the preincubation period with metabolic inhibitors (NaF and NaN_3), protease inhibitors (Tos-Lys-CH_2Cl, phenylmethylsulfonylfluoride, Trasylol®) and a lysosomotropic agent (NH_4Cl). None of these agents influenced HCG binding (data not shown). Compared with untreated control membranes NaF and NaN_3 are potent inhibitors of the LH/HCG-receptor inactivation at a concentration of 10 and 100 mM. A similar effect was accomplished with NH_4Cl. The other agents tested did not have any effect on the receptor inactivation process (table).

A time course study under inhibiting conditions (10 mM NaN_3) was performed using crude testicular membranes and ovarian membranes of pregnant animals. Under this condition the inactivation process is extremely delayed and reduced, but there is no complete inhibition of receptor loss (figure, d). The incomplete inhibition of receptor degradation could be explained by an additional unspecific temperature effect. Since sodium azide has been shown as an inhibitor of many enzymes, the effect of the metabolic agents tested on the receptor inactivation might be related to an inhibition of membrane associated enzyme-action and/or to an inhibition of receptor aggregation in the plasma membrane. If the free receptor is inactivated in the same way as the hormone-receptor complex, the aggregation is a necessary step for the processing and degradation of the receptor.

1 This work was supported in part by the Deutsche Forschungsgemeinschaft (Si 185/2). We thank Mrs Rita Rudolph for skilful technical assistance.
2 Author for reprint requests.
3 A.R. Midgley, Jr, Adv. exp. Med. Biol. *36*, 365 (1973).
4 J.S. Richards and A.R. Midgley, Jr, Biol. Reprod. *14*, 82 (1976).
5 K.J. Catt, J.P. Harwood, G. Aguilera and M.L. Dufau, Nature *280*, 109 (1979).
6 P.M. Conn, M. Conti, J.P. Harwood, M.L. Dufau and K.J. Catt, Nature *274*, 598 (1978).
7 J.P. Merlie, J.P. Changeux and F. Gros, Nature *264*, 74 (1976).
8 R. Bellisario and O.P. Bahl, J. biol. Chem. *250*, 3837 (1975).
9 F. Leidenberger and L.E. Reichert, Jr, Endocrinology *91*, 135 (1972).
10 Th.A. Bramley and R.J. Ryan, Endocrinology *91*, 135 (1972).
11 J.W. Siebers, F. Peters, M.T. Zenzes, J. Schmidtke and W. Engel, J. Endocr. *73*, 491 (1977).
12 G. Scatchard, Ann. N.Y. Acad. Sci. *51*, 660 (1949).
13 M. Ascoli and D.J. Puett, J. biol. Chem. *253*, 4892 (1978).

Continuous optical assay of sucrase and other glucosidases

P. Vanni[1] and G.M. Hanozet[2,3]

Laboratorium für Biochemie, Eidgenössische Technische Hochschule Zürich, Universitätsstrasse 16, CH-8092 Zürich (Switzerland), 22 November 1979

Summary. A continuous optical method for the assay of glucose-releasing hydrolases is reported. Particular emphasis is given to the assay of purified sucrase from rabbit small intestine. The procedure requires glucose dehydrogenase and mutarotase. In the presence of the latter enzyme, the initial lag is substantially shortened when glucose is released as α-anomer. Under the test conditions used, the method shows a good proportionality up to an activity of 0.2 units/3 ml and may also be applied for measuring the activity in crude homogenates.

The possibility of a continuous optical measurement of an enzymic reaction rate by recording the formation or disappearance of a chromogen with a recording photometer generally offers advantages in accuracy, precision and rapidity and allows a better measurement of kinetic behaviour. The methods most used until now for the assay of disaccharidases and other glucosidases are discontinuous 2-step[4-7] or 1-step[8] methods, in which the reaction is stopped at suitable times and the glucose released is measured by glucose oxidase/peroxidase[4-8], hexokinase/glucose-6-phosphate dehydrogenase[6] or glucose dehydrogenase[7]. A method of isomaltase assay with a coupled system glucose-6-phosphate dehydrogenase/NADP has been described in the past[9], but it has not been used since. With the most commonly used glucose oxidase method, lags of up to 15 min have been observed before the reaction attains a constant rate[8]. Such lags are principally due to the non-enzymic conversion of glucose from the α-form to β-form, the latter being the substrate of glucose oxidase.

Purified preparations of NAD-dependent glucose dehydrogenase and mutarotase for the assay of glucose have recently been made commercially available[10]. In the present paper we describe a method which uses such preparations for the measurement of the activity of the enzyme sucrase-isomaltase. The same procedure may be employed for the determination of other glucosidases, either in crude extracts or in purified preparations.

Materials and methods. α-glucosidase and β-fructosidase from yeast, β-glucosidase from sweet almonds, hexokinase/glucose-6-phosphate dehydrogenase from yeast, ATP and $NADP^+$, were purchased from Boehringer, Mannheim, FRG. Glucose dehydrogenase, mutarotase, NAD^+, sucrose, palatinose, maleic acid and other chemicals were from Merck, Darmstadt, FRG. D-salicin was purchased from Fluka, Buchs, Switzerland.

For the preparation of the small intestine crude extracts, frozen rabbit small intestine was homogenized twice for 30 sec at 20% (w/v) in 50 mM sodium maleate buffer, pH 6.8+5 mM EDTA in a Waring Blendor at 4 °C. The homogenate was filtered through a cheese-cloth and the measurements of the enzyme activities were carried out on the filtrate. The protein content was determined according to Lowry et al.[11].

The sucrase-isomaltase complex was isolated from rabbit

Activities of some disaccharidases in a crude homogenate of rabbit small intestine

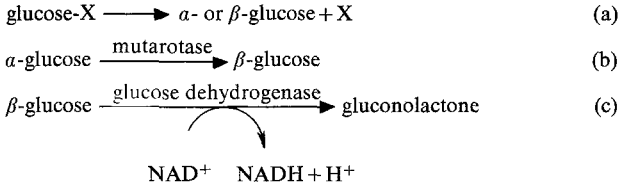
Substrate	Continuous method (U/mg protein)	Discontinuous methods (HK+G6PD)	(MR+GD) (U/mg protein)
Sucrose	0.228±0.012	0.228±0.019	0.239±0.012
Maltose	0.518±0.037	0.610±0.026	0.660±0.020
Trehalose	0.102±0.008	0.120±0.007	0.122±0.007

Test conditions for continuous assay are as indicated in figure 2; for discontinuous methods as indicated under 'materials and methods'. HK = hexokinase; G6PD = glucose-6-phosphate dehydrogenase; MR = mutarotase; GD = glucose dehydrogenase. Each value is the mean of 4 measurements ± SD.

small intestine by papain solubilization, according to Cogoli et al.[12], or by Triton X-100 solubilization, according to Sigrist et al.[13].

Discontinuous assay of disaccharidases, used as a comparison with the present method, was carried out at 37 °C in 33 mM sodium maleate buffer, pH 6.8 and 33 mM substrate. After 5 min of incubation the reaction was stopped by heat denaturation and the glucose released was determined by the hexokinase/glucose-6-phosphate dehydrogenase method[14] or by the mutarotase/glucose dehydrogenase method, with Merckotest 3389. The continuous optical determinations were carried out at 37 °C and at 366 or 340 nm, with a recording Unicam spectrophotometer. 1 unit of enzyme (U) corresponds to the enzymatic activity that transforms 1 µmole of substrate per min.

Results. The principle of the continuous optical method is based upon the following order of reactions:



In the case of intestinal sucrase, where X is fructose, the presence of mutarotase (reaction b) in the test mixture was proved to be absolutely essential. Figure 1, A, shows the time course of the sucrase reaction in the presence (+MR) and in the absence (−MR) of mutarotase. As intestinal sucrase liberates D-glucose in the α form[15], it is evident that in the absence of mutarotase the non-enzymic conversion of α- into β-glucose is rate limiting. On the contrary, in the case of β-glucosidase (figure 1, B) the anomer released is β-glucose and thus mutarotase is not necessary.

The concentrations of glucose dehydrogenase, mutarotase and NAD^+ indicated in figure 1, A, ensure that the reaction catalyzed by sucrase shows a very short lag of about 30 sec and that the pH optimum of the assay corresponds to that previously reported for sucrase, i.e. pH 6.8[16], and not to that of the auxiliary system, which would be higher, about 7.6[10].

By applying the continuous optical method, the K_ms of the sucrase-isomaltase complex for sucrose and palatinose were calculated. They were 4.53±0.21 mM and 3.79±0.18 mM respectively in agreement with those previously reported, obtained with a discontinuous method[16]. According to these data, the following composition of the mixture for the routine continuous assay of sucrase-isomaltase is suggested: 33 mM Na maleate buffer, pH 6.8, 33 mM sucrose or palatinose, 2 mM NAD^+, 0.2 U/ml mutarotase, 9.6 U/ml glucose dehydrogenase, in a final volume of 3 ml. Figure 2 shows the effect of the increase of enzyme concentration on sucrase activity. A good linearity of response is obtained up to an activity of 0.2 µmoles/min, corresponding to a ΔE_{340}/min of 0.42 (in a volume of 3 ml). At higher activities there is a deviation from linearity. From the linear portion of the curve in figure 2 (and taking into account the concentration of sucrose used and the corresponding value of K_m) it is possible to calculate the turnover number of the rabbit intestinal sucrase, that is 45 sec^{-1}, assuming a mol. wt of 200,000.

A comparison between the continuous optical assay of sucrase and 2 discontinuous methods has been performed (figure 3). Linear regression analysis shows that there is a good correlation in both cases, the coefficient of correlation being equal to 1.

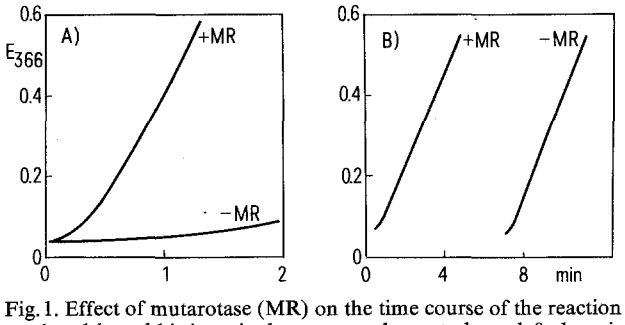


Fig. 1. Effect of mutarotase (MR) on the time course of the reaction catalyzed by rabbit intestinal sucrase and sweet almond β-glucosidase. *A* Sucrase. Test conditions were: 33 mM sodium maleate buffer, pH 6.8; 33 mM sucrose; 2 mM NAD^+; 9.6 U/ml glucose dehydrogenase; ±0.2 U/ml mutarotase; 65 µg sucrase in a final volume of 3 ml. *B* β-Glucosidase. Test conditions were the same as above, with 33 mM D-salicin instead of sucrose and 5 µg of β-glucosidase in a final volume of 3 ml. Temperature 37 °C.

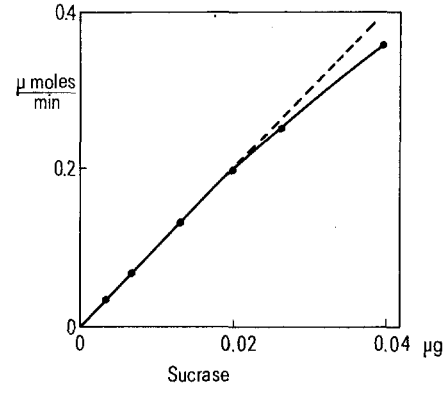


Fig. 2. Effect of the enzyme concentration on sucrase activity assayed with the continuous optical method. Test conditions were: 33 mM sodium maleate buffer, pH 6.8, 33 mM sucrose, 2 mM NAD^+, 0.2 U/ml mutarotase, 9.6 U/ml glucose dehydrogenase and the indicated amounts of the enzyme in a final volume of 3 ml. Temperature 37 °C.

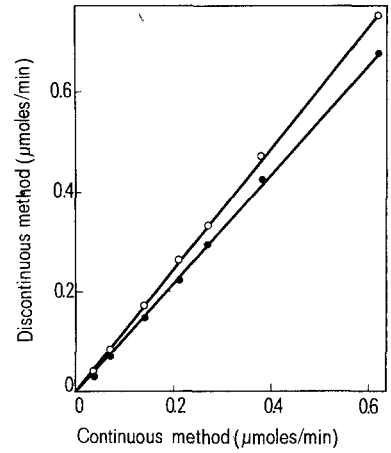


Fig. 3. Comparison between the sucrase activity determined with continuous optical method (x-axis) and with 2 discontinuous methods (y-axis). (○) Discontinuous method with hexokinase/glucose-6-phosphate dehydrogenase. (●) Discontinuous method with mutarotase/glucose dehydrogenase. Test conditions for the continuous method are as in figure 2; those for discontinuous methods are as in 'materials and methods'. Results of linear regression analysis are as follows: (○) $a_0 = 0.00094$; $a_1 = 1.2249$; $s_0 = 0.00194$; $s_1 = 0.0067$; $r = 1.00$; $s_{y,x} = 0.003665$. (●) $a_0 = -0.00262$; $a_1 = 1.1042$; $s_0 = 0.00203$; $s_1 = 0.0069$; $r = 1.00$; $s_{y,x} = 0.003828$.

As well as sucrase, other glucose-releasing hydrolases can be assayed by the continuous method. Experiments were carried out with purified α-glucosidase and β-fructosidase from yeast and β-glucosidase from sweet almonds. In the case of β-glucosidase, on the basis of the results shown in figure 1, B, mutarotase has been omitted from the reaction mixture. For α- and β-glucosidase a proportionality has been observed between the measured activity and the amount of enzyme used, up to a value of 0.2 µmoles/min. For β-fructosidase a proportionality that reaches 0.6 µmoles/min has been obtained.

The continuous method can also be usefully applied to crude homogenates. The table shows the activities of some disaccharidases in a crude homogenate of intestine, determined with the continuous method and compared with 2 discontinuous methods. With sucrose as substrate there is a good agreement between the activities determined by the present procedure and these determined by discontinuous methods. With trehalose and maltose as substrates, the activities determined by discontinuous methods are slightly higher.

Discussion. The present work describes a continuous optical method for the assay of sucrase activity, which can be applied to all other glucose-releasing hydrolases. The presence of glucose dehydrogenase in the auxiliary system has 2 main consequences. Firstly, the interference of other enzymes possibly present in the crude extracts and/or in the auxiliary system is greatly reduced. For instance, the assay of sucrase with hexokinase/glucose-6-phosphate dehydrogenase can be greatly affected by the presence of glucose-phosphate isomerase or 6-phosphogluconate dehydrogenase. Secondly glucose dehydrogenase is specific for the β-anomer of glucose. Therefore, whenever the α-anomer of glucose is released, in the absence of mutarotase an exceedingly long lag will be observed. In our case, with the addition of mutarotase to the reaction mixture, the time lag is noticeably shortened, becoming about 30 sec.

It is also known that disaccharidases, besides their hydrolytic activity, are endowed with a transglucosidase activity[6], that may interfere in the assay and in the determination of kinetic parameters. With the present method, the glucose formed is oxidized by the auxiliary system and therefore cannot become an acceptor for the transferase activity.

In conclusion, when compared with the discontinuous methods used until now, the present method has noticeable advantages of rapidity and accuracy, especially in kinetic studies, in which it is essential to determine the initial rate of the reaction.

1 Present address: Istituto di Chimica biologica, University of Florence, viale Morgagni 50, Florence, Italy.
2 Present address: Istituto di Fisiologia generale e di Chimica biologica, University of Milan, via Saldini 50, Milan, Italy. To whom correspondence should be sent.
3 It is a pleasure to acknowledge Professor G. Semenza for his continued interest in this work and for critical reading of the manuscript. Glucose dehydrogenase was kindly supplied by Dr N. Hennrich (E. Merck, Darmstadt), to whom due thanks are addressed.
4 A. Dahlqvist, Analyt. Biochem. *7*, 18 (1964).
5 A. Dahlqvist, Analyt. Biochem. *22*, 99 (1968).
6 G. Semenza and A.K. von Balthazar, Eur. J. Biochem. *41*, 149 (1974).
7 M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Müller and G. Semenza, Biochim. biophys. Acta *506*, 136 (1978).
8 S. Messer and A. Dahlqvist, Analyt. Biochem. *14*, 376 (1966).
9 J. Larner and C.M. McNickle, J. biol. Chem. *215*, 723 (1955).
10 D. Banauch, W. Brümmer, W. Ebeling, H. Metz, H. Lang, K. Leybold and W. Rick, Z. klin. Chem. Biochem. *13*, 101 (1975).
11 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
12 A. Cogoli, H. Mosimann, C. Vock, A.K. von Balthazar and G. Semenza, Eur. J. Biochem. *30*, 7 (1972).
13 H. Sigrist, P. Rommer and G. Semenza, Biochim. biophys. Acta *406*, 433 (1975).
14 H.U. Bergmeyer, E. Bernt, F. Schmidt and H. Stork, in: Methods of Enzymatic Analysis, vol.3, p.1196. Ed. H.U. Bergmeyer. Verlag Chemie, Weinheim 1974.
15 G. Semenza, H.Ch. Curtius, O. Ranhardt, P. Hore and M. Müller, Carbohydr. Res. *10*, 417 (1969).
16 J. Kolínskà and G. Semenza, Biochim. biophys. Acta *146*, 181 (1967).

The distribution of pyrroline carboxylate reductase and proline oxidase in the larva of the blowfly, *Aldrichina grahami*

S. Tsuyama, T. Higashino and K. Miura

Laboratory of Biochemistry, Department of Agricultural Chemistry, University of Osaka Prefecture, Sakai-City, Osaka 591 (Japan), 27 December 1979

Summary. P5C reductase and proline oxidase in the larva of the blowfly, *Aldrichina grahami,* were found to be localized mainly in the fat body mitochondrial matrix and the muscle, respectively.

Arginine is required in the diet of all insects, and during the larval growth of the blowfly, *Aldrichina grahami,* it is metabolized mainly to proline in vivo[1]. Δ^1-Pyrroline-5-carboxylate (P5C) has been suggested to be an intermediate formed during this conversion. It is well known that P5C is metabolized to proline by P5C reductase and that proline oxidase is its reverse enzyme. In insects, it has been reported that P5C reductase is localized in the cytosol of the fat body of the adult silkmoth, *Hyalophora gloveri*[2], and that proline is oxidized in the mitochondria of the flight muscle of the blowfly, *Phormia regina*[3]. However, the distribution of these enzymes during larval growth has not been elucidated. In this paper, we report on the distribution of P5C reductase and proline oxidase in the larva of the blowfly, *A. grahami.*

Materials and methods. Blowflies were reared aseptically on semi-synthetic diets at 25 °C as described previously[4]. The 4-day-old larvae were dissected in a cold saline solution [120 mM NaCl, 5 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.2 mM $NaHCO_3$, 0.13 mM NaH_2PO_4 and 5 mM N-2-hydroxyethylpiperadine-N′-2-ethanesulfonic acid (HEPES), pH 7.4] and the fat body, gut, malpighian tube, haemolymph, muscle and other tissues containing cuticle and trachea were isolated. These isolated organs were homogenized in 9 volumes of 0.4 M sorbitol solution containing 1 mM ethylendiamine tetracetic acid, 0.2%