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DETERMINATION OF THERMODYNAMIC DATA BY MICROCALORIMETRY: THE MICHAELIS CONSTANT OF GLUCOSE OXIDASE IMMOBILIZED ON VARIOUS CARRIERS

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ABSTRACT

Microcalorimetry and polarography were chosen for the investigation of dissolved and immobilized enzymes. Glucose oxidase and catalase were used bound to polyacrylamide or were immobilized by attaching them to nylon fibers which had been treated with triethyloxonium-tetrafluoroborate, diaminohexane and glutaraldehyde according to MORRIS, CAMPELL and HORNBY (1975). The latter method assured that the enzymes are located in a side chain to the polyamide structure. If, and to what degree, this immobilization could have changed the properties of the enzymes was tested by determining the MICHAELIS constant of dissolved and immobilized enzyme. Polarography, in the case of the dissolved enzymes, microcalorimetry and polarography in the case of the immobilized enzymes, led to the same K_m-value. Replacement of air by oxygen increased the final heat output rate, but had no influence on the K_m-value. Irradiation of the immobilized enzyme decreased the heat output rate, the K_m-value remained unchanged. These results prove that once a glucose oxidase molecule is damaged it looses all activity rather than remaining partially active.

I. INTRODUCTION

In a previous paper (SCHACHINGER et al., 1985) it was shown by polarographic measurements, that immobilization of glucose oxidase (GOD) on an "activated" nylon carrier did not alter its affinity for its substrate, expressed by the MICHAELIS constant. A corresponding result was reached by Rekharsky et al. (1980), who compared the reaction enthalpy of formate dehydrogenase in soluble form and covalently bound to an "aminated, porous support" by microcalorimetry. The reaction enthalpy and its dependence on the glucose-concentration were exactly the same for both forms of the enzyme. SCHMIDT, KRISAM and GRENNER (1976) carried out quantitative investigations on the reaction enthalpy of glu-

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cose oxidation by various preparations of glucose oxidase (see discussion). These authors also determined the catalase-content of their preparations and found, that none of them was free of it.

We were interested in the effect of irradiation on the MICHAELIS constant and the reaction enthalpy of the glucose oxidation:

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconate} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{Catalase}} 1/2\ O_2 + H_2O$$

and we considered microcalorimetry as another very usefull tool for its investigation.

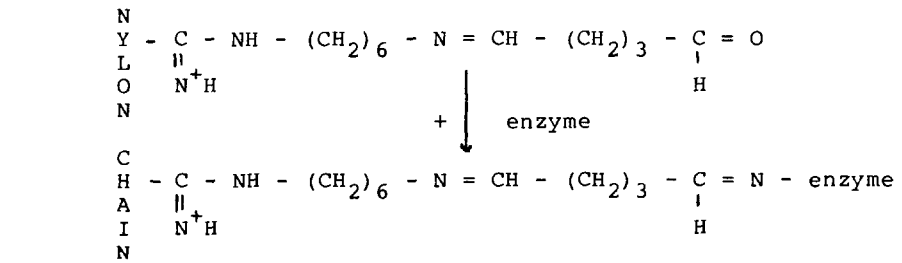
II. EXPERIMENTAL

II.1. MATERIAL

a) Soluble enzymes: The glucose oxidase (purity I, M=186000) and catalase (M=250000) were purchased from Boehringer, Mannheim.

b) Immobilized enzymes: GOD-Enzygel$^{(R)}$ was also purchased from Boehringer, Mannheim. The preparation used for most of the experiments took the series Nr. 1425-305.

c) Immobilization of enzymes on nylon: Because the absorption vessel was broken several times by the expansion of the gel due to swelling, we changed to another kind of immobilization, to the fixation of the enzymes on a nylon carrier. We used the method of MORRIS, CAMPELL and HORNBY (1975), leading to the formation of an "activated" form of nylon. In contrast to the above authors, we used a multifilament woven nylon fabric in the following experiments (referred to in the text as "nylon tissue").



The formula of this "immobilized enzyme" shows that it can be attached covalently to a solid support (for definition, see KLIBANOV, 1983). Since the reactive group of the "activated" nylon is the aldehyde one, lysine should be the reactive amino acid for binding to the protein part of the enzyme (LANG, 1971).

II.2. METHODS OF INVESTIGATION:

II.2.1 Microcalorimetry:

The microcalorimetry of glucose oxidase and catalase immobilized as "Enzygel(R)" or on nylon was carried out as described by JOHANSSEN et al. (1973) and by SCHMIDT, KRISAM, and GRENNER (1976)*. A LKB microcalorimeter was equipped with a sorption-flow cell and connected to a LINSEIS recorder via an intermediate amplifier (KEITHLY Microvolt Ammeter). An adaptor to integrate the instantaneous signal allowed the simultaneous recording of heat flux and total heat evolved. The flow rate of the buffer was 4 ml per hour (Figure 1).

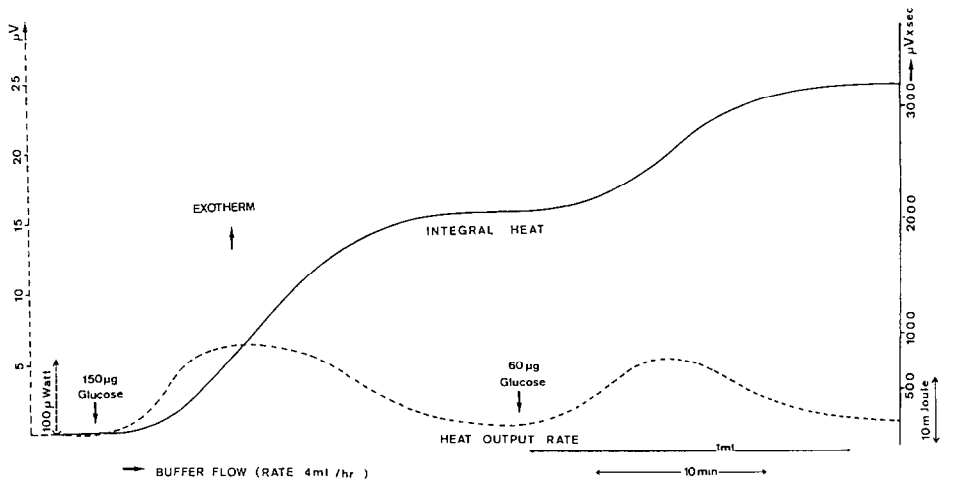


Fig. 1. Heat production during the oxidation of glucose by immobilized glucose oxidase.

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For the addition of the glucose two different methods were used:

Method A: 30 µl glucose solutions of various concentrations were introduced into the buffer stream by means of a CHROMATRONIX microvalve. The calculation of the maximal glucose concentrations in the reaction cell was based on the dilution of the 30 µl added to the volume of the sorption cell, 0.5 ml less the volume of the immobilized enzyme. The latter ranged between 25 and 40 mg. Thus, generally a dilution factor of 30:470 = 1:15.7 was assumed. This method was advantageous in calculating the reaction enthalpy.

Method B: The other method exposed the immobilized enzyme to a continuous stream of the substrate containing buffer flowing through the reaction cell, which leads to a steady state of heat production. This method was prefered for the determination of the MICHAELIS constant because the actual concentrations are better defined. On the other hand, for the calculation of the amount of glucose available per unit time the flow rate has to be taken into account, which sometimes showed slight deviations.

The results given in Joule min^{-1} or mJoule μMol^{-1} were corrected with the factor 1.13 for reduced heat transfer from the inside of the absorption cell compared to the built-in heater used for calibration (SCHACHINGER et al., 1983).

II.2.2 Irradiation:

Irradiation was carried out with a Philips X-ray apparatus, Mikro 1130, equipped with a X-ray tube, Type 2184/00. The absorption cell was removed from the calorimeter for irradiation and placed into a cylindric vessel of polyethylene of 1.4 cm diameter and about 1 cm higher than the absorption cell. The vessel was filled with the same buffer as used for the experiments. By that procedure care has been taken to avoid that the Enzygel could get dry and change its structure, combined with a possible change in affinity. In order to improve the uniformity of irradiation the plastic vessel was located in constant rotation during irradiation by means of a synchron motor at 60 cycles per min.

The dose-rate was determined by ferrous sulfate dosimetry as 27.3 Gy min^{-1}.

III. RESULTS

III.1 Effect of immobilization on the MICHAELIS constant

Figure 2 shows on the left side the dependence of the heat flux on the glucose concentration for two differently immobilized enzymes: GOD-Enzygel(R) and GOD on "activated" nylon. The right side demonstrates the corresponding LINEWEAVER-BURK plot. Both curves meet the X-axis at the same point leading to a k_M value of 2×10^{-3} M. This is the same value as found for soluble enzymes, GOD alone and GOD + catalase and for the nylon-bound enzymes by polarography (SCHACHINGER et al., 1985).

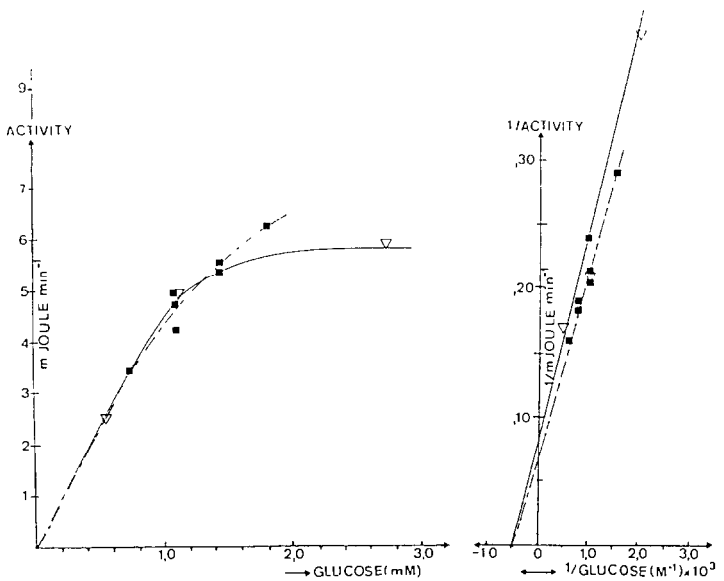

Fig. 2. Effect of immobilization on the MICHAELIS constant of GOD
■ GOD immobilized on Enzygel(R)
▽ GOD + catalase immobilized on "activated" nylon

Replacement of air (concentration of dissolved oxygen 265 µM) in the buffer by saturation with O_2 (concentration of dissolved oxygen 1.36 mM; D'ANS et LAX, 1943; HODGMAN et al., 1962) increased the final activity reached at higher glucose concentrations, but did not alter the activity at low concentrations, nor the value of the MICHAELIS constant.

III.2 Effect of irradiation on the MICHAELIS constant

Figure 3 shows the effect of irradiation on the dependence of the activity of another sample of immobilized enzyme Enzygel(R) on the glucose concentration. While the activity decreases at the same rate as reported for the effect of irradiation on soluble enzymes (SCHACHINGER et al., 1985) the MICHAELIS constant remains unchanged, as can be seen from the right side of Figure 3.

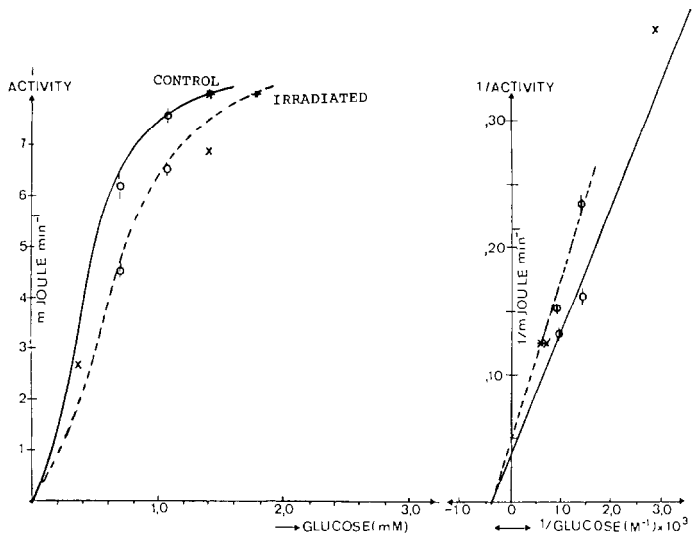

Fig. 3. Effect of irradiation on the activity of immobilized enzymes, Enzygel(R) [Dose rate 27.3 Gy min^{-1}, dose 137 Gy].
x single experiments;
ϕ mean values (irradiated: 5 exp. each; control: 3 exp. each)

III.3 Reaction enthalpy and the effect of irradiation thereupon

Figure 4 illustrates the reaction enthalpy for the oxidation of glucose by immobilized GOD. With the two methods, (A) and (B) (see II.2.1) a different dependence was found on glucose concentrations: In the case of method (B) the concentration, to which the single measured points are related, are the actual ones for several minutes, in the case of method (A) the concentrations refer only to those at the maximum of the heat output curves and not of the total area (see Figure 1). Both curves showed the same interaction with the ordinate at 250 mJoule/µMol.

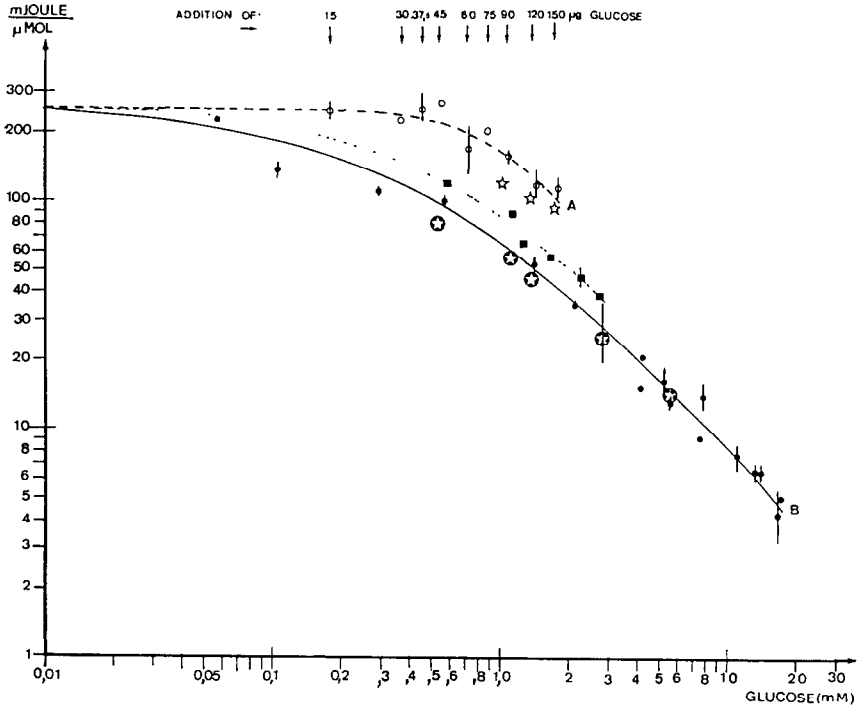

Fig. 4. Enthalpy of glucose oxidation determined with immobilized GOD and GOD + catalase.

○ GOD Enzygel(R) control Method A (see II.2.1)
☆ GOD Enzygel(R) irradiated Method A
Dose-rate 27.3 Gy min^{-1}, dose 137 Gy.

● GOD Enzygel(R) control Method B (see II.2.1)
✪ GOD Enzygel(R) irradiated Method B
Dose-rate 27.3 Gy min^{-1}, dose 430 Gy.

■ GOD + Catalase, immobilized on "activated" nylon, Method B.

Only at very low glucose concentrationst the measurement of the reaction enthalpy is exact. Above 10^{-2} M the value declines, due to the limited availability of oxygen and to the insufficient activity of the enzymes.

We also show measurements using method B with GOD-Enzygel(R) and GOD + Catalase, bound to nylon tissue by us. The small difference between these two immobilized enzyme-preparations allows us to assume that GOD-Enzygel(R) contains a re-

markable amount of catalase, which was also observed by SCHMIDT et al. (1976).

Finally, some values of the reaction enthalpy of glucose oxidation by irradiated Enzygel(R) are shown in Figure 4. They where positioned on the same curves as the unirradiated samples.

IV. DISCUSSION

Microcalorimetry is an excellent tool for the determination of thermodynamic data from kinetic studies. The fact that affinity constants of enzymes are not changed by immobilization regardless of the carrier encourages the use of matrix-bound enzymes as models for membrane- and structure-bound enzymes in the living organism.

SCHMIDT et al. (1976) determined the reaction enthalpy of glucose oxidation by glucose oxidase + catalase to be 49.5 Kcal/mol, corresponding 207 mJoule/μMol. Taking into account the correction (f=1.13) for lowered heat transfer through the glass wall (SCHACHINGER et al., 1983) one would expect a value of 238 mJoule/μMol for a glucose addition of 50 μg, which is in good agreement with our results at 0.59 mM final concentration.

Subtraction of the reaction enthalpy of the decomposition of H_2O_2 by catalase - 30.5 Kcal/mol - (WEAST, see SCHMIDT et al., 1976), corresponding to 127.5 mJoule/ μMol, leads to a reaction enthalpy for the gluconate formation of 122 mJoule/ μMol.

Irradiation of the GOD-Enzygel(R) did not change the k_M-constant of the enzyme and affected the molar enthalpy very little despite the fact that the activity of GOD + catalase was decreased to 85% by 137 Gy and to approximately 45% (extrapolated) by 430 Gy. These results prove that once a glucose oxidase molecule is damaged it looses all activity rather than remaining partially active. The k_M value of certain other enzymes (e.g. glutamate dehydrogenase) is reported to be influenced by irradiation. The mechanisms underlying these differences in the effect of irradiation will be discussed elsewhere.

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