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Cellulose beads: a weak leaking affinity support

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ABSTRACT

The release of immobilized ligands from cellulose beads was studied by solvolytic detachment of glycine, DL-leucine, azocasein and leucine aminopeptidase, which were coupled to carbonochloridate-activated supports. It is demonstrated with immobilized glycine and leucine that the urethane linkage formed between the matrix and ligands is very stable in the pH range 2–10. Comparison of the leakage with corresponding Sepharose conjugates shows the superiority of conjugates based on cellulose beads. The release of immobilized amino acids from bead cellulose was less than 1% for 1000 h at room temperature. For the immobilized proteins it is shown that the initial leakage is due to the desorption of a small amount of non-covalently bound protein (< 10%). Leakage of the proteins multivalently bound to the matrix by the urethane bond was not detectable.

INTRODUCTION

Immobilized enzymes or other biologically active compounds are of growing interest for biotransformation processes, *e.g.*, in enzyme reactors, for the chromatographic purification of biomacromolecules, *e.g.*, in affinity chromatography of antibodies, lectins and enzymes, and for desorption processes in clinical medicine, *e.g.*, extracorporeal detoxification. The application of supports with covalently bound ligands for these purposes depends on the stability of the matrix, their binding capacities and an unimpaired manifold reuse. Moreover, it is essential to check their liability to leakage because the detached ligand may contaminate the purified product or decrease the binding capacity in the further employment of the adsorbent.

In addition to attrition of the matrix, Lasch *et al.* [1] discussed a number of possibilities as reasons for ligand leakage, for instance, cleavage of polymer bonds and of anchoring and ligand bonds. All three types of cleavage have been confirmed by Johansson *et al.* [2] in the case of cross-linked Phenyl-Sepharoses and Phenyl-Superose.

Ligand leakage was most often studied from affinity supports based on agarose. A partial or complete release of ligands coupled to cyanogen bromide (CNBr)-activated matrices by solvolytic detachment was described in several studies [3–8].

To minimize the release of ligands, more stable matrices, *e.g.*, cross-linked agaroses [2,9] or cellulose beads [7], and various activation methods were used. Several studies [1,8–16] have shown that for ligands covalently attached by a more stable (*e.g.*, ether, secondary amine, urethane) bond the leakage was significantly decreased in comparison with ligands immobilized via an isourea linkage obtained with CNBr-activated supports.

After developing a novel technique to bind ligands via a urethane bridge [15,17] in high yield to supports containing hydroxyl groups, we examined in this study the stability of this linkage by monitoring the leakage of low-molecular-weight ligands ([^{14}C]glycine and [^{3}H]leucine) and of proteins (azocasein, M_r 22 000, and leucine aminopeptidase, M_r 300 000, immobilized by multi-point attachment) from cellulose beads (Divicell) and agarose supports (Sepharose).

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Naturally, single and manifold moorings of ligands lead to different leakage kinetics [6,18], which can be modelled theoretically by a leakage function derived earlier [19]. It has been shown [18] that in the case of proteins non-covalent binding by unusually strong adsorption has to be taken into account.

EXPERIMENTAL

Materials

Sepharose 4B, Sepharose CL-6B and CNBr-activated Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden). Divicell (cellulose beads, particle size 80–200 μm) was obtained from Leipziger Arzneimittelwerk (Leipzig, Germany), 5-norbornene-2,3-dicarboximido carbonochloridate (Cl-CO-ONB) from the Institute of Drug Research (Berlin, Germany), carbonyldiimidazole (CDI) from Merck (Darmstadt, Germany) and [^{14}C]glycine and [^{3}H]leucine (specific activity 1.11 GBq/mmol and 2.38 TBq/mmol, respectively) from the Central Institute of Nuclear Research (Dresden, Germany). Azocasein was a gift from Dr. J. Langner (Institute of Biochemistry, Martin Luther University, Halle/Saale, Germany). Leucine aminopeptidase was prepared according to Hanson *et al.* [20]. All other reagents were of analytical-reagent grade.

Preparation of activated supports

Sepharose CL-6B and Divicell were activated with Cl-CO-ONB as described by Boeden *et al.* [17][a].

The activation of Sepharose 4B was carried out catalytically with Cl-CO-ONB in the presence of 4-dimethylaminopyridine within 20 min in dry acetone at room temperature [21].

Divicell epoxy was prepared by conversion of Divicell with epichlorohydrin as described recently [17].

CDI-activated cellulose beads were prepared by activation of Divicell with CDI according to Bethell *et al.* [22], but only one third of the CDI amount per millilitre of gel was used.

The activation level was determined for Cl-CO-ONB-activated supports to be 18–30 μmol/ml gel [15], for Divicell epoxy 30 μmol/ml gel [23] and for CDI-activated Divicell about 23 μmol/ml gel by UV spectrophotometric determination of the CDI concentration in solution at 260 nm prior to and after activation.

Immobilization of glycine

A mixture of non-labelled and ^{14}C-labelled glycine (specific activity 2.64 MBq/mmol) was coupled to the Cl-CO-ONB-activated supports at room temperature in 0.1 *M* borate buffer (pH 8.3) for 20 h, to CDI-activated cellulose beads in 1 *M* sodium carbonate buffer (pH 10) for 20 h, to Divicell epoxy in 0.1 *M* NaOH for 30 h (35°C) and to CNBr-activated Sepharose 4B in 0.1 *M* borate buffer (pH 8.3) for 2 h. The molar ratio between activated groups and added glycine was 1:1.5. The coupling yield related to the active groups was 40% (CDI-activated), 45% (epoxy-activated) and 60–80% (Cl-CO-ONB-activated). The coupling yield was calculated by the difference in the radioactivity of [^{14}C]glycine in coupling solutions before and after immobilization or by determination of the immobilized glycine after blocking with ethanolamine and washing (see below) according to the combustion method of Gács *et al.* [24], counting the [^{14}C]carbon dioxide formed (absorbed with methoxypropylamine). The found values for the two variants were in fair accordance.

The residual active groups were blocked by treatment with 1 *M* ethanolamine (pH 10) for 16 h at room temperature in all samples. The supernatant was then sucked off and the supports were washed three times with a five-fold volume of coupling buffer and finally with water on a sintered-glass funnel, followed by shaking of the gel with water for at least 2 h at room temperature until [^{14}C]glycine was no longer detected in the washings. The total amount of [^{14}C]glycine shed during the washing period with water was $\leqslant 0.1\%$ relative to the initial amount of glycine for all supports.

Immobilization of DL*-leucine*

A mixture of DL-leucine and ^{3}H-labelled leucine (specific activity 39.7 MBq/mmol) was coupled to Divicell ONB-carbonate with 19 μmol active groups/ml gel in 0.1 *M* borate buffer (pH 10.7) for 3 h at room temperature. The molar ratio between active groups and added leucine was 1:5 and the coupling yield about 50% relative to the active

[a] Cl-CO-ONB-activated Divicell = Divicell ONB-carbonate.

groups. After extensive washing of the gel with a twenty-fold volume of coupling buffer and finally with water the content of immobilized leucine was determined by the combustion method of Gács *et al.* [24] and measurement of the radioactivity of the water formed.

Immobilization of proteins

The coupling of proteins to Divicell ONB-carbonate with 30 μmol active groups/ml sedimented gel in 0.1 *M* borate buffer (pH 9.1) was done as described by Büttner *et al.* [15].

Azocasein was monitored spectrophotometrically at 366 nm [25]. Depending on the lot, the absorbance was $A_{0.5\%}$ = 10.7–12.0. The amount bound was estimated from the balance of azocasein added and that recovered in the filtrate and washings. The washings included the carrier inactivation with 0.2 *M* glycine buffer (pH 8.5) and two washings with 1 *M* NaCl and with borate buffer overnight. On average, 1.5 mg of azocasein per millilitre of sedimented gel were found, *i.e.*, the coupling yield was about 30%.

In order to trace the enzymatically active leucine aminopeptidase, it was determined by its catalytic activity and expressed in nkat/ml. Coupling and the washings were as with azocasein, but the buffer was 0.05 *M* Tris–HCl (pH 8.5). The substrate was 0.01 *M* L-leucine *p*-nitroanilide (LPNA). It was found that 48% of the added activity was bound, corresponding to 3.3 nkat per 5 ml settled cellulose beads.

Leakage of immobilized glycine from agarose and bead cellulose supports

A 1-g amount of suction-dried support with covalently bound glycine (*ca.* 1.5 ml of gel) was shaken with 3 ml of buffer containing 0.03% of sodium azide for 1000 h (see Table I and Fig. 2). At specified times, 25-μl aliquotes of the supernatant were withdrawn and the radioactivity of [^{14}C]glycine was measured by liquid scintillation counting (PW 4700 counter; Philips, Eindhoven, Netherlands).

To determine the on-column ligand leakage, some of the supports were tested in a continuously working column system (low-pressure conditions). Buffer (6 ml) was circulated through a column (5 cm × 1.0 cm I.D.) with 2 g of wet support (*ca.* 3 ml of gel) at a flow-rate of 3 ml/h for 100 h at room temperature.

Leakage of immobilized DL*-leucine from cellulose beads*

The release of leucine immobilized to Divicell ONB-carbonate (coupled amount 9.7 μmol/ml gel) was investigated during storage of 1 ml of sedimented gel in 3 ml of buffer containing 0.04% of sodium azide for 270 days at room temperature (with occasional shaking). Samples of 80 μl of the supernatant were taken and assayed for radioactivity at specified times.

At the end of the leakage experiments the radioactivity of the supports was checked by the combustion method [24].

Leakage of immobilized proteins

The cellulose–support conjugates, *e.g.*, 25 ml of gel suspension (corresponding to 5 ml settled gel), were shaken in 0.1 *M* borate buffer (pH 9.1) containing 0.02% of sodium azide at room temperature for up to 10 days.

The percentage leakages were calculated from the time course of the absorbance in the supernatant: $A(t) - A(0) = A_{corr}$; $C_x\,(t)/C_s = A_x/A_s$ [x denotes the unknown, s a standard solution (500 mg/ml) and *C* concentration]. It follows that $(m/V)/(500/100) = A_{corr}/A_{0.5\%}$, where *m* denotes the milligrams of azocasein per actual total sample volume and *V* the actual sample volume. Hence,

$$m = (A_{corr}/A_{0.5\%}) \cdot 500(V/100) \quad (1)$$

where $V/100$ adjusts the volumes of standard and sample and

$$\text{leakage } (\%) = (m \times 100)/B \quad (2)$$

where *B* denotes the amount of protein bound (mg).

The leakage of leucine aminopeptidase was followed by the enzymatic activity that was liberated into the supernatant as a percentage of the activity bound, *i.e.*, *B* in eqn. 2 was replaced with the bound activity in nanokatals and *m* by the total activity in the actual volume[a].

[a] Using in all calculations the actual volumes means that they were corrected for withdrawn volumes.

RESULTS AND DISCUSSION

Stability of immobilized amino acids

The use of cellulose beads, their activation, the immobilization of ligands and application to the chromatographic purification of proteins have been described recently [15,17]. Supports based on cellulose beads are a suitable chromatographic material because of their higher mechanical, thermal and chemical stability compared with agarose supports [17]. For investigations of ligand leakage we chose activation with 5-norbornene-2,3-dicarboximido carbonochloridate (Cl-CO-ONB), which leads to a urethane (carbamate) linkage between the ligand and matrix after coupling. The stability of these conjugates was compared with that of supports with covalently bound ligands via an isourea (coupling to CNBr-activated Sepharose 4B) and an ether and a secondary amine linkage, respectively (coupling to epichlorohydrin-activated cellulose beads). The three different coupling structures result in different leakage products, as represented schematically in Fig. 1 for the corresponding glycine conjugates.

The glycine leakage, including the portion resulting from the cleavage of glycosidic bonds of the matrix, was determined at various pH values at room temperature by calculation of the amount of the released ligand from the [^{14}C]glycine content of the cleavage products in the supernatants. The results of solvolytic detachment of immobilized glycine are summarized in Table I. The comparison of times after which 1% leakage has occurred shows that the stability of all conjugates increases with decreasing pH. It is noteworthy that the leakage from conjugates prepared with non-cross-linked Sepharose types increases steeply with increasing pH. At pH 4 the support on the basis of CNBr-activated Sepharose 4B is twenty times more stable than at pH 10. However, the stability of conjugates derived from Sepharose CL-6B and Divicell gels is only 1.2–1.3 times higher.

These findings are in accordance with results obtained by Johansson *et al.* [2] and Hearn *et al.* [12], who found a higher stability of agarose supports in water and weakly acidic media (*ca.* pH 4) than at basic pH values using cross-linked Phenyl-Sepharoses or -Superose (ether linkage) and [^{14}C]glycine immobilized onto CDI-activated Sepharose CL-6B (urethane linkage), respectively.

As expected, the highest leakage was found for the support based on CNBr-activated Sepharose 4B and the lowest for the Divicell epoxy derivative. The stability of the Sepharose CL-6B conjugate is considerably higher than that of Sepharose 4B conjugates, by a factor of 9 at pH 10, but only 1.6 times higher at pH 4. Divicell conjugates are extremely stable. It is obvious that the stability of coupled gels, which contain glycine immobilized to Cl-CO-ONB-activated supports, depends strongly on the matrix and increases in the order Sepharose 4B $<<$ Sepharose CL-6B $<$ Divicell. This means that the leakage caused by cleavage of glycosidic bonds is enormously decreased by cross-linking the agarose matrix. A similar result was obtained by Johansson *et al.* [2] for Phenyl-Sepharoses. The high stability of Divicell (a non-cross-linked support) can be explained by the existence of crystalline structures in the cellulose matrix [26].

At pH 10, the release of glycine bound to Divicell ONB-carbonate is diminished by a factor of 10 and 100 compared with that found for Cl-CO-ONB- and CNBr-activated Sepharose 4B, respectively. At pH 8.3 and 4 these differences in stability are smaller. Especially for the CNBr-activated gel this is due to the higher stability of the isourea linkage at lower pH. Glycine bound to Sepharose CL-6B by the urethane linkage is detached more slowly (ca. 25 times

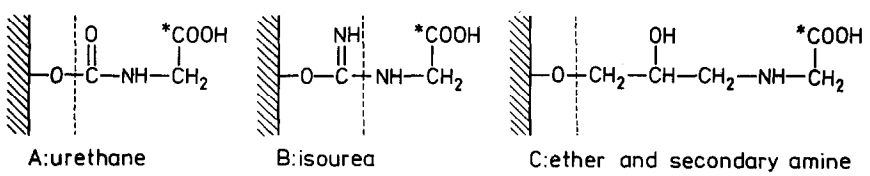

Fig. 1. Expected cleavage of the anchoring bonds. The dashed lines indicate the bonds split preferentially. Radioactive atoms are labelled with asterisks. Leakage products are (A) glycine and carbon dioxide, (B) glycine or N-carbamoylglycine, depending on the pH [3] and (C) N-(2,3-dihydroxypropyl)glycine.

TABLE I

LEAKAGE OF IMMOBILIZED GLYCINE FROM BEAD CELLULOSE AND AGAROSE SUPPORTS

After coupling of glycine (tracer [^{14}C]glycine) to activated supports the gels were treated with appropriate buffers and shaken gently at room temperature (see Experimental). For released glycine the detection limit was about 15 nmol/ml gel; error = ±4.4%.

Activated support	Immobilized glycine (μmol/ml gel)	Time for leakage of 1% glycine (h)[a]		
		pH 10.0[b]	pH 8.3[c]	pH 4.0[d]
CNBr-activated Sepharose 4B	17.0 ± 0.2	10	40	220
Cl-CO-ONB-activated Sepharose 4B	20.4 ± 0.3	90	250	630
Cl-CO-ONB-activated Sepharose CL-6B	10.2 ± 0.1	820	1050	1070
Divicell ONB-carbonate	24.1 ± 0.4	970	1350	1300
Divicell epoxy	13.5 ± 0.2	1400	1500	1600

[a] Release of glycine was determined up to 1000 h; values for > 1000 h were estimated by graphical analysis.
[b] 1 *M* sodium carbonate.
[c] 0.1 *M* sodium tetraborate.
[d] 0.1 *M* sodium acetate.

at pH 8.3) than that from the corresponding support on the basis of CNBr-activated Sepharose 4B. A similar proportion was reported by Hearn *et al.* [12] for the release of ligands covalently attached to CNBr- and CDI-activated agarose.

The great differences in stability of the tested supports are also obvious from the time-dependent detachment of immobilized glycine which is shown in Fig. 2. A linear dependence was found for all supports. The rate of ligand leakage of glycine bound to CDI-activated bead cellulose is identical with that found for glycine immobilized to Divicell ONB-carbonate. When glycine is bound to Sepharose CL-6B via the urethane linkage (immobilization to Cl-CO-ONB-activated gel), the rate of solvolytic detachment is of the same order of magnitude as that determined by Hearn *et al.* [12] for [^{14}C]glycine immobilized to CDI-activated Sepharose CL-6B, *e.g.*, at pH 10 *ca.* 1.2 and 2% of glycine, respectively, were released during 1000 h of hydrolysis.

Provided that the rate of leaking does not change for a longer period, a total detachment of the immobilized glycine should be attained after 1000 h or about 1 year at pH 10 for supports derived from CNBr- and Cl-CO-ONB-activated Sepharose 4B, respectively. For the two bead cellulose supports 100% release of bound glycine could be expected after 11 and 16 years, respectively.

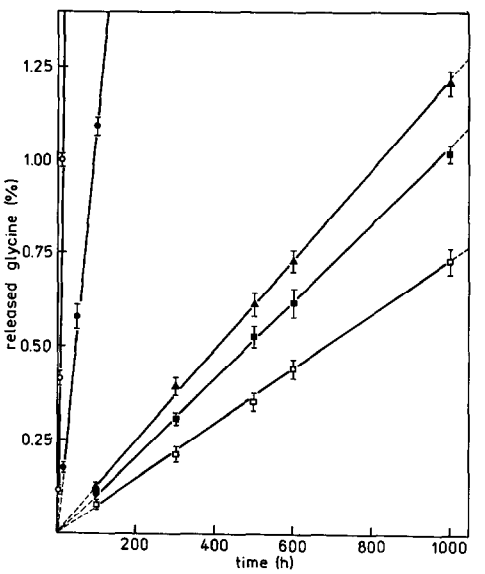

Fig. 2. Release of immobilized glycine (tracer [^{14}C]glycine) with time. The conjugates, as in Table I, were shaken in 1 *M* sodium carbonate (pH 10) containing 0.03% of sodium azide at room temperature. The percentage of released glycine was calculated from the radioactivity measurements in the supernatants related to the total amount of immobilized glycine via the specific radioactivity. Supports used: ○ = CNBr-activated Sepharose 4B; ● = Cl-CO-ONB-activated Sepharose 4B; ▲ = Cl-CO-ONB-activated Sepharose CL-6B; ■ = Divicell ONB-carbonate; □ = Divicell epoxy.

The results found for glycine leakage by circulation of the buffer solution through a gel column of the conjugates based on CNBr- and Cl-CO-ONB-activated Sepharose 4B and Divicell ONB-carbonate (data not shown) for a period of 100 h at pH 10 and room temperature were similar to those obtained from the batch experiments presented.

The leucine leakage was determined from the content of [^{3}H]leucine in the supernatants after incubation of the gels with an appropriate buffer at room temperature. The results are shown in Table II and again indicate that a maximum release of immobilized ligand occurs at basic pH. The amount of leucine detached was 7.0% after storage of the coupled gel for 270 days at pH 10.5. No substantial differences were found between pH 7.5 and 2.0. In either instance the leakage rate is approximately half of that at pH 10.5. A leaking of 1% of the immobilized leucine was obtained after 820 h (pH 10.5), 1800 h (pH 7.5) and 1440 h (pH 2.0). These values are comparable to those mentioned above for released glycine immobilized to Divicell ONB-carbonate.

It was estimated from the results at pH 2.0 that the leucine leakage (cleaving of the urethane linkage) was so small that only about half of the immobilized ligand would be detached after 11 years, assuming linear kinetics. In comparison with this, Johansson *et al.* [2] reported a half-life of 15 years at pH 2.0 for Phenyl-Sepharose CL-4B (cleavage of the ether linkage).

Leakage of immobilized proteins

The release of azocasein, shown in Fig. 3, is nonlinear: fast leakage within 5 days is followed by stability. The same result is obtained with the immobilized enzyme (Fig. 4). In all experiments with fixed proteins the total release did not exceed 10%. Although shaking of the gel is different from chromatographic flow, we obtained similar results when we used a low-pressure chromatographic flow instead of shaking.

As postulated earlier [18], there are at least three populations of immobilized proteins: one released by solvolytic cleavage of anchoring bonds and/or attack of present nucleophiles, a second which is extremely strongly adsorbed by multiple, chelate-like interactions and a third which is stable (leakage-resistant). The present results corroborate this classification. Despite prolonged extensive washings prior to the experiment until the tests for immobilized ligands in the filtrate were negative, about 8% of the fixed proteins were liberated under "operational conditions" within several days and then no further leakage, detectable by the analytical methods used, occurred.

These results are qualitatively in accordance with previous leakage experiments using leucine aminopeptidase immobilized to Sepharose 6B [6] and azocasein covalently bound onto Eupergit C [11].

Naturally, the strongly adsorbed protein can be removed more rapidly by means of more drastic and thorough washings, *e.g.*, as described by Ngo

TABLE II

LEAKAGE OF IMMOBILIZED LEUCINE FROM CELLULOSE BEADS

After coupling of DL-leucine (tracer [^{3}H]leucine) to Divicell ONB-carbonate, the supports were stored in buffer at room temperature (see Experimental). For released leucine the detection limit was 1 nmol/ml gel; error = ±6.2%.

Buffer	pH	Period of storage (days)	Leucine leakage	
			μmol/ml gel	%
0.1 *M* Sodium tetraborate	10.5	120	0.369	3.8
0.1 *M* Sodium tetraborate	10.5	270	0.678	7.0
0.01 *M* Sodium phosphate[a]	7.5	120	0.155	1.6
0.01 *M* Sodium phosphate[a]	7.5	270	0.339	3.5
0.1 *M* Glycine–HCl	2.0	120	0.194	2.0
0.1 *M* Glycine–HCl	2.0	270	0.330	3.4

[a] Containing 0.15 *M* NaCl.

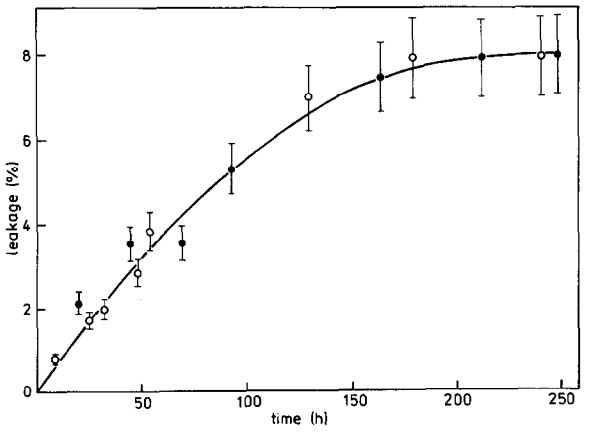

Fig. 3. Leakage of azocasein from cellulose beads. The conjugates, (●) 1.4 mg protein/ml settled gel and (○) 1.5 mg protein/ml, were shaken at room temperature in borate buffer (pH 9.1) and the liberated protein was determined in the filtered supernatant photometrically.

[16] for the complete washing out of non-covalently bound protein from supports after the coupling procedure. However, the applied washing solutions containing 1.5 *M* potassium thiocyanate, 8 *M* urea and 10% sodium dodecyl sulphate are not suitable for most proteins owing to impairment or abolition of their biological activity.

The much more sensitive method of counting the release of radioactively labelled compounds reveals an essentially slower solvolytic liberation of an immobilized low-molecular-weight ligand (single-point attachment) than of macromolecular ligands such as proteins (multi-point attachment) during the first 200 h. We interpret this finding as circumstantial evidence that the initial leakage of immobilized azocasein and leucine aminopeptidase is due to unusually strongly adsorbed protein molecules and not to cleavage of the urethane anchoring bond.

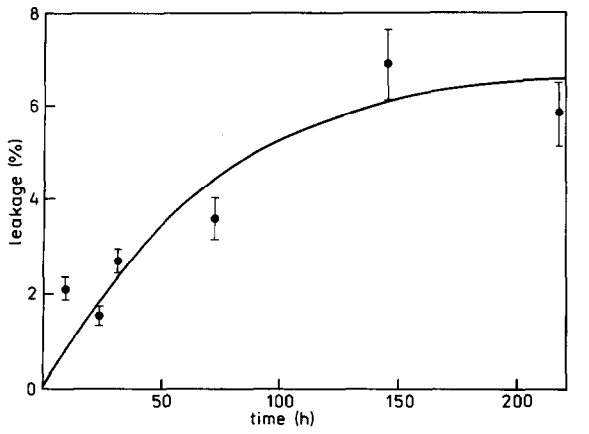

Fig. 4. Leakage of leucine aminopeptidase from cellulose beads. The conjugate, 0.67 nkat/ml settled gel, was shaken as the samples in Fig. 3.

CONCLUSIONS

Cellulose beads (Divicell) represent a more stable matrix than Sepharose 4B and Sepharose CL-6B. The stability of the urethane linkage between the matrix and the monovalently immobilized ligand is substantially higher than the isourea bond and comparable to that of an ether or secondary amine linkage. After the immobilization of proteins, a small part of the attached protein is non-covalently bound to the matrix and leaks very slowly. The results show that the proteins polyvalently bound to cellulose beads via the urethane linkage are extremely stable, and the leakage can be neglected.

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