetics of its reaction with notatin can only be carried out in presence of a high concentration of enzyme which simultaneously ensures a reasonably rapid oxidation of the α-form and the virtually instantaneous oxidation of the β-form that appears by mutarotation. In this way the O_2 uptakes can be corrected for the oxidation of β-glucose formed from initially pure α-glucose. Fig. 4 (*b*, *c* and *d*) summarizes the results of experiments carried out in presence of notatin concentrations varying from

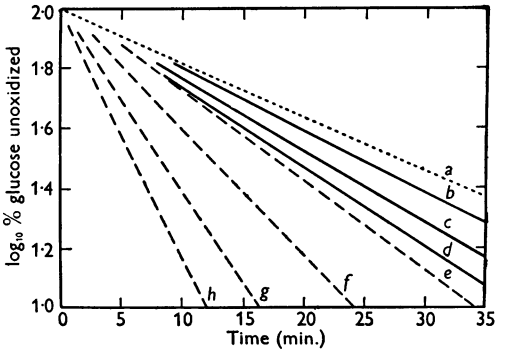

Fig. 4. The oxidation of α-glucose by different concentrations of notatin. 0·04 ml. catalase, 3 mg. α-glucose from dangling cup, 0·2 M-phosphate buffer (pH 5·6) to 3·3 ml. $T = 20°$. Experiments carried out in Barcroft differential manometers in quadruplicate. Full lines (notatin *Y*): *b*, 0·333; *c*, 0·666; and *d*, 0·999 mg. Pecked lines (notatin *Z*): *e*, 0·165; *f*, 0·333; *g*, 0·666; and *h*, 0·999 mg. Dotted line *a*: theoretical line for oxidation β-glucose produced by mutarotation of α-glucose if the latter is not oxidized by notatin.

0·333 to 0·999 mg./3·3 ml. solution in the manometer flasks. In these experiments a small steady-state concentration of β-glucose is rapidly established, after which the rate of the α → β-change and the oxidation of β-glucose become equal. The dotted line (Fig. 4*a*) represents the theoretical course of oxidation of the β-glucose that would be formed by mutarotation of 3 mg. α-glucose on the assumption that α-glucose is not oxidized and that mutarotation is unaffected by the presence of the enzyme preparation. Any increase in the rate of oxidation of α-glucose with increase of notatin concentration can only be ascribed to the catalysis by notatin of either mutarotation, or the direct oxidation of α-glucose, or both. Since, as will be shown later, the sample *Y* (410–417) of notatin does not catalyse mutarotation the increase in the rate of oxidation of α-glucose with notatin concentration must be due to a direct although slow catalytic oxidation of α-glucose.

From the slopes of the lines *b*, *c* and *d* of Fig. 4 the apparent velocity constants K''_α of oxidation of α-glucose by notatin *Y* can easily be calculated (Table 4). The real velocity constant $K'_\alpha = K''_\alpha - k_\alpha$, where $k_\alpha = 0{\cdot}0418$ min.$^{-1}$ is the reaction velocity constant of the forward reaction in the equilibrium

$\alpha \rightleftharpoons \beta$ (see p. 334). The catalytic activity of notatin *Y* in the direct oxidation of α-glucose (A_α) is thus 20·7 (Table 4).

Table 4. *Velocity constants K'_α for oxidation of α-glucose by notatin samples Y and Z and the activities A_α of notatin*

(Data obtained from Fig. 5. The apparent velocity constant $K''_\alpha = K'_\alpha + k_\alpha$, where k_α (=0·0418 min.$^{-1}$) is the velocity constant of the $\alpha \rightarrow \beta$ change in 0·2 M-phosphate buffer pH 5·6 at 20°. Activity of notatin towards α-glucose: $A_\alpha = K'_\alpha$/g. enzyme.)

Sample of notatin	Notatin (mg./3·3 ml.)	Slope $\left(-\frac{K''_\alpha}{2{\cdot}303}\right)$	K''_α	K'_α	A_α
Y	0·333	−0·0212	0·0489	0·0071	21·3
	0·666	−0·0241	0·0555	0·0137	20·6
	0·999	−0·0269	0·0620	0·0202	20·2
				Mean	**20·7**
Z	0·165	−0·0302	0·0695	0·0277	168
	0·333	−0·0421	0·0970	0·0552	166
	0·666	−0·0624	0·1437	0·1019	153
	0·999	−0·0832	0·192	0·150	150
				Mean	**159**

The catalytic activity of notatin *Y* towards α-glucose expressed as a percentage of its activity towards β-glucose is therefore $100 \times 20{\cdot}7/3250 = 0{\cdot}64$ %, which is of the same order as that previously given for mannose and xylose at equilibrium (Keilin & Hartree, 1948*a*).

The effect of substrate concentration upon the rate of reaction at 20° was determined manometrically with 10 μg. notatin *Y* (added from a dangling cup)

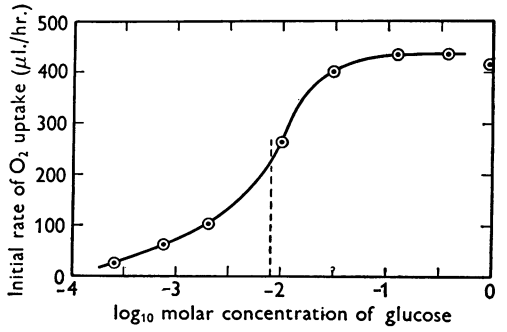

Fig. 5. Determination of Michaelis constant of glucose oxidase at 20°. 10 μg. notatin *Y*, 0·04 ml. catalase. Notatin (in 0·2 ml. buffer) added from dangling cup to catalase + glucose (0·909–0·00025 M) made up to 3·1 ml. with 0·2 M-phosphate buffer pH 5·6.

and glucose concentrations varying from 0·00025 to 0·909 M. From the results thus obtained, which are summarized in Fig. 5, the Michaelis constant K_m is 0·0096 M. It can be argued that because the results were obtained with equilibrium glucose the true K_m for β-glucose is in fact about 64 % of the value given.

The low concentration of glucose that was necessary in the above kinetic experiments (3 mg./flask, or 0·005 M), being less than K_m, lies well within the range where the rate of oxygen uptake is proportional to substrate concentration (Fig. 5). The oxidation of glucose in these experiments is therefore a first-order reaction. Since the true substrate of notatin is β-glucose the above considerations cannot be applied to the much slower oxidation of α-glucose. Although there is no satisfactory method of determining K_m for α-glucose the fact that it does not inhibit the oxidation of β-glucose competitively indicates that α-glucose has a low affinity for notatin and that its oxidation will therefore be a first-order reaction. The same applies to the kinetics of the apparent rate of oxidation of α-glucose depending partly upon mutarotation which is also a first-order reaction.

Oxidation of glucose catalysed by notatin *Z*

The results of manometric experiments on the oxidation of α-, β- and equilibrium glucose catalysed by notatin *Z* are summarized in Fig. 6. These results,

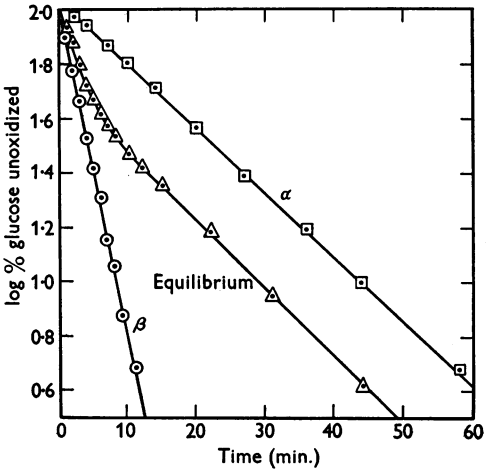

Fig. 6. The oxidation of α-, β- and equilibrium glucose by notatin *Z*. Conditions as in Fig. 2 except that 300 μg. notatin *Z* was used instead of notatin *Y*.

which are very similar to those with notatin *Y* (Fig. 2), clearly show that the enzyme catalyses the oxidation of β-glucose much more rapidly than that of α-glucose. The rate of oxidation of the former is the same with either sample of notatin, but α-glucose is oxidized by notatin *Z* at a rate which is significantly faster than the oxidation by notatin *Y*. This difference is more clearly established by the experiments described in the next paragraph. In the case of equilibrium glucose there is also a break in the curve corresponding to the change from

oxidation of β- to oxidation of α-glucose. This inflexion is reached here within 6 min., the time during which the β-form is removed by oxidation, and therefore the rate of the subsequent oxidation becomes dependent upon the rate of mutarotation $\alpha \rightarrow \beta$ and also upon the rate of oxidation of the α-form.

The oxidation of α-glucose by notatin Z. For this study the concentrations of notatin varied from 0·165 to 0·999 mg./3·3 ml. buffer solution in the

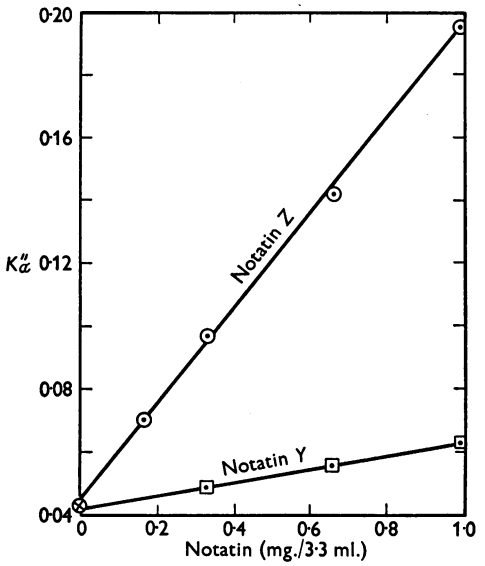

Fig. 7. Relationship between the apparent rate of oxidation of 3 mg. α-glucose expressed as the first-order reaction constants (K''_α) and concentration of notatin *Y* and *Z* (based on data of Fig. 4 and Table 4). Both lines cut the K''_α axis at 0·0418 which is the value of k_α the velocity constant of the change $\alpha \rightarrow \beta$ in absence of notatin.

manometric flasks. The results of these experiments are summarized in Fig. 4. From the slopes of the lines *e*, *f*, *g* and *h* the velocity constants K''_α and K'_α as well as the catalytic activity A_α are calculated as before (Table 4). Thus the enzyme activity (A_α) of notatin *Z* towards α-glucose is about 159 which is eight times higher than the corresponding activity of notatin *Y*. This difference between notatin *Y* and *Z* can be clearly seen in Fig. 7 in which the apparent velocity constants K''_α are plotted against enzyme concentration. In this figure both lines cut the K''_α axis at 0·0418 (k_α).

Polarimetric studies of mutarotation in presence of notatin preparations Y and Z

Although notatins *Y* and *Z* display the same very high catalytic activity in the oxidation of β-glucose, they differ in their catalytic activities towards α-glucose. This suggested that sample *Z* contains an additional component which can catalyse either a direct oxidation of α-glucose or its mutarotation to the very easily oxidizable β-glucose. The latter possibility could only be tested polarimetrically.

In order to eliminate the considerable errors that would result from the loss of asymmetry at carbon atom 1 on oxidation of glucose to gluconic acid, the polarimetric measurements were carried out under anaerobic conditions using for this purpose the apparatus shown in Fig. 1. A solution of 9·0 mg. notatin *Y* or *Z* in 18 ml. 0·2 M-phosphate buffer was pipetted into *A* after the required amount of α- or β-glucose had been introduced into *B*. When the sugar had been dissolved anaerobically the mutarotation was followed as previously described.

Table 5. *Mutarotation constants of α- and β-glucose in presence of 0·2 M phosphate buffer*, pH 5·6, *and notatin Y or Z at 20° under anaerobic conditions*

(Notatin concentration 0·5 mg./ml. Experiments carried out in a jacketed 22 cm. polarimeter tube as shown in Fig. 1. S.D. = standard deviation (%).)

Glucose				
Isomers	Concentration (%, w/v)	Notatin sample	$10^3 K$ (min.$^{-1}$)	S.D.
α	3·98	None	65·8	Data from Table 2
β	4·01	None	66·4	
α	3·99	*Y*	66·2	0·90
β	4·00	*Y*	66·5	0·72
α	3·96	*Z*	131·4	1·00
β	4·01	*Z*	116·5	1·86

The mean values of the mutarotation constants obtained in these experiments (Table 5) show that whereas mutarotation is not influenced by notatin *Y*, it is markedly accelerated by notatin *Z*. These results provide an explanation of the different action of these two samples in the oxidation of α-glucose as shown in Figs. 4 and 7. Moreover, since sample *Y* has no influence on mutarotation, the increasing rates of oxidation of α-glucose by increasing concentrations of notatin (Fig. 4, *b–d*) must be a true measure of the direct oxidation of α-glucose. With notatin *Z* the extra increase in the rate of oxidation of α-glucose is due to the increased mutarotation of α- to β-glucose catalysed by an additional substance present in this sample. Having at their disposal only the notatin sample *Z* ('233–237') Bentley & Neuberger (1949) concluded: 'There is thus suggestive, but not conclusive evidence that notatin catalyses the mutarotation of glucose.' Our experiments, however, have shown that the acceleration of mutarotation is due to a factor distinct from glucose oxidase. The properties of this mutarotation catalyst in its relation to notatin will be examined in a separate paper (Keilin & Hartree, 1951).

Further studies on the specificity of notatin for glucose

Taking the activity of notatin towards β- or equilibrium glucose as 100 the slight degree of oxidation of 6-methyl glucose (1·85) and of 4:6-dimethyl glucose (1·22) together with a few other results previously recorded (Keilin & Hartree, 1948*a*) suggest that it is the 1-, 2- and 3-positions in the glucose molecule which must be free before activation by the enzyme can occur. This is corroborated by our finding that the activity of notatin towards 4:6-benzylidene glucose is 1·90.

Table 6 gives the activity of notatin towards all eight aldohexoses. The various sugars were allowed to equilibrate in solution before being tested, and although the α/β ratios will vary from one sugar to another this can have no serious effect on the overall picture of the high specificity of notatin.

Table 6. *Activity of notatin Y towards* D-*aldohexoses determined manometrically*

(30 mg. monosaccharide; 300 μg. notatin *Y* (only 10 μg. used for oxidation of glucose); 0·04 ml. catalase; 0·2M-phosphate buffer, pH 5·6; $T = 20°$. Sugar solutions equilibrated overnight before being used.)

Sugar	Activity
Equilibrium glucose (≡β-glucose)	100
Mannose	0·98*
Altrose	0·16
Galactose	0·14*
Talose (two samples)	0·015,† 0
Allose	0
Gulose	0
Idose (syrup)	0·018†

* From Keilin & Hartree (1948*a*).
† These activities cannot be regarded as significant.

It has recently been found by Monod (1950) that improvements in the method of purification of notatin from culture medium filtrates of *P. notatum* yield a product that will not oxidize trehalose and that the slight activity (0·28) that we recorded previously was due to the presence of trehalase. Indeed in the case of trehalose such an explanation is the only possible one since this sugar is a non-reducing one with 1-1′ linkage of the glucose residues. However, from the progress of its oxidation, it was obvious that the oxygen uptake was not due to contamination with glucose. Considering that substitution in the 4- and 6-positions of glucose does not entirely abolish the oxidation by notatin and that with the exception of trehalose the disaccharides tested were either 4- or 6-substituted glucoses, it is conceivable that in some cases at least the observed oxidations of disaccharides were genuine.

THE MOLECULAR WEIGHT OF NOTATIN

According to the published determinations of sedimentation velocity and diffusion constant the molecular weight of notatin is 152,000 (Cecil & Ogston, 1948*a*). Following an investigation into the accuracy of the ultracentrifugal method which involved corrections for errors in cell temperature (Cecil & Ogston, 1948*b*) Dr R. Cecil has re-examined notatin sample *Y* (410–417) and has amended $S_{20} \times 10^{13}$ from 8·27 to 8·09 which corresponds to a molecular weight of 149,000.

DISCUSSION

It is now well established that glucose oxidase or notatin is a chromoprotein enzyme of molecular weight about 149,000 and with two prosthetic groups (alloxazine-adenine dinucleotide) per molecule of protein. In any discussion of the mechanism of the enzyme activity the role of this prosthetic group is of primary importance. All that can be said at present is that the enzyme forms a complex with its highly specific substrate (glucose) which becomes activated. The prosthetic groups acting as hydrogen acceptors are then reduced while glucose undergoes oxidation. In fact the well known anaerobic reduction of flavoprotein enzymes by their substrates has also been demonstrated in the case of notatin (Keilin & Hartree, 1948*a*). In other words, the kinetics of the enzymic oxidation of glucose can be followed spectroscopically under strictly anaerobic conditions. Whether the two atoms of hydrogen are transferred stepwise from glucose to flavine it is difficult to say. Although flavines readily form coloured free radicals by accepting one H (Michaelis, Schubert & Smythe, 1936; Michaelis & Schwarzenbach, 1938), it is unlikely in the case of such an active enzyme as notatin that any intermediate would have an appreciable life.

Once the molecule of glucose is oxidized and the prosthetic group of the enzyme reduced to dihydroflavine, the latter can become reoxidized by a suitable hydrogen acceptor, such as molecular oxygen which becomes reduced to hydrogen peroxide, or under anaerobic conditions, by 2:6-dichlorophenol-indophenol which becomes reduced to a leucoindophenol. In all known cases of oxidation reactions catalysed by autoxidizable flavoprotein enzymes the hydrogen peroxide arises from the reduction of molecular oxygen by the dihydroflavine prosthetic group of the enzyme.

All this shows that glucose oxidase or notatin is a typical aerobic dehydrogenase and this has been confirmed by isotopic experiments carried out in presence of $H_2{}^{18}O$ and $^{18}O_2$ by Bentley & Neuberger (1949). It has long been known that the final product

of the oxidation of glucose catalysed by notatin is gluconic acid. However, Bentley & Neuberger (1949) have demonstrated by estimations of total and free gluconic acid at different stages of the reaction, and by polarimetric experiments, that the first product of oxidation is the neutral δ-gluconolactone which undergoes spontaneous hydrolysis to gluconic acid.

The comparative manometric study of the catalytic activities of notatin in the oxidation of α- and β-glucose required careful correction for mutarotation of α-glucose. An added complication was the presence in some samples of notatin of a catalyst which accelerates mutarotation. It has, however, been possible to eliminate these effects and to establish that the relative rates of oxidation of the two isomers are $\beta : \alpha = 100 : 0{\cdot}64$. In other words, the catalytic activity of notatin in the oxidation of α-glucose is of the same order of magnitude as that previously obtained for some other sugars such as mannose and xylose.

The apparent high activity of notatin towards α-glucose (as high as 77% of that of β-glucose) recorded by Bentley & Neuberger (1949) can only be due to the fact that under their conditions the formation of β-glucose by mutarotation of the α-form proceeded almost as rapidly as the direct oxidation of β-glucose.

The notatin used in our present experiments was taken from two batches labelled '410–417' and '233–237' and referred to in this paper as *Y* and *Z* respectively. Manometric study of the kinetics of oxidation of α- and β-glucose by these samples of notatin gave strong indications of the presence in notatin *Z* but not in notatin *Y* of an additional component capable of catalysing the mutarotation of glucose. This was confirmed by polarimetric measurements carried out under strictly anaerobic conditions. The mutarotating catalyst present only in *Z* increased its apparent activity in the oxidation of α-glucose about eight times and thus raised the rate of its oxidation to about 5% of that of β-glucose.

All eight D-aldohexoses have now been tested with notatin. If its activity towards β- (or equilibrium) glucose = 100, the relative rates for other sugars are: mannose = 0·98, altrose = 0·16, galactose = 0·14, whereas allose, idose, gulose and talose are not oxidized.

In cases where notatin shows an activity towards different sugars one must keep in mind the possibility of the presence in the sample under investigation of glucose as an impurity. This was, for instance, the case with a commercial galactose which in presence of notatin gave a very rapid initial oxygen uptake followed by a slow but steady oxidation of galactose. By extrapolating to zero time we were able to determine that the sample of galactose contained about 4% glucose (Keilin & Hartree, 1948*a*, Fig. 9). It is only when the oxygen uptake shows no sudden change in rate and when it persists over a long period that the sugar can be considered susceptible to direct catalytic oxidation by notatin.

The previous tests with about fifty sugars and their derivatives (Keilin & Hartree, 1948*a*), together with the examination of aldohexoses described in this paper, as well as the fact that the first product of oxidation is δ-gluconolactone (Bentley & Neuberger, 1949), give a clear indication that notatin can be considered as an oxidase highly specific for β-D-glucopyranose.

Every change in configuration and any substitution in the —OH groups either reduces the catalytic activity of notatin to a very low level or abolishes it altogether. So far no compound has been found showing a susceptibility to oxidation by notatin which is intermediate between that of β-glucose (100) and those of 6-methyl glucose (1·85) or 4:6-benzylidene glucose (1·90).

It is perhaps surprising that notatin shows even a slight activity towards α-glucose, considering that stereospecificity is a very general property of enzymes. Inversion at $C_{(2)}$, $C_{(3)}$ or $C_{(4)}$ of β-glucose (to give mannose, allose and galactose respectively) reduces the activity of notatin by at least 99%. But since it is the H atoms associated with $C_{(1)}$ which undergo transfer to the flavine groups of notatin it would be expected that inversion at this point would more effectively interfere with the oxidation.

That an enzyme may form complexes with several substances belonging to the same family as the substrate does not imply that all such substances will undergo activation. The existence of compounds which combine with, but are not activated by, an enzyme is revealed by the competitive inhibition they exercise upon the reaction between the enzyme and its true substrate. Indeed numerous cases of such interesting competitive inhibitors are known for xanthine oxidase, uricase, succinic dehydrogenase and others. The affinity of the enzyme for a competitive inhibitor is sometimes much greater than for the true substrate.

The fact that of the many sugars tested with notatin none has exhibited any appreciable inhibition of oxidation of β-glucose may be taken to indicate that all except β-glucose have either a very small or no affinity for the enzyme. It is conceivable that it is mainly the difficulty in forming a sufficiently stable enzyme-substrate complex that is primarily responsible for the high specificity of the enzyme for β-glucose.

Careful consideration of specificity data recorded in the present and the previous paper (Keilin & Hartree, 1948*a*) gives no clear indication of the

mode of attachment between notatin and its substrate. All that can be said at present is that whereas substitution in the 1-, 2- or 3-OH groups of glucose abolishes enzyme activity entirely, substitution at 4 or 6 or both positions reduces the activity to about 1–2 % of that of β-glucose itself.

In view of the sharp specificity of notatin for the β-form of glucose it becomes important to review the methods we have previously described (Keilin & Hartree, 1948*b*) for the estimation of glucose in terms of oxygen uptake in presence of notatin. When glucose is to be estimated in a static system, i.e. one where glucose is not being formed, the differential rates of oxidation of the two isomers will not affect the final oxygen uptake. As we have already demonstrated, the theoretical uptake is rapidly attained at 39° and must eventually be reached at lower temperatures.

Naturally-occurring derivatives of glucose often have the α-glucopyranose structure (e.g. sucrose, Cori ester) and glucose will be liberated from these in the α-form. There is thus a possibility that the rate of its oxidation may lag behind the rate of its production. At 39° we invariably found that the course of glucose-producing reactions as estimated with notatin could be closely checked through periodic estimations of glucose by independent methods. Even at 20° we found it possible, by using a high concentration of notatin, to obtain a picture of the inversion of sucrose which could be confirmed by the Hagedorn & Jensen procedure. Such agreement may well have been due partly to the presence in the notatin (*Z*) of the component catalysing mutarotation and partly to the high concentration of the oxidase. In fact, even the low activity of notatin towards α-glucose under the above conditions corresponds to a Q_{O_2} of about 300. On the other hand, the experiments that we carried out at 22° on the enzymic hydrolysis of amygdalin and sinigrin presented no complication of this nature, since glucose is present in these glucosides in the β-form and is therefore immediately oxidized by notatin as it is liberated.

Thus the form in which the glucose is liberated will not affect its estimation in terms of oxygen uptake by notatin at 39°. At lower temperatures, however, preliminary manometric experiments must be carried out in order to adjust the concentration of the components of the system, so as to ensure that liberation of glucose and not its oxidation by notatin will limit the rate of the overall reaction.

SUMMARY

1. The catalytic oxidation by notatin of β-glucose is much faster than that of α-glucose.

2. Two samples of notatin (*Y* and *Z*), which were of equal catalytic activity in the oxidation of β-glucose, showed very different activities in the oxidation of α-glucose. Sample *Z* oxidized α-glucose about eight times faster than sample *Y*.

3. Manometric study of the oxidation of α-glucose by these samples of notatin indicated that sample *Z* contained an additional component capable of catalysing mutarotation.

4. This was confirmed by polarimetric study of the effect of the two samples of notatin on mutarotation of glucose under strictly anaerobic conditions, the experiments being carried out in an apparatus specially devised for this purpose.

5. Making use of notatin *Y*, which did not influence mutarotation, the rates of the direct oxidations of α- and β-glucose were obtained by correcting the manometrically observed rates of oxygen uptake for the calculated mutarotation of the α- to the easily oxidizable β-form under the influence of the buffer solution. The ratio of oxidation rates thus obtained is $\beta : \alpha = 100 : 0{\cdot}64$ at 20°.

6. The bearing of this result on the use of notatin for estimation of glucose formation in biological systems is discussed. The oxygen uptake in presence of notatin is a reliable measure of glucose formation at any temperature if glucose is liberated in the β-form, while at 39° the mutarotation is fast enough to ensure that the oxygen uptake is a true measure of the rate of appearance of either isomer. At lower temperatures, if glucose is liberated in the α-form the concentration of notatin must be carefully adjusted so that the oxidation of glucose does not limit the rate of the overall reaction.

7. All eight D-aldohexoses have been tested with notatin. If the rate of oxidation of β-glucose = 100, those of the other sugars are: mannose = 0·98, altrose = 0·16 and galactose = 0·14, while allose, gulose, idose and talose are not oxidized.

8. The high specificity of notatin for β-glucose is indicated by the fact that optical inversion at any carbon atom or substitution in the hydroxyl groups of the substrate either reduces the catalytic activity of notatin to about 1 % of its original value or abolishes it altogether.

9. Notatin can now be considered as a specific β-D-glucopyranose oxidase.

We wish to thank Dr A. Gottschalk and Dr D. J. Bell for much valuable advice, and Dr R. Cecil for re-examining notatin in the ultracentrifuge. We are greatly indebted to Prof. C. S. Hudson, Prof. T. Reichstein and Dr H. S. Isbell for their gifts of rare monosaccharides.

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Biological Catalysis of Mutarotation of Glucose

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(*Received* 23 *May* 1951)

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.).