

FLOW INJECTION DETERMINATIONS OF GLUCOSE AND ADENOSINE-5'-TRIPHOSPHATE (ATP) BASED ON A PACKED-BED GLUCOKINASE REACTOR

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ABSTRACT. A 100- μ l immobilized glucokinase (GK) packed-bed reactor was optimized for flow injection determinations of glucose and ATP in a two-channel flow injection system. ATP phosphorylated glucose in the GK reactor. A second reactor consisting of co-immobilized pyruvate kinase (PK) and L-lactate dehydrogenase (LDH) regenerated ATP from ADP in presence of phosphoenolpyruvate (PEP). The pyruvate formed was then reduced by nicotinamide adenine dinucleotide (NADH). Injection of glucose or ATP produced absorbance or current peaks, negative to the NADH base-line and proportional to their concentrations. The linear ranges were 5 - 600 μ M for glucose and 5 - 500 μ M for ATP with sample through-puts of 20 - 25 h⁻¹, at a flow rate of 0.35 ml min⁻¹. The detection limits for both species were 2 μ M with the amperometric and 3 μ M with the spectrophotometric methods. The reactors were used for five months without significant loss of activities. Storage of the reactors was made in phosphate buffer (pH 7.0) at 4°.

INTRODUCTION

Significant progress in fast and reliable assays for D(+)-glucose (glucose) has been achieved with immobilized enzyme reactors in flow systems because of its clinical and industrial importance. Enzyme reactors commonly employed for detecting glucose in such systems are based on glucose oxidase, GOD [1-5], glucose dehydrogenase, GDH [6-9], or hexokinase-glucose-6-phosphate dehydrogenase, HK-G6PDH [10 - 12]. Detections are usually made by measuring the consumption of oxygen or production of hydrogen peroxide in the case of GOD, and production of NADH in the latter two. All the three systems catalyze the reactions of β -D(+)-glucose and, during the enzymatic reaction, the α - β equilibrium shifts to the β -anomer. A variety of flow through detectors for glucose are employed in flow systems which includes spectrophotometry [1 - 3, 6, 10] and amperometry [4, 7 - 9].

Another enzyme which is not widely used in the assay of glucose is glucokinase (GK) from *Bacillus stearothermophilus*. This is an enzyme which possesses very high selectivity towards D(+)-glucose catalyzing the reactions of both the α - and the β -anomers [13]. Unlike HK, it does not promote phosphoryl transfer to hexoses such as fructose, mannose and glucoseamine [13]. GK is also much more glucose-selective than GDH since the latter catalyzes oxidations of some hexoses and pentoses [14].

Applications of GK in analysis include homogeneous enzymatic assays for glucose [13, 15] and creatine kinase [13, 16, 17] monitored by NADPH, via catalysis of G6PDH. A colorimetric assay of glucose at 570 nm was reported using the same enzymes with final

detection of a formazan [18]. Glucose sensors with GK have also been devised with electropolymerized polypyrrole on a field effect transistor, FET [19] and with physical immobilization on a pH base sensor [20]. To our best knowledge, it seems that there are no reports on the analytical applications immobilized GK reactors.

Apart from its favorable selectivity to glucose, an attractive feature of this enzyme is its thermal and storage stability. In solutions, HK loses its activity above 40° while GK is fully active at 60°, when tested for one hour [13]. The enzyme in the FET glucose sensor was reported to function even at 80°. At room temperature, its activity in buffer was retained after about a month [13].

In a recent work, we showed that a PK-LDH reactor can, favorably, be used for a flow injection determination of ADP [21] based on a final NADH-consuming reaction. From an equilibrium point of view, phosphoryl transfer from ATP to glucose is favorable (K_{eq} is 6.45×10^3 at pH 7.4) [22]. The PK-LDH system can, therefore, be coupled to the ADP-producing GK catalysis, giving way not only to glucose detection but also to ATP. The reactions are summarized in Figure 1.

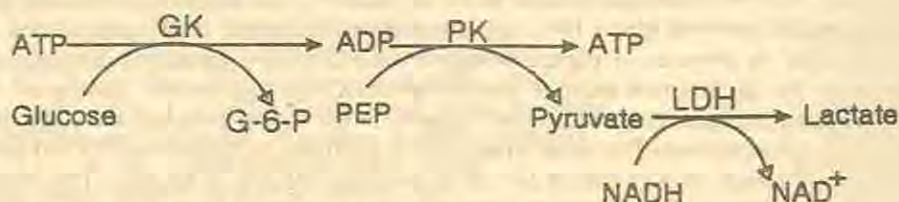


Figure 1. Reaction scheme for the determination of glucose and ATP.

Coupling an immobilized GK reactor with a G6PDH reactor should be a feasible glucose-detecting system because the reactions have favorable equilibria towards the products as shown for serially arranged HK and G6PDH reactors [10-12]. Investigation of a GK reactor in series with a PK-LDH reactor is, however, proposed because the system can be easily extended to sequential or simultaneous determinations of glucose and other ADP-producing substrates with parallel arrangement of other kinase reactors. In a recent work we applied a sequential FI system for creatinine and creatine with creatinine amidohydrolase and creatine kinase (CK)-PK-LDH reactors [23]. Multi-component FIA based on a common detection reaction, e.g., oxygen consumption, has been increasingly applied in the last decade because of easy automation of handling the systems. Reports on FI systems with common detectable products from enzymatic reactions include for samples containing as much as six biological compounds [12, 24-26].

This report presents the application of immobilized GK and PK-LDH reactors for FI detection of glucose via consumption of NADH monitored with a flow-through UV-Vis spectrophotometric cell or an amperometric cell with a phenoxazine-modified graphite electrode. The system is also useful for quantitative detection of ATP.

EXPERIMENTAL

Enzymes and reagents. The following enzymes and reagents, all from Sigma, were used as received. Glucokinase (E.C. 2.7.1.2, from *Bacillus stearothermophilus*, G-8887), pyruvate kinase (E.C. 2.7.1.40, P-9136) and L-lactate dehydrogenase (E.C. 1.1.1.27, L-1254) from

rabbit muscle, D(+)-glucose ($\alpha_D = +52.5^\circ$), ATP, ADP, PEP, NADH, sodium pyruvate, magnesium acetate, potassium acetate and imidazole. All the other reagents used were of analytical grade.

Double-distilled and degassed water was used as sample carrier as well as to prepare buffer and reagent solutions. An aqueous solution of D(+)-glucose (10 mM) was used as stock and prepared every week. Reagent solutions for glucose determinations were prepared each day with the following composition: ATP (1.25 mM), PEP (1.0 - 1.5 mM), NADH (0.30 mM), Mg^{2+} (10 mM), and K^+ (15 mM), all in 100 mM imidazole-acetate buffer at pH 7.5. The effects of reagents and pH were studied by variation of each factor while maintaining the others constant as specified here. 3.0 mM glucose replaced ATP in the reagent when the latter was the analyte.

Preparation of enzyme reactors. Controlled porosity glass, CPG, (CPG-10, pore size 500 Å, particle size 120 - 200 μm , Serva) was initially silanized with aminopropyl(triethoxy)silane in water-free toluene and activated with glutaraldehyde according to the recommended procedure [27]. Before application, glutaraldehyde was pre-treated with activated charcoal and filtered to remove possible polymeric products. 0.8 mg of GK (200 U) in 2.5 ml of 0.1 M phosphate buffer (pH 7) was added to 80 mg of the activated CPG and the enzyme was allowed to react with the derivatized glass surface at reduced pressure and room temperature (30 min) and at 4° overnight. The immobilization yield was 82% as estimated from the absorbances of the enzyme solution at 280 nm before and after the immobilization. A 100- μl Plexiglass tube (i.d. 2 mm) was finally packed with about 45 mg of the enzyme-bound glass.

To prepare a 50- μl packed-bed PK-LDH reactor (i.d. 2 mm), 2.6 mg of PK (1000 U) and 2.0 mg of LDH (1740 U) in 2.5 ml of the same buffer were also immobilized on 120 mg of the activated CPG with the same procedure (immobilization yield 52%).

The flow injection system. The two-channel FI assembly consisted of a Gilson Minipuls 2 peristaltic pump which propelled the carrier and the reagents. During the flow, the two streams were mixed in a knitted Teflon tubing (i.d. 0.5 mm) before reaching the first (GK) reactor. Unless specified the total flow rate after the mixing was 0.35 ml min⁻¹. Other connections in the system were made with Teflon tubings of i.d. 0.5 mm. Sample injection to the carrier was made with a 50- μl loop by a pneumatically controlled injection valve (Cheminert, type SVA). The consumption of NADH in the reactions was monitored with an LKB 2151 flow through spectrophotometer (at 340 nm) or with an amperometric detector (Zäta LC 4, Höör, Sweden).

The wall-jet amperometric cell, described earlier [28], consisted of a chemically modified graphite working electrode, a saturated calomel electrode (Radiometer K 701) and a built-in platinum counter electrode. One end of approximately 6 cm graphite rod (Ringsdorff-Werke, GMBH, type RWO, diameter 3.1 mm) was modified by adsorption applying a few drops of 1,4-bis(5-carboxylamino-9-diethylamino-benzo[a]phenoxazin-7-ium)benzene (Mirak's Blue) in acetone. The potential of the chemically modified electrode was held at 50 mV vs SCE. Before modification of the graphite surface, cleaning and heat-treatment were made according to a recommended procedure [29]. The phenoxazine mediator was first synthesized and used to construct a glucose biosensor for FI application [9]. It strongly adsorbs on graphite surface and promotes the electrocatalytic oxidation of NADH. The mechanism of reaction is tentatively assumed to be the same as those of other phenoxazines presented elsewhere (e.g. Meldola's Blue [30]). A chemical oxidation step of NADH takes place at the modified electrode surface followed by fast electrochemical regeneration of the mediator.

The base-line from the flow-through detector is the steady state absorbance or the electrocatalytic oxidation current of NADH in the reagent. The detector and the recorder were adjusted so that the steady state response to NADH was suppressed to zero base-line. The polarity of the recorder was reversed and the peaks from the NADH base-line due to glucose (or ATP) injections were recorded positive.

RESULTS AND DISCUSSIONS

pH Dependence. Plots of flow injection absorbance peak against pH, for different glucose concentrations, is shown in Figure 2. It represents the combined effect of pH on the two reactors. The curves in the figure, with an optimum range pH 7.5 - 8.0 shows that the system is practically insensitive to changes in pH within this range. The optimum pH ranges of the enzymes in homogenous systems are 9 for GK [31], 7.4 - 8.4 for PK [32] and pH 7.4 - 7.9 for LDH (in the direction of L-lactate formation) [33]. The reported measurements were made spectrophotometrically in different buffers. The results, therefore, indicate that the overall shift in the optimum pH is not influenced by the co-immobilization of the two enzymes.

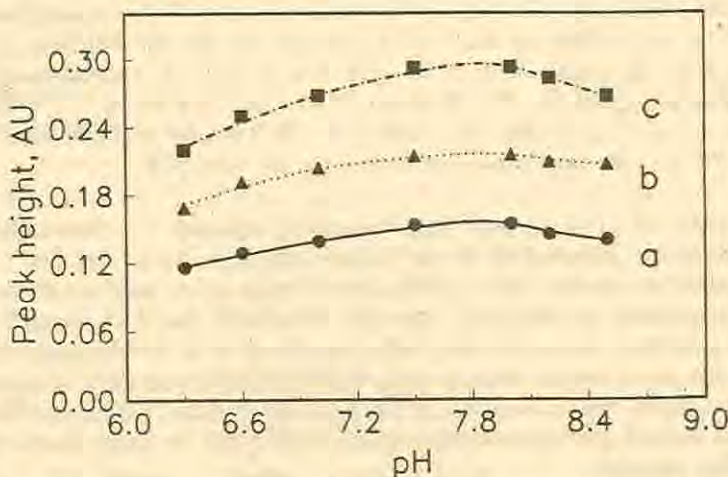


Figure 2. The effect of pH on the FI peak height of (a) 200, (b) 300 and (c) 400 μM glucose using spectrophotometric detection. Reagents and flow rate as described in the Experimental.

The effect of pH on the amperometric glucose signals was also examined. The modified electrode response showed an increase with pH to a maximum at pH 7.5, followed by a very gradual decline up to pH 9.1 (Figure 3). The response variation with pH should not only be dependent on the outputs from the reactors but also on the phenoxazine electrocatalysis at the electrode surface. The current response of the modified electrode to NADH decreases from pH 5 upwards [9]. The shape of the current-pH curve will therefore be the combined effects of pH on the conversion efficiency of the enzyme reactions in the two reactors and the electrocatalysis at the modified electrode.

Reagents and activators. To optimize the system, the effects of the reagent concentrations on glucose peaks were examined spectrophotometrically. 0.3 mM NADH was used in the reagent which was sufficient enough to obtain linear responses up to 600 μM glucose. The steady state response of the two-channel system to 0.3 mM NADH was about 0.8 absorbance units (AU). Responses to glucose above 600 μM were not linear even after increasing the NADH concentration. Linear responses to 1000 μM ADP and 1200 μM pyruvate were, however, obtained after raising NADH to 0.5 mM. The observed and non-linear response beyond the upper limit for glucose at this condition should be due to limited activity of the enzyme in the first reactor.

Variation of PEP up to 0.5 mM showed increasing peaks when tested with 300 and 400 μM glucose which then became constant up to the studied limit, 2.0 mM. 1.0 - 1.5 mM of the reagent was used to produce stoichiometric excesses for high analyte concentrations.

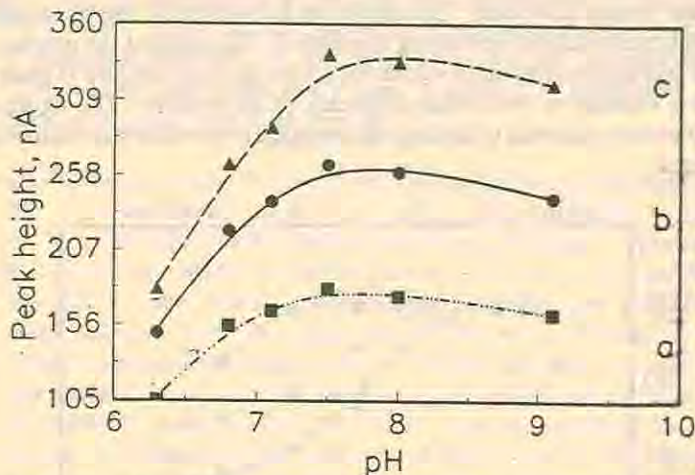


Figure 3. The effect of pH on the FI peak heights of (a) 200, (b) 300 and (c) 400 μM glucose with the amperometric method.

ATP was varied between 0.1 - 2.25 mM. The result showed a constant response within 1.0 - 1.5 mM ATP, with declining responses below and above this range. Equilibrium was not attained at the flow rate employed (0.45 ml min^{-1}) and the response to glucose injections when ATP was between the optimum range should correspond to maximum activity. The declining response when ATP was beyond 1.5 mM is expected to be due to ADP inhibition on GK [34] or ATP inhibition on PK [35], or both. Similar inhibitory effect of ATP was observed earlier in studying a CK-PK-LDH reactor [23]. During phosphoryl transfer from ATP to glucose, there is no product inhibition from G-6-P at low concentrations [34], implying an additional advantage of GK over the use of HK.

Potassium (K^+) activates PK [36] while magnesium (Mg^{2+}) activates both PK [36] and GK [34]. Maximal peak responses to glucose injections were obtained when the reagent contained 15 - 45 mM potassium and 6 - 10 mM magnesium acetates.

Flow rate effects. Since flow rate is inversely related to the residence time of the substrates and products within the enzyme reactor [37], decreasing the flow rate will increase the conversion efficiency of the reactor. Hence, there is a minimum flow rate required for equilibrium to set-in, depending on the activity of the enzyme in a reactor. The effect of flow rate in this system was studied by injection of $300 \mu\text{M}$ glucose and comparisons were made with the same concentrations of NADH, pyruvate and ADP, but for the latter the reagent contained no ATP. Both spectrophotometry and amperometry were employed in the study. The peak heights of injected NADH and substrates, though of opposite polarity, were directly compared.

The FI absorbance peaks against flow rate, were plotted for glucose, ADP, pyruvate and NADH (not shown). The NADH curve showed a more gradual decrease with flow rate due to increasing dispersion in the system. The peak for NADH decreased by about 15% from 0.18 to 0.72 ml min^{-1} . The glucose, ADP and pyruvate curves departed from the NADH curve at different points, with glucose departing earliest (lowest flow rate). Dispersion in the reactors depends on the molecular weights of the substrates but the differences are assumed negligible for the present discussion. The decline of the curves for the substrates from that of NADH are thus attributable to decreasing conversion efficiencies of the reactors. The same data are used to plot relative responses against flow rate for each substrate (Figure 4) and this can be used as a measure of conversion efficiency of the reactors. The figure shows that conversion of glucose to products is limiting and complete conversion was attained only at less than 0.35 ml min^{-1} .

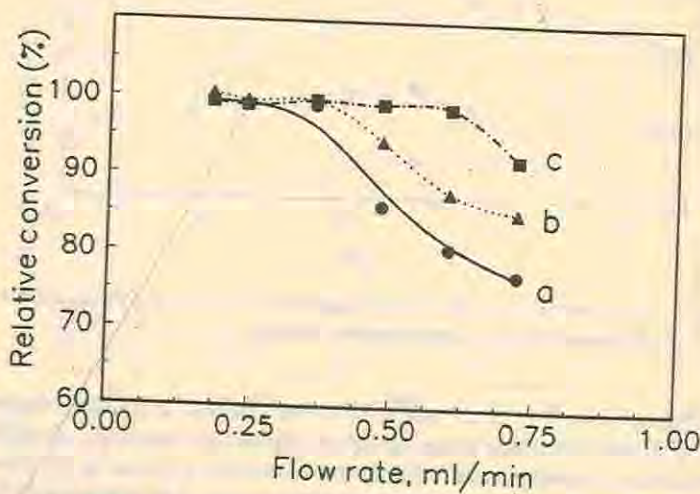


Figure 4. The relative conversion of $300 \mu\text{M}$ each of (a) glucose, (b) ADP and (c) pyruvate against flow rate, using spectrophotometric detection (340 nm).

The relationship between the peak current and the flow rate was also examined with injections of $300 \mu\text{M}$ glucose, pyruvate and ADP and NADH. The NADH curve increased with flow rate as expected since mass transport is enhanced due to formation of thinner diffusion layer at the wall-jet electrode surface. The pyruvate ADP and glucose curves depart from that of NADH at different points. Since the final detected species is NADH in all cases, mass transport effect at the electrode surface is the same for all at a given flow rate.

Consequently, the points of departure of the pyruvate, ADP and the glucose curves, from that of NADH, correspond to the flow rates at which equilibria did not set-in, assuming again the species would experience the same dispersion in the reactors. On the basis of these results, the conversion of glucose by the GK reactor is the most limiting followed by PK, as also reflected in the spectrophotometric study. Immobilization of more of these two enzymes on CPG should help attain more efficient conversions at higher flow rates.

Stability of reactors. Both the GK and PK-LDH reactors were used almost every day for the first two months and every week for the next three months. Between applications, the reactors were stored in 0.1 M phosphate buffer (pH 7) at 4°. The response to glucose, about 0.8 AU mM⁻¹ at 0.45 ml min⁻¹, decreased by about 10% during the first ten days which is very likely due to leaching of adsorbed GK from the glass support. In the rest of the five months, the system response to glucose had a sensitivity of 0.70 - 0.73 AU mM⁻¹ at the same flow rate. The result confirms high operational and storage stability of the thermostable enzyme.

The sensitivity of the flow system to 300 μM ADP and pyruvate for the PK/LDH reactor was about 1.0 AU mM⁻¹ at less than 0.45 ml min⁻¹. The enzyme loading in the second reactor is high (52% immobilization) and any activity decay of the two enzymes may have passed unnoticed. Tests at high flow rates would have revealed this. Previously, we recorded a stability of eight months for a PK-LDH reactor [21].

Calibrations with glucose and ATP. The FI system was calibrated with standard glucose and ATP using the prescribed reagents at pH 7.5. Typical calibration curves for glucose with the amperometric and spectrophotometric methods are shown in Figure 5. The sensitivities of the two methods for ATP are similar to that of glucose, i.e. 1.0 nA/μM and 0.82 mAU/mM ATP. Both species have same detection limit, 2 μM for the spectrophotometric and 3 μM for the amperometric methods. The linear ranges are 5 - 600 μM for glucose and 5 - 500 μM for ATP. The relative standard deviation is 1.0 - 1.7% for 200 glucose or ATP. The results reflect that the system can be favorably used for the determination of these two species in samples. The sample through-puts (20 - 25 h⁻¹) are, however, relatively low compared with other glucose and ATP detecting FI systems. The efficiency of the GK reactor, and thereby the through-put, can be increased by using more enzyme in subsequent immobilizations.

For assays of glucose in real samples, the system is expected to be interference-free from other hexoses [13], unlike HK-based systems [38]. In GDH-based methods, glucose determinations suffer interferences that arise from the reactions of xylose, mannose and cellobiose [14]. The high selectivity of the system characterized by GK should thus be attractive to monitor glucose in complex samples such as fermentation products. With the same viewpoint, serum glucose may be assayed with the proposed method to diagnose diabetic patients. Glucose in serum is 3.6 - 6.1 mM in normal humans [39] which, for the present system, entails dilution of samples by a factor of more than 10. The upper limit of the calibration curve can, of course, be increased by decreasing the size of the sample loop, e.g. 10 μl.

It is evident that the presence of pyruvate would interfere with the glucose-detecting system. The effect is additive to glucose signals at low flow rates and the pyruvate contribution could be subtracted from the total response (glucose + pyruvate) by measuring the pyruvate response with the reagent stream containing NADH alone. The pyruvate error should not, however, be serious in measuring serum glucose since its level is normally 0 - 110 μM [40]. This could even be considerably lowered during dilution, even below its detection limit. The detection limits of pyruvate and glucose are about the same in this system.

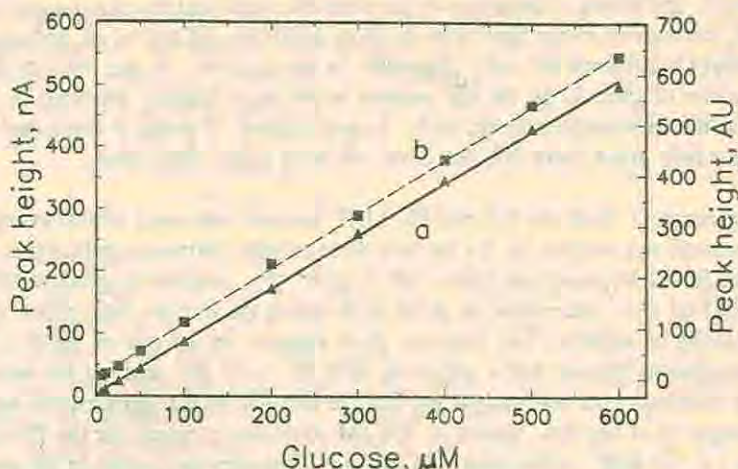


Figure 5. Calibration curves for glucose with the (a) spectrophotometric and (b) amperometric methods.

A number of FI methods for ATP are available. This includes systems with HK and G6PDH in one or separate reactors. The usual detection limit for the nucleotide is in the order of a few μM with FI-spectrophotometry. The ATP-detecting system in our work may, therefore, be alternative to the existing FI methods.

CONCLUSIONS

The results of the flow injection experiments show that a GK reactor could be applied to glucose determinations. In immobilized form, the analytical applications of this thermostable enzyme is quite attractive because of its stability in the stated storage and working conditions. The specificity of the enzyme could be employed to discriminately detect glucose in samples containing other monosaccharides. In parallel combination of the glucose-converting reactor with other ADP-producing reactors, a multi-analyte determination system for a sample can be set up e.g. creatinine and creatine. The system can also be applied to assay ATP. The low sample through-put of the method with the equilibrium mode, can be easily improved by increasing the loading of GK and PK during subsequent immobilizations.

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