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Note

Measurement of the ligand content of affinity gels by an "in-gel" method

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There is a clear need for routine methods of analysis of the ligand concentration in affinity gels. Convenient methods are required for (a) monitoring the success of synthetic procedures, (b) studying leakage or other deterioration during the use of the gel and (c) studying the effect of ligand concentration on separation, which is particularly important in developing mathematical models for affinity chromatography[1].

Applicable methods can be based on either hydrolysis of the gel and subsequent analysis of the ligand in the hydrolyzate, or on direct "in-gel" analysis. The hydrolytic methods have some drawbacks: the hydrolysis of sugar polymers generally results in a variety of reactive and undefined substances and causes the polymers to turn black at the temperatures needed for the hydrolysis. Accuracy will also be impaired due to the many steps in handling. In the case of labile ligands, the analytical difficulties after the hydrolysis may also be considerable.

Quantitative in-gel methods are based mainly on the potentiometric titration of rather large gel volumes[2], direct spectroscopic measurements in the case of suitable ligands[3] or the addition of a specific reagent to a gel batch and estimation of the consumption of the reagent from the solvent after completion of the reaction[4]. The last method is inaccurate for the estimation of the dilution of the reagent when added to the gel. Labelled ligands needed for radioactive methods are seldom available commercially.

We describe here an in-gel method which is based on the elution of a suitable reagent through a small column containing the gel sample. As an example, we show how aldehyde groups can be measured. In-gel methods like this avoid excessive handling of the gel. Moreover, very small samples of the gel are needed, the gel volume may be observed directly in a packed state, little effort is required due to the automation of the method and reliable results are obtained. Because the method is simple and flexible we suggest that it could be adopted for routine use.

EXPERIMENTAL

Materials

The preparation of a Sepharose 4B gel containing the ligand group $-NH(CH_2)_3 \cdot CHO$ has been published[5]. O-Benzyloxyamine was purchased from Aldrich Europe (Beerse, Belgium). An Ismatec mini-2 peristaltic pump and a Beckman DU-equipped Gilford Model 2220 spectrophotometer were used.

Apparatus

The apparatus is shown in Fig. 1. It contains a small column and a recording spectrophotometer with a flow cell (volume *ca.* 0.5 ml). A siphon-operated flow meter was constructed for this system (see Fig. 1). In the course of filling the siphon the eluent makes contact between the platinum wires 7 and 8 and a relay partially short-circuits the recorder input, so that the recording level changes slightly. The filling time to the wire 8 is not dependent on possible variations in the siphoning which are detectable from the width of the transient interruption in the recording of a real absorbance. The width of this "break" peak is controlled by the screw 6. The flow meter, although very practical, is not necessary if the flow-rate is otherwise measured and shown to be constant. The electric circuit of Fig. 1 is reliable and can be constructed using a minimum of materials.

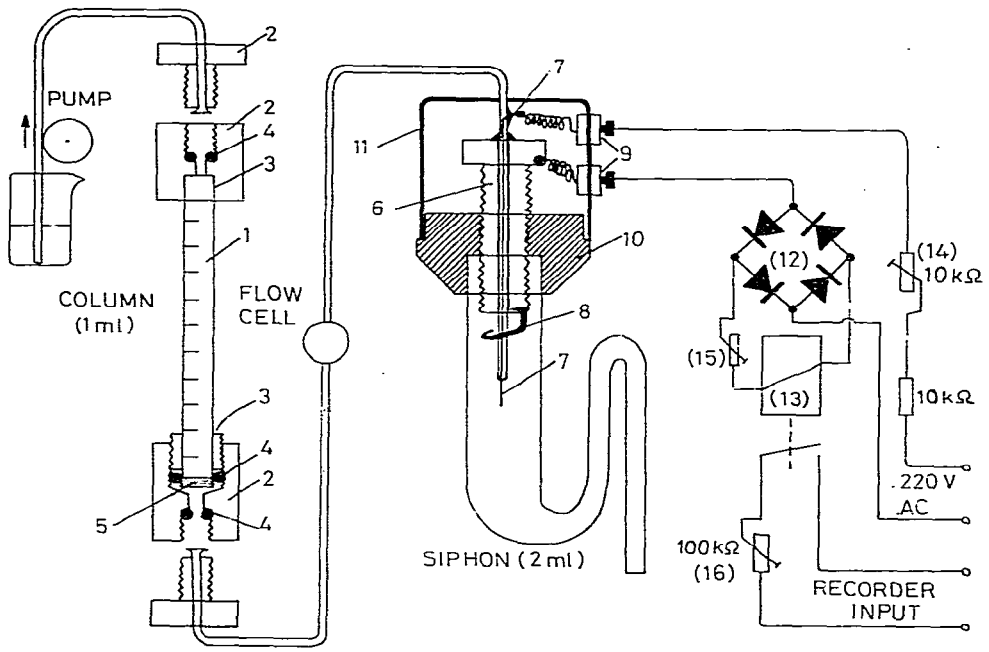

Fig. 1. The apparatus used in the measurement of the ligand concentration of a substituted agarose. 1 = Column made from a 1-ml graduated pipette; 2 = plastic connectors; 3 = pipette glued to 2; 4 = O-ring; 5 = glass filter (part number 782-1424 for Perkin-Elmer KLA-5 amino acid analyzer, diameter 1/4 in.); 6 = stainless-steel screw with a hole bored through the centre; 7 = platinum wire inserted in the tube; 8 = platinum wire connected to 6; 9 = plugs; 10 = mounting of 6, removable from the siphon; 11 = plastic cover; 12 = rectifier, *e.g.* 250 V, 0.1 A; 13 = d.c. relay, *e.g.* 24 V; 14 = potentiometer for controlling the contact sensitivity between 7 and 8; 15 = potentiometer for adjusting the current in 13; 16 = potentiometer for adjusting the height of the break peak on the recorder (the resistance value is dependent on the output mode of the spectrophotometer).

Procedure for ligand measurement

Benzyloxyamine has a UV absorption peak at 260 nm and reacts with aldehydes almost irreversibly[6]. The gel samples (0.4–1 ml) were introduced into the small column with the aid of an injection syringe. Air was removed from the column and tubes, and the peristaltic pump was connected. After washing for 30 min with 0.5 *M*

NaCl containing 0.2 *M* sodium phosphate, pH 5.0, the gel volume was observed and the feeding tube was transferred to a beaker containing 1 m*M* benzyloxyamine supplemented with the buffer and NaCl mentioned above. When the maximum absorbance of the solution was reached (step A), the washing was carried out again until the baseline absorption was obtained (step B). Step A was then repeated (step C). The area between the curves obtained for steps A and C was estimated by weighing, and the consumption of benzyloxyamine per millilitre of gel was calculated from the flow-rate (*ca.* 0.2 ml/min).

RESULTS

Fig. 2 shows the recorder curves of one analysis obtained with the above procedure. A slight absorption in the 2–7 ml range of curve A shows that the reaction is not fast enough to consume all the benzyloxyamine in the eluent. This does not cause any difficulty, however, since it is taken into account in the integration of the area. The reaction of benzyloxylamine is almost quantitative as indicated by the almost complete symmetry of B and C.

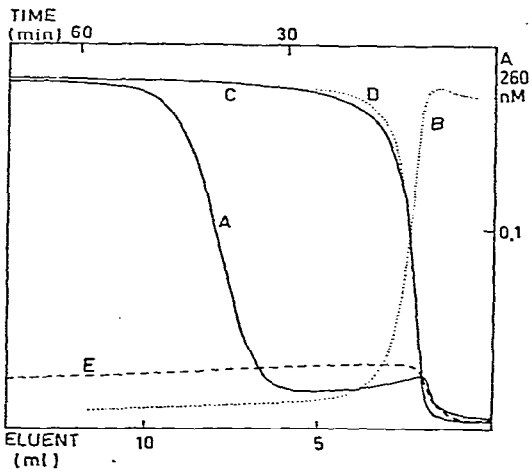

Fig. 2. Spectrophotometric recorder curves obtained when a benzyloxyamine solution was eluted through an agarose column, containing aldehydic substituents (A), when the same gel was washed (B) and when benzyloxyamine was again applied (C). Curve D is the mirror image of B. Curve E was obtained after steps C, B and then elution with a hydroxylamine solution. Curves A and C (or D) are needed for the measurement of the aldehyde ligand concentration. For details, see Experimental.

The integration of the area between A and C yielded a ligand concentration of 9.7 m*M* (S.D. = 0.4 m*M*, $n = 3$). A gas chromatographic method[5] gave a value of 8.4 m*M* for a settled volume of gel. Thus the results are quite consistent. The unsubstituted Sepharose did not absorb benzyloxyamine.

When the above procedure has been performed once carefully, it is sufficient in subsequent analyses to carry out only step A, providing the same gel volumes are used

and the internal volume of the apparatus is kept constant. The ligand concentration is then obtained by comparing the elution volumes with those of the first analysis.

As shown by curve E (Fig. 2), it is possible to elute benzyloxyamine from the column with 0.1 *M* hydroxylamine (benzyloxyamine recovery *ca.* 100%), but measurement of the re-appearance of benzyloxyamine in the eluent is not practical due to the slow exchange reaction between hydroxylamine and benzyloxyamine. The baseline absorption was attained after 8–12 h. The concentration of benzyloxyamine in the eluent was selected in such a way that the elution volumes of A and C could be accurately measured. It was estimated that gel samples as small as 0.1–0.2 ml would still give quantitative results.

DISCUSSION

The applications of the principle that an immobilized ligand (or protein, membrane, cell, etc.) absorbs a reagent irreversibly from the eluent are wide in biochemistry Many biological processes function *in vivo* under similar conditions.

When using this analytical principle it is important to be able to distinguish between specific and nonspecific sorptions. This is usually made possible by the continuous monitoring of the eluent. If the recorder curves show unwanted sorption, the composition of the elution buffer should be altered. In addition to the concentration of functional groups, further information about possible buried groups may be extracted from the forms of the elution curves. It is reported[7] that a considerable portion of the functional groups in affinity gels may also resist secondary substitution.

In the case of affinity ligands, the availability of suitable irreversible reagents seems to be adequate (see, for example, solid-phase syntheses). A mixture of activating and masking reagents may be used. An additional colour-producing reagent may be introduced after the column, if a photometer is to be used as the monitor. The exceptional stability of agaroses makes it possible to readily modify the elution conditions[8] If the reagent selected is not stable in water, organic solvents such as methanol or dimethylformamide can be used[9]. In case of sluggish reactions, temperatures up to 100° can be used to shorten the elution times[8]. The extent of secondary substitution to spacer arms can be estimated from the decrease in the number of free spacers.

Thus, the method presented here is very flexible and seems to be applicable to a large variety of affinity gels.

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