A Quick and Simple Bioassay System for Effectors of Cell Metabolism Using Biospecifically Immobilized Cells: Assay of Thiamine and Amphotericin Using Yeast Cells *

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Bioassays have usually been performed in plate cultures of microbial cells.[1] Such assays are very specific and often very sensitive.[2] But a drawback of these bioassays is that they are substantially slower than modern chromatographic and immunologic techniques. If they could be speeded up, the inherent characteristics of the bioassay systems would make bioassays an attractive alternative when analysing many biomolecules.

In a recent paper[3] we described that reversible biospecific immobilization can be used in assays of the number of viable cells in a sample. The sample is passed over a bed of biospecific sorbent and the number of cells captured is estimated from their metabolic activity when treated under standardized conditions with a defined substrate. This paper reports the use of affinity immobilization of cells prior to bioanalysis of effectors of cell metabolism, a positive – thiamine, and a negative – amphotericin-B.

Materials and Methods. Chemicals used: Amphotericin-B was a generous gift from Squibb and Sons, London, UK., Thiamine, thiamine pyrophosphate and acid phosphatase (from potato E.C. 3.1.3.2. grade II) were purchased from Sigma Chemical Co., St. Louis, Mo, USA, neutral red and bromophenol blue from E. Merck, Darmstadt, Concanavalin A Sepharose (Con A Sepharose) from Pharmacia Fine Chemicals AB, Uppsala, Sweden, and thiamine assay medium was obtained from Difco, Detroit, Mi, USA. All other chemicals used were of analytical grade.

Yeast cells were obtained from a local source.

Cultivation of yeast cells (Saccharomyces cerevisiae) for thiamine assay: One hundred ml of sterile thiamine assay medium (8.5 % w/v) were inoculated with 2 mg of a lyophilized yeast preparation and then incubated on a shaker at 37 °C for 36 hrs. The cells were collected by centrifugation and washed three times with water giving 1 g of yeast cells (wet paste).

Immobilization procedure. The cells were biospecifically bound to Con A Sepharose according to a standardized procedure. Aliquots of Con A Sepharose (0.5 ml sedimented gel) were filled into disposable plastic syringes equipped with a nylon net (25μ mesh) at its outlet.

The cells were suspended in 0.1 M acetate buffer containing 137 mM NaCl, 0.95 mM $CaCl_2$, 5.35 mM KCl, 0.80 mM $MgSO_4$, 1.0 mM $MnCl_2$, pH 4.5, and a fixed volume of the buffer was sucked into each of the syringes. As earlier, capture is very fast[3], but for practical reasons a defined incubation time of 20 min was used. The sorbent was washed by filling and emptying each syringe 4 times with the above described acetate buffer prior to use in the bioassays.

Exposure to thiamine – containing solutions. Standard solutions of thiamine were prepared in the above described acetate buffer. To improve the uptake of thiamine by the cells glucose (to 1 mM) was added to the medium. The same buffer was used when real samples were analyzed.

Exposure to amphotericin-B. The fungicide, which is poorly soluble in water, was first dissolved in *N,N*-dimethylformamide (to 6.66 mg/ml) and then added to the above acetate buffer. Final concentration of *N,N*-dimethylformamide was maximally 0.5 %. After exposure to amphotericin the metabolic activity of the immobilized cells was measured.

Assay of metabolic activity. After having been exposed to the substance to be quantified the immobilized cells were carefully washed with the buffer making up the assay medium, except for glucose and the indicator, before incubation in a glucose-rich buffer containing an indicator, either a redox or a pH-indicator.

The buffer containing the redox indicator consisted of: 50 mM glucose in the above described acetate buffer. pH was set at 4.5. 100 ml of this buffer was mixed with 1.0 ml of a solution obtained by dissolving 0.1 g of brom phenol blue in 14.9 ml 10 mM NaOH, which then was diluted to 265 ml.

The other assay solution was based on the pH-indicator neutral red. It consisted of a buffer containing 0.004 mM KH_2PO_4, 0.041 mM $NaHCO_3$, 0.055 mM Tris, 0.95 mM $CaCl_2$, 5.35 mM KCl, 137 mM NaCl, 0.80 mM $MgSO_4$ and 50 mM glucose, pH 7.4. To 100 ml of this glucose-containing buffer was added 4.0 ml of a solution of neutral red obtained by dissolving 10 mg of indicator in 50 ml of ethanol and then diluting the

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mixture with 50 ml of water.

The cells were incubated in their respective assay media by filling the syringes with a fixed volume (1.0 ml). The incubation time was usually 2 hrs, except when low concentrations of amphotericin were quantified when 4 hrs incubation was used.

After exposure the solvent was pressed out of the syringe into a cuvette and assayed spectrophotometrically at 585 nm (bromophenol blue) and at 528 nm (neutral red).

Results and discussion. The yeast cells were immobilized by affinity interactions between mannans on the cell surface and the immobilized lectin, concanavalin A bound to Sepharose 4B. The substrate used in this assay was glucose dissolved in buffer and the presence of an indicator. The uptake of the vitamin is pH-dependent and the yield of thiamine in the final assay step was improved by addition of glucose to the thiamine-containing medium.

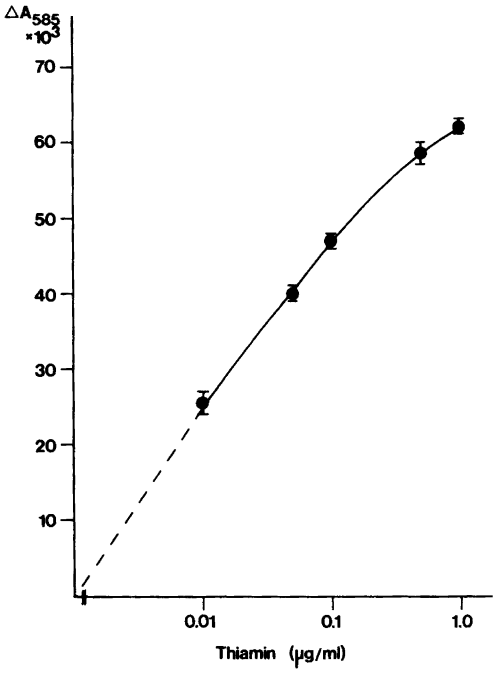

Fig. 1. Calibration curve for thiamine. The metabolic response registered as a function of thiamine concentration. All measurements were performed in triplicate. The bars in the figure denote the range of variation of the results of the assays. The syringes used were all preexposed to 6×10^6 cells. The figures are corrected for blank reactions registered in the blank sample. Experimental details as given in the text.

Furthermore, the duration of exposure of the cells to the vitamin solution is important. The metabolic stimulation from the preceeding step was maximal when the exposure lasted 30 min. In a typical assay 6×10^6 cells were added to each syringe. Fig. 1. shows a typical standard curve. All the measurements were made in triplicate and the bars in the figure denote the range of variation of the assays. Some food samples were analysed as well, and compared to results from the conventional procedures using fluorescence assay.[4] It was, however, found that the analytical results differed markedly, but those from the bioassay agreed well to those obtained with a yeast electrode.[5] The difference between the analytical results of the present method and those of the conventional spectrofluorimetric may be ascribed to the fact that the bioassay method is extremely specific, whereas a chemical method is less so. Some recovery experiments were performed to check that the response reflected a thiamine-related activity. Recovery was approximately 90 %.

The effect of thiamine pyrophosphate (TPP) on the system was showed. TPP as such has no effect on the yeast cells but it lowers the response on thiamine. This could be interpreted in terms of specific inhibition of vitamin uptake. Treatment of TPP with acid phosphatase, showed that the inhibiting effect disappeared together with a concomitant increased recovery of the added TPP in the analysis.

The sensitivities in the thiamin – assay are well within the range of interest in *e.g.* food analysis, and are better than those reported for bioassays based on microbe electrodes.[6]

Analysis of amphotericin-B. In a similar way as the effect of a stimulus may be registered it should also be possible to assess the effect of inhibitors. Since the effect of amphotericin is said to be due to its effect on biological membranes, one could expect that at low concentrations the damage to the cell membranes would be small and consequently only marginally influence the metabolism.

Earlier work on yeast cells has shown that pH-indicators[3] can be used when quantifying the metabolic activity of the cells. Using a redox indicator showed a large change whereas no effect was observed on neutral red.

The change in the assay with bromphenol blue must be due to factors other than changes in metabolism, unless the indicators influence the cells in markedly different ways – which has not been observed in former studies.

The amphotericin molecules dissolve constituents of the membrane and thereby make it easy for brom phenol blue to pass through into the

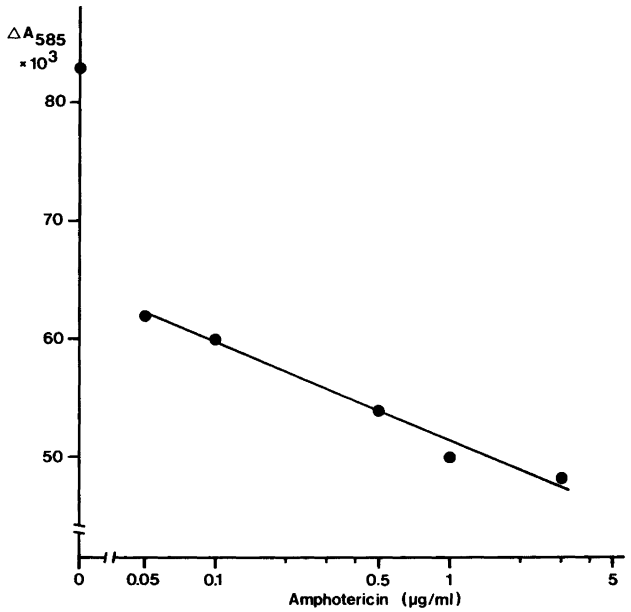

Fig. 2. Response of affinity bound *Saccharomyces cerevisiae* to treatment with amphotericin according to the procedure given in the text.

membrane. The difference between the results of the experiments with the different indicators can then be ascribed to differences in their affinity for the membrane.

A calibration curve towards amphotericin is shown in Fig. 2. The concentration range in which the assay reported in this paper operates is well within that of clinical interest.

The use of redox indicators instead of pH-indicators eliminates the need of very weak buffers in the incubation step. In the experiments reported here 0.1 M buffer was used throughout. Using such a strong buffer gives a better pH-stability and reduces the need of washings between the exposure of the cells to the systems to be quantified and the final registration of the metabolic activity.

1. Solberg, O. and Hegma, I. K. *Methods Enzymol. 62* (1979), 201
2. Daly, J. R. (1978) in *Recent Advances in Clinical Biochemistry* ed. K.G.M.M. Alberti, Churchill Livingstone, pp. 129.
3. Mattiasson, B. and Johansson, P.-A., *J. Immunol. Methods* 52 (1982), 233.
4. Airth, R. L. and Foester, G. E. *Methods Enzymol. 18* (1970), 81.
5. Mattiasson, B., Larsson, P.-O., Lindahl, L. and Sahlin, P. *Enzyme Microbe Technol. 4* (1982), 153.
6. Matsunaga, T., Karube, I., and Suzuki, S. *Anal. Chim. Acta 98* (1978), 25.

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