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GLUCOSE RELEASE MEASUREMENTS FROM LIPOSOMES WITH AN OXYGEN ELECTRODE

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SUMMARY

A method for measuring glucose release from liposomes by means of an oxygen electrode is described. The glucose concentration is determined from the initial rates of oxygen reduction catalysed by glucose oxidase. The reduction is directly proportional to either glucose (0.066–3.0 mM) or enzyme concentration (0.3–10 μg). The enzyme does not penetrate lipid bilayers and free glucose can be measured directly. Trapped glucose can be released and measured in the presence of detergents which do not interfere with the assay. By using appropriate calibration curves, glucose concentrations could be determined under extreme conditions of pH (3–9.5), temperature (10–51 °C), and in the presence of various salts and buffers. The application of polarographic methods for studies of the permeability of amino acids and other sugars is discussed.

INTRODUCTION

The permeability of liposomes to a non-electrolyte such as glucose, as well as the effect of various substances on the release of this compound from the liposomes might be used to gain a better understanding of the physico-chemical properties of the liposomes. Two main methods have been used to measure glucose release from liposomes: The release of the confined radioactive glucose[1] or enzymatic determination of glucose utilizing auxiliary enzyme system consisting of hexokinase, glucose-6-phosphate dehydrogenase, ATP, $MgCl_2$ and NADP[2,3]. In the present work, glucose release from liposomes is measured directly in a polarograph, in the presence of glucose oxidase.

THEORETICAL BACKGROUND

Glucose oxidase catalyses the oxidation of glucose to δ-gluconolactone and the initial reaction rate, measured by oxygen consumption ($-dO_2/dt$), is related directly to glucose concentration. This reaction is in two steps of which the oxidation of glucose to δ-gluconolactone (the first step) is rate limiting[4]. The K_m for glucose is very high (about 0.12 M) relative to that of oxygen which is much lower (0.21–0.83 mM depending on the temperature)[4]. For these reasons, the reaction rate at constant temperature follows first order kinetics over a wide range of glucose concentrations.

This enables the determination of glucose concentration from a first order equation, $y = ax + b$, where y is $-dO_2/dt$, a is the slope, x is the glucose concentration and b is the intercept.

MATERIALS AND METHODS

Materials

The solutions of D-glucose (BDH, analytical grade) were prepared 24 h before use to achieve complete mutarotation. Glucose oxidase from *Aspergillus niger* was purchased from Sigma (type V 1090 units/ml, 5 mg protein/ml). The preparation as supplied contained about 100 units of catalase per ml, according to the manufacturer. Dicetyl phosphate was purchased from Sigma. Egg lecithin was prepared according to Rhodes and Lea[7], and its phosphorus concentrations determined according to the method of Bartlett[8]. Triton X-100 was purchased from BDH. Dodecyldimethylamine oxide was a gift from Dr M. Shinizky of the Weizmann Institute. [6-^{3}H]glucose was purchased from the Radiochemical Centre, Amersham, Bucks, Great Britain. All other reagents were of analytical grade.

Experimental procedure

The polarographic assay was performed using a 2 μA oxygen electrode (Yellow Spring Instrument Company Model 4004). This was connected to a polarograph (built by the electronics laboratory of the Hebrew University-Hadassah Medical School, Jerusalem, by Engineer D. Levi). The electrode was covered by a 0.001 inch teflon membrane and was immersed in a 1.8 ml glass cell. A polarizing voltage of 0.7 V was applied across the electrodes. The cell was equipped with a magnetic stirrer and the temperature controlled by immersion in a water bath connected to a controlled temperature circulator. A routine reaction was carried out at 37 °C; temperature equilibration was achieved before the reaction was started. The final volume of the reaction mixture was 1.5 ml (constituents indicated in the legends of the figures). The reaction was started by the addition of 0.3–20 μg glucose oxidase. The time course rate of oxygen consumption was monitored graphically using a Servogor potentiometric recorder model RE 512.

The response time of the oxygen electrode was related to the glucose concentration. Thus the response time varied from 6 to 25 s for glucose concentrations ranging from 2.5–0.25 μmoles. A reduction in response time may be possible with thinner membranes through which the rate of oxygen diffusion would be faster.

The concentration of glucose is determined from the initial rate of oxygen uptake ($-dO_2/dt$). The units are described by the slope (cm/min) calculated for a recorder sensitivity of 20 mV and a chart speed of 10 mm/min.

Preparation of liposomes. Multibilayered liposomes were prepared from a mixture of egg lecithin and dicetyl phosphate in a molar ratio of 10 to 1 according to Bangham *et al.*[9] in either 50 mM KCl or 0.3 M glucose. Untrapped glucose was removed by dialysis against 4–5 changes of 100 vol. iso-osmotic buffer (usually 0.15 M potassium phosphate, pH 6.5). This was reduced to less than 0.05% of the original concentration after 2–3 h. In some experiments liposomes were prepared in 0.3 M [6-^{3}H]glucose (1 Ci/mole).

Determination of radioactivity. Radioactivity of liposome dispersion (or, alter-

natively, the millipore filters containing liposomes) were determined by scintillation spectroscopy in a mixture consisting of 2 ml ethanol–Triton X-100 (2:1, v/v) and 10 ml of toluene containing 50 mg 2,5-diphenyl oxazole and 1.30 mg 1,4-bis-(2-4 methyl-5-phenyloxaxolyl) benzene.

RESULTS

(1) Calibration curve. Fig. 1 shows that the initial reaction rate was related

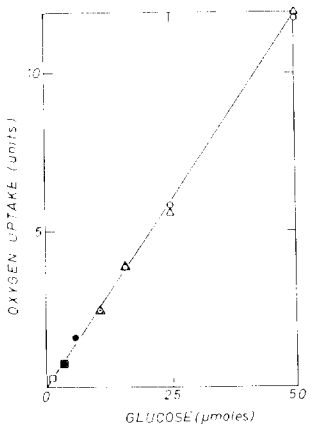

Fig. 1. Effect of glucose concentration on the initial rate of oxygen reduction. The measurements were done as described under Methods using 0.05 M of sodium acetate buffer, pH 5.6, and 10 μg of enzyme (2.2 units of activity). ▲—▲, sensitivity 50 mv, chart speed 10 cm/min; ●—●, sensitivity 20 mv, chart speed 10 cm/min; ○—○, sensitivity 20 mv, chart speed 40 mm/min; □—□, sensitivity 5 mv, chart speed 10 mm/min. The initial rate of $-dO_2/dt$ is described by the slope (cm/min), calculated for a recorder sensitivity of 20 mv and a chart speed of 10 cm/min.

directly to glucose concentration in the range of 0.1–5.0 μmoles glucose. Using a linear regression analysis, the relationship between $-dO_2/dt$ and glucose concentration may be described by the following equation:

$$-dO_2/dt = (2.22 \pm 0.035)\ [Glc] + (0.242 \pm 0.091)$$

with a correlation coefficient of 0.998.

(2) Reaction rates were directly proportional to enzyme concentration in the range 0.6-20 units of enzyme activity (according to the manufacturer).

$$-dO_2/dt = (0.91 \pm 0.045)\ [Enz] + (0.1 \pm 0.05)$$

Glucose release from liposomes was studied under various conditions of pH, salt concentration and temperature, and also in the presence of a number of substances likely to interact with the liposomes and modify their permeability to glucose. The effect of the above on glucose oxidase activity was studied.

(a) Effect of pH on glucose oxidase activity: The pH of the reaction mixture (range 3.5–7) had little effect on the initial rate of oxygen uptake (Fig. 2); however, measurable rates could be obtained over a wider spectrum of pH (3–9.5). None of the buffers used had any unusual effects on the reaction rate.

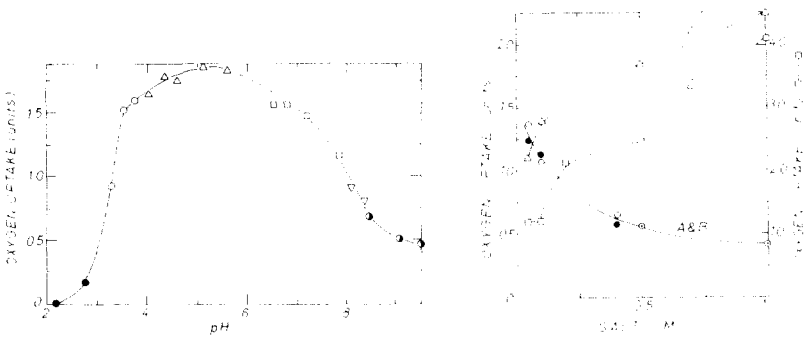

Fig. 2. The effect of pH on the initial rates of oxygen uptake. Measurements were done as described under Methods using the following buffers (each at a concentration of 0.05 M). ●—●, glycine HCl, pH 2.2–2.8; ○—○, sodium lactate, pH 3.25–3.75; ▽—▽, sodium acetate, pH 4.05–5.65; □—□, potassium phosphate, pH 6.5–7.8; ▽—▽, Tris–HCl, pH 8.05–8.13; ◐—◐, Diethanolamine, pH 8.4–9.5. Reaction mixtures contained 1 μmole glucose and 10 μg of glucose oxidase (2.2 units of activity).

Fig. 3. Effect of salts on the initial reaction rates. The measurements were carried out as described in Methods. Reaction mixtures contained 1 μmole of glucose and 10 μg of enzyme (2.2 activity units). The left ordinate describes the following: ▽—▽, increasing concentration of KCl (A); ●—●, increasing concentration of NaCl (B); □—□, increasing KCl concentration in 0.05 M potassium phosphate, pH 6.5 (C). Right ordinate describes effect of increasing concentration of sodium acetate buffer, pH 5.6. (D).

(b) Effect of salts on glucose oxidase activity: At pH 5.6 the reaction rate was markedly affected by salt concentration. There is a progressive inhibition of the reaction with increasing salt concentration (Curves A and B, Fig. 3) and the rate decreases by 75% between 0.05 and 1 M with either KCl or NaCl: no differences were apparent between either salt up to 1 M salt; the results with NaCl or KCl were almost identical. Addition of 0.05 M sodium acetate buffer, pH 5.6, abolished the inhibition. When the sodium acetate buffer was replaced by 0.05 M potassium phosphate buffer, pH 6.5, increasing either the KCl or the NaCl concentration enhanced the enzymic activity as shown in Curve C of Fig. 3.

Increasing the concentrations of potassium phosphate buffer at pH 6.5 did not markedly affect the reaction rate. However, increasing concentrations of potassium acetate buffer (pH 5.6) caused a 3.5 fold increase in activity over the concentration range, 0.05–1 M (Fig. 3, Curve D).

The complex effect of salts on the initial reaction rates of oxygen uptake ruled out the possibility that the effect was only due to changes in oxygen solubility.

(c) Effect of temperature: Fig. 4 shows that the initial reaction rates were increased 14-fold between 15 °C and 50.5 °C.

(d) Effect of substances which modify glucose permeability of liposomes: The following substances in the range of concentrations indicated had no effect on the initial rate of oxygen uptake: glycerol (0.1–10%, v/v), ethylenglycol (0.1–10%, v/v), EDTA (1–10 mM), $CaCl_2$ (1–10 mM), Triton X-100 (0.1–4 mM), dodecyldimethylamine oxide (0.1–10 mM) and venom of *Crotalus adamanteus* and *Naja naja* (0.1–1.4 mg).

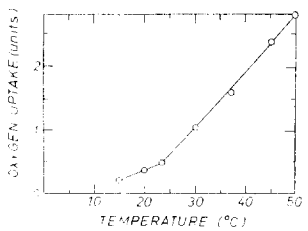

Fig. 4. Effect of temperature on the initial reaction rates. Measurements were made as described under Methods, reaction mixtures contained 1 μmole of glucose and 10 μg of enzyme (2.2 activity units).

The application of the polarographic determination of glucose for measuring glucose release from liposomes

(I) Determination of total glucose concentration. The total glucose concentration of liposome dispersions (GLc_{total}) were determined by two methods. Polarographic measurements were performed after releasing trapped glucose from the liposomes by treatment with Triton X-100 (3 μmoles per μmole lecithin) or extraction of glucose with 4 vol. chloroform–methanol (2:1 by vol.) and recovering the glucose from the upper aqueous phase[12]. Electron microscopy and light scattering of Triton X-100 treated liposomes confirmed complete disruption of the structures (refs 10, 11 and Hertz R. and Barenholz Y., unpublished). Separate experiments using [6-^{3}H]glucose confirmed the results obtained by polarographic assay of total glucose concentrations.

(II) Determination of untrapped glucose. Experiments were designed to determine if only untrapped glucose (GLc_{free}) reacted with glucose oxidase in intact dispersions of liposomes and whether or not liposomes themselves affected the assay. The latter was checked by adding either glucose containing liposomes or liposomes prepared in solutions of 50 mM KCl to reaction mixtures containing various concentrations of glucose. The results (Fig. 5) show that the measurement of untrapped glucose is not affected by the presence of liposomes.

All untrapped glucose was oxidized by preincubation of liposome dispersion 10 min in the presence of glucose oxidase. Measurement of glucose after Triton X-100

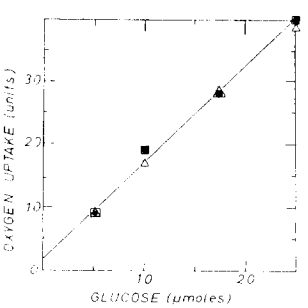

Fig. 5. Effect of liposomes on the initial rate of glucose oxidation. Measurements were made as described in Methods. Incubation mixture contained 10 μg of enzyme (2.2 activity units). ● ●, no additives; □---□, effect of liposomes prepared in 50 mM KCl (2 μmoles phospholipids); ▲ —▲, effect of liposomes prepared in 0.3 M glucose (1 μmole lecithin, 1.95 μmoles glucose per μmole lecithin).

or chloroform–methanol treatment gave results which were identical to those obtained for the amount of glucose trapped inside the liposomes ($GLe_{liposomes}$) before the preincubation (1.95 μmoles glucose/μmole lecithin). The amount of glucose trapped by liposomes varied between 1.8 and 2.5 μmoles/μmole lecithin and over a range of 0.2 to 3.0 μmoles liposome phospholipid the same value of untrapped glucose μmole lecithin was obtained. This was confirmed by measuring the radioactivity of dialysed liposomes prepared in the presence of 0.3 M, 1 Ci mole[6-^{3}H]glucose and measuring glucose polarographically after Triton X-100 treatment. Dialysed liposomes containing trapped[6-^{3}H]glucose were also separated from the dispersion medium by millipore filtration (0.05 μm) using iso-osmotic buffer. The amount of [6-^{3}H]glucose retained on the millipore filter was the same as above using the polarographic assay for glucose.

DISCUSSION

This work describes a method for measuring glucose released from liposomes. Using an oxygen electrode, the concentration of the released glucose is deduced from the initial rates of oxygen reduction using glucose oxidase from *Aspergillus niger*. The method is based on the kinetic of glucose oxidase reaction[4] in which the initial reaction rate is of first order with respect to glucose concentration.

The use of the above method for measuring both glucose released and glucose trapped in liposomes has the following advantages:

(1) Direct measurements of glucose trapped inside the liposomes and in the medium can be obtained without having to separate liposomes from the external medium. (2) Optical disturbance caused by turbidity or by the presence of chromophores has no effect on the measurement. (3) Glucose release can be measured accurately over short time periods. (4) The method detects glucose over a concentration range of at least 0.066–3 mM. (It is very likely that replacing the 2 μA electrode with a 0.25 μA electrode would increase the sensitivity.) (5) Only small amounts of the commercial enzyme are required (10 μg or less) for each assay because the specific activity of the enzyme is high. (6) The properties of the enzyme such as its stability toward heat and sulphydryl reducing reagents[4–6] enables assays to be performed under extreme conditions of pH and temperature. Using an appropriate calibration curve, measurement can be made over a pH range of 3–9.5, temperatures of 10–51°C and in the presence of various salts or buffers in very wide ranges of concentrations. (7) Many substances which may modify glucose permeability of liposomes such as glycerol, ethyleneglycol, Ca^{2+}, EDTA and snake venoms do not effect glucose determination. (8) One of the main advantages of this method is that cofactors such as Mg^{2+} or ATP, which might change the permeability of liposomes, are not needed for the assay. It is well known that ions might interact with the hydrophilic region of the liposomal membrane[9–13]. This will change parameters such as the potential which affects the permeability of liposomes. (9) The enzyme is very stable toward the anionic detergent sodium dodecylsulphate[5] and to the anionic detergents Triton X-100 and dodecyl dimethylamine oxide. The insensitivity to detergents permits measurements of the amount of glucose trapped inside the liposomes, and in addition enables study of liposome-detergent interaction[10].

It should be noted that Weissman *et al.*[14] used Glucostat for measuring glucose

released from liposomes, but this was done after separating liposomes containing trapped glucose from media containing free glucose. This work describes measurement of glucose outflux from liposomes, but it should be noted that a similar method might be used for measuring glucose influx into liposomes (Barenholz and Hertz, unpublished results). This was done by preparing the liposomes in a solution containing glucose oxidase. The external untrapped enzyme is removed by ultracentrifugation or by gel filtration; glucose is then added to the medium and its permation into the liposomes can be followed by its oxidation.

An analogous method might be used for measuring glucose release from erythocytes. While this work was in preparation, a similar method was described by Taverna and Langdon[15] for measuring glucose flux inside human erythrocyte ghosts.

Similar methods could be employed for measuring release from liposomes of substances that are oxidized by atmospheric oxygen such as galactose, D-amino acids, L-amino acids, xanthine, *etc.* using the appropriate enzymes.

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