In the cow in which the foetus was given cortisol, like those receiving Synacthen, foetal plasma TSH was greatly depressed during the period of treatment (Figure 3). However the prenatal depression of TSH was not sufficient to inhibit the postnatal rise in TSH which occurred within 30 min after birth (Figure 3), although endogenous cortisol secretion remained very low, not demonstrating the usual post-natal rise in the newborn calf[2].

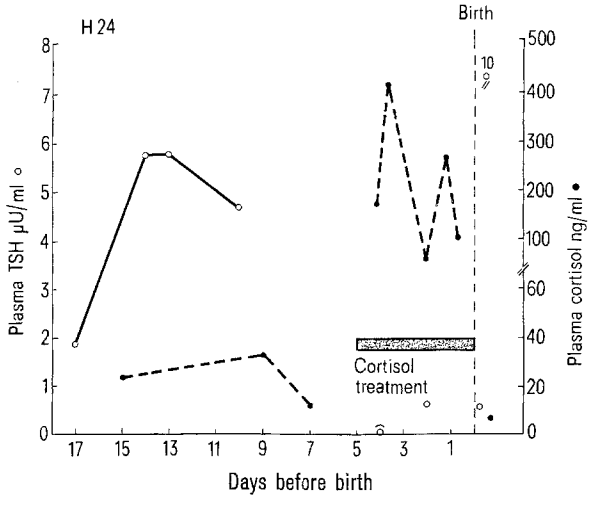

Fig. 3. Changes in plasma TSH and cortisol during initiation of parturition with cortisol (100 mg/day) for 5 days to a foetal calf. Delivery occurred at 258 days gestation.

The present findings in the calf foetus under chronic conditions suggest that the pronounced fall in foetal plasma thyroxine which occurs before birth in this species is preceded by a drop in foetal plasma TSH. These changes contrast with the situation in the foetal lamb in which the picture is far less clear cut: HOPKINS and THORBURN[4] reported a prenatal fall in plasma thyroxine but no change in plasma TSH, whereas stable plasma thyroxine levels were observed in foetal lambs in this laboratory up to the time of parturition[3].

Résumé. On a mesuré le taux de thyrotrophine (TSH), thyroxine et cortisol dans le plasma du foetus du veau pendant les 18 jours précédant la naissance. La diminution du TSH a précédé de 6 jours et s'est poursuivie en même temps que celle du taux de thyroxine.

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[4] G. D. THORBURN and P. S. HOPKINS, (1973) *Foetal and Neonatal Physiology*. Proceedings of the Sir Joseph Barcroft Centenary Symposium (Ed. R. S. COMLINE; Cambridge University Press), p. 488.

[5] We are grateful to the Medical Research Council and the Milk Marketing Board for financial support.

[6] The TSH preparation used for iodination in these studies was kindly provided by Dr. J. PIERCE, and the antiserum to Pierce bovine TSH was provided by Dr. G. D. THORBURN. Synacthen was kindly provided by Dr. D. M. BURLEY, CIBA Laboratories, Horsham, Sussex and Efcortelan by Dr. E. S. SNELL,Glaxo Laboratories, Middx.

THEORIA

Ligand-Leakage in Affinity Chromatography, a Mathematical Approach

Although cellulose-bound, highly specific substrates have already been used by CAMPBELL and LERMAN[1,2], about 20 years ago, for the isolation and purification of biological macromolecules, the large increase in the application of this technique, named affinity chromatography[3], only started in 1967. The introduction of the cyanogen bromide activation of insoluble polysaccharides for the coupling with ligand molecules by AXÉN et al.[4-6], and the use of beaded agarose[7] as the solid support[8] have certainly been important stimuli for this overwhelming development[8-12]. A further improvement of this technique was obtained by the insertion of spacer-molecules between the ligand and the matrix[3,9].

Several alternatives for agarose as the solid support have been proposed, e.g. glass beads, nylon fibres, polyacrylamide, cellulosederivatives, ethylene-maleic

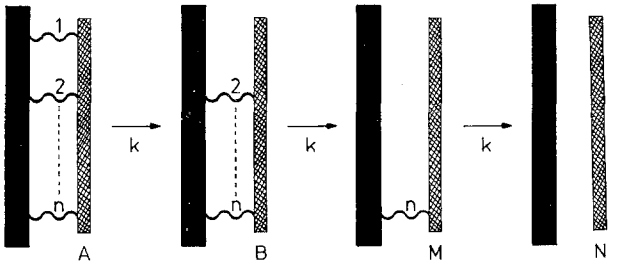

Fig. 1. Schematic representation of the detachment of a multiply bound ligand-molecule from a matrix. ■, matrix; ▒, ligand; *k*, detachment rate constant.

[1] D. H. CAMPBELL, E. L. LUESCHER and L. S. LERMAN, Proc. natn. Acad. Sci., USA *37*, 575 (1951).

[2] L. S. LERMAN, Proc. natn. Acad. Sci. ,USA *39*, 232 (1953).

[3] P. CUATRECASAS, M. WILCHEK and C. B. ANFINSEN, Proc. natn., Acad. Sci., USA *61*, 636 (1968).

[4] R. AXÉN, J. PORATH and S. ERNBACK, Nature, Lond. *214*, 1302 (1967).

[5] J. PORATH, R. AXÉN and S. ERNBACK, Nature, Lond. *215*, 1491 (1967).

[6] R. AXÉN and S. ERNBACK, Eur. J. Biochem. *18*, 351 (1971).

[7] S. HJERTÉN, Biochim. biophys. Acta *79*, 393 (1964).

[8] P. CUATRECASAS and C. B. ANFINSEN, A. Rev. Biochem. *40*, 259 (1971).

[9] P. CUATRECASAS, C. B. ANFINSEN, *Methods in Enzymology* (Ed. W. B. JAKOBY; Academic Press, New York and London 1971), vol. 22, p. 345.

[10] I. H. SILMAN and E. KATCHALSKI, A. Rev. Biochem. *35*, 873 (1966).

[11] P. CUATRECASAS, *Biochemical Aspects of Reactions on Solid Supports* (Ed. G. R. STARK; Academic Press, New York and London 1971), p. 79.

[12] H. GUILFORD, Chem. Soc. Rev. *2*, 249 (1973).

anhydride copolymers. Recently TURKOVÁ et al.[13] introduced a hydroxyalkylmethacrylate gel which is claimed to combine the outstanding properties of agarose with a high chemical and mechanical stability. Finally BRÜMMER[14] has re-introduced cellulose. A high degree of substitution with ligand molecules, and a much improved mechanical stability in comparison with agarose, are said to be the advantages of this material. At present, however, cyanogen bromide-activated agarose remains the most frequently used support in affinity chromatography.

Besides many succesful purifications realized in this way, there is now a growing number of authors who report difficulties in applying this method, as a consequence of ligand-leakage. TESSER et al.[15] found that a derivative of c-AMP, coupled by cyanogen bromide activation to agarose, could be detected in the column-eluate even after thoroughly washing. LUDENS et al.[16] reported leakage of deoxycorticosterone and estriol from agarose conjugates by washing with cytosol. WILCHEK[17] established that ε-DNP-lysine, coupled directly to cyanogen bromide activated agarose, was detached from the matrix at a rate of 15% in 3 months (pH 8, room temperature). DAVIDSON et al.[18] mentioned a slow but continuous leakage of insulin from agarose-insulin preparations. Finally, also SICA et al.[19] pointed out that the basic linkage between cyanogen bromide-activated agarose and a primary amine is cleaved at a finite and significant rate.

One of us suggested the use of polyvalently bound ligands, to arrive at a higher stability of the ligand-matrix fixation, which was felt as essential. Most relevant experiments have been carried out independently by WILCHEK[17], who found in fact a much increased stability.

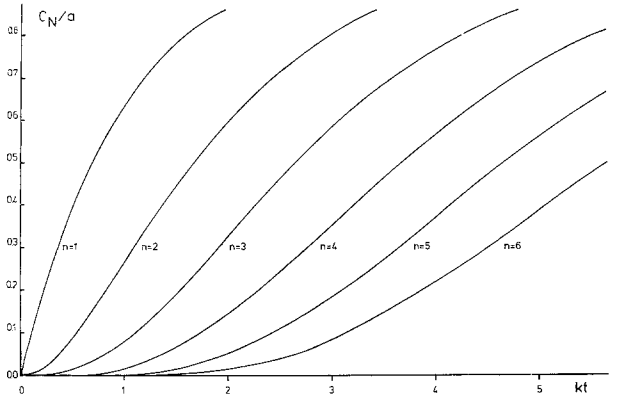

Fig. 2. Graphic representation of C_N/a as a function of kt, for $n = 1, .., 6$. (cf. equation (2)).

Table I. Numerical results of the solution of equation (3), for $n = 1, .., 6$

n	$k\tau_n$
1	0.69
2	1.68
3	2.67
4	3.67
5	4.67
6	5.67

In connection herewith we designed a mathematical model for the hydrolytic detachment of polyvalently bound ligands from a solid support. The main results are presented schematically in this paper, and will be used in further investigations into alternatives for the cyanogen bromide activation.

The hydrolytic detachment of a multiply bound ligand molecule from a matrix can be described as a consecutive reaction (Figure 1). With the assumptions of pseudo-first-order kinetics, which is compatible with the proposed detachment mechanism[20], and of reaction constants of the same magnitude at all steps, an expression for the concentration of free ligands (C_N) can be derived. If the concentration of totally fixed ligand is given by a (μmole/ml wet gel) then: $a = C_A + C_B + .. + C_N$ (1). The rupture of the first bond (A → B) is described by the following equations:

$$-dC_A/dt = k \cdot C_A \rightarrow \int dC_A/C_A = -k \int dt \rightarrow C_A = a \cdot e^{-kt}. \quad (1)$$

Rupture of the second bond (B → C):

$$dC_B/dt = k \cdot C_A - k \cdot C_B = k \cdot a \cdot e^{-kt} - k \cdot C_B$$

or:

$$(dC_B/dt) + k \cdot C_B = k \cdot a \cdot e^{-kt}$$

Solution of this differential equation, and of analogous equations for the reactions C → D, D → E, etc., by the method of TEORELL[21] leads to:

$$C_A = a \cdot e^{-kt} = a \cdot \frac{(kt)^0}{0!} \cdot e^{-kt}$$

$$C_B = a \cdot kt \cdot e^{-kt} = a \cdot \frac{(kt)^1}{1!} \cdot e^{-kt}$$

$$C_C = \frac{1}{2} \cdot a \cdot (kt)^2 \cdot e^{-kt} = a \cdot \frac{(kt)^2}{2!} \cdot e^{-kt}$$

$$C_M = \frac{1}{2} \cdot \ldots \frac{1}{(n-1)} \cdot a \cdot (kt)^{n-1} \cdot e^{-kt} = a \cdot \frac{(kt)^{n-1}}{(n-1)!} \cdot e^{-kt}$$

Combination of these equations with eq. (1), leads to the expression:

$$C_N = a - a \cdot e^{-kt} \cdot \sum_{p=0}^{n-1} \frac{(kt)^p}{p!} \quad (2)$$

For each value of n a curve can be constructed for C_N/a as a function of kt. Results for $n = 1, .., 6$ are represented in Figure 2. It is clear that a time-lag exists between the start of the detachment reactions and the appearance of the first free ligand molecules, in the case of $n > 1$. This interval increases with increasing values of n and with

[13] J. TURKOVÁ, O. HUBÁLKOVÁ, M. KŘIVÁKOVÁ and J. ČOUPEK, Biochim. biophys. Acta *322*, 1 (1973).
[14] W. BRÜMMER, Merck Kontakte 1/74, p. 23–29.
[15] G. I. TESSER, H. U. FISCH and R. SCHWYZER, FEBS Lett. *23*, 56 (1972).
[16] J. H. LUDENS, J. R. DE VRIES and D. D. FANESTIL, J. biol. Chem. *247*, 7533 (1972).
[17] M. WILCHEK, FEBS Lett. *33*, 70 (1973).
[18] M. B. DAVIDSON and A. J. VAN HERLE, N. Engl. J. Med. *289*, 695 (1973).
[19] V. SICA, E. NOLA, I. PARIKH, G. A. PUCA and P. CUATRECASAS, Nature New Biol. *244*, 36 (1973); J. biol. Chem. *248*, 6543 (1973).
[20] G. I. TESSER, H. U. FISCH, R. SCHWYZER, Helv. chim. Acta, to be published; see also ref [12].
[21] T. TEORELL, Archs Int. Pharmacodyn. Thér. *57*, 205 and 226 (1937). – A. GOLDSTEIN, L. ARONOW and S. M. KALMAN, *Principles of Drug Action* (Harper & Row, Publishers, New York, Evanston, London 1969), p. 318.

Table II. Values of kt at varying values of n and C_N/a, as the result of computer-calculation

C_N/a	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}
$n=1$	0.10×10^{-1}	0.10×10^{-3}	0.10×10^{-5}	0.10×10^{-7}	0.10×10^{-9}	0.10×10^{-11}
$n=2$	0.15×10^{0}	0.14×10^{-1}	0.14×10^{-2}	0.14×10^{-3}	0.14×10^{-4}	0.14×10^{-5}
$n=5$	0.13×10^{1}	0.44×10^{0}	0.17×10^{0}	0.66×10^{-1}	0.26×10^{-1}	0.10×10^{-1}
$n=10$	0.41×10^{1}	0.22×10^{1}	0.13×10^{1}	0.77×10^{0}	0.47×10^{0}	0.29×10^{0}
	kt	kt	kt	kt	kt	kt

decreasing values of k. From equation (2) a leakage-halftime (τ) can be calculated by setting C_N equal to $a/2$:

$$e^{-k\tau}\cdot\sum_{p=0}^{n-1}\frac{(k\tau)^p}{p!}=\frac{1}{2} \tag{3}$$

Because this equation is transcendental, no exact analytical solution can be given. Graphic solution, however, leads to the results presented in Table I. A less time-consuming and more generally useful solution can be attained by application of the Newton-Raphson procedure, with the assistance of an appropriate computer program (copies of this program are available upon request). Apparently the following generalization is allowed:

$$\tau_n\sim\{(n-1)+\ln 2\}/k. \tag{4}$$

It is also possible to calculate a value for the time-lag, mentioned before. By substituting chosen values of C_N/a and n, the same computer program provides the accessory values of kt (Table II). In accordance with expectations, there is a decrease of kt at decreasing values of C_N/a and constant n, and an increase of kt at increasing values of n and constant C_N/a. A remarkable result is that for increasing values of n the difference between the values of kt at the extrema of C_N/a (first and last column) diminishes. Replacement of a monovalently by a bivalently coupled ligand gives much more profit than, for example, the change from $n=5$ to $n=10$. From the experimental results of TESSER et al.[15], a value can be computed for the detachment rate constant. In their experiments at pH 8 and room temperature, this constant appears to be 0.25×10^{-4} min^{-1}. This value combined with data from Table II leads to the result that after 2–3 sec (!) the concentration of free ligand molecules will reach a value of 2 picomoles/ml wet gel, when originally 2 μmoles/ml wet gel were coupled, monovalently. This order of magnitude is in agreement with ligand-leakage rates found by the authors mentioned before[16–19]. At the same value of k the 2–3 sec, mentioned above, will change in 1 h ($n=2$), 5 days ($n=5$) and 5–6 weeks ($n=10$). This trend corresponds with the findings of WILCHEK[17].

In many cases, this rather minimal leakage will not be prohibitive for the isolation of biological macromolecules by affinity chromatography. However, it will be disastrous in the case of isolation of minute amounts of material out of large volumes, or in receptor localization[18]. In this context we feel that the given mathematical approach to the problem of ligand-leakage may be useful in the interpretation of experimental results and in the development of new coupling-procedures, which is the subject of our current investigations.

Zusammenfassung. Es wird ein theoretisches Modell beschrieben für die Abspaltung polyvalent gebundener Liganden von einem, wasserunlöslichen Träger. Die Annahme einer konsekutiven Reaktion führt zu einer Abschätzung der zu erwartenden Zeitverzögerung.

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PRO EXPERIMENTIS

Lanthanum Staining of the Intermediate Region of the Cell Wall in *Escherichia coli*

The cell wall of gram-negative bacteria basically consists of an outer double track membrane (wall membrane) and an intermediate region which contains the mucopeptide (peptidoglycan) layer responsible for wall rigidity[1]. This region is difficult to see by ordinary electron microscopy of thin sections, its preservation being greatly affected by the nature of the fixatives[2]. In order to improve its preservation and preferential staining, we tried several experiments with alcian bleu, lanthanum salts and simple fixatives in *Escherichia coli*.

Methods. E. coli strain B, was used throughout this study. It was grown in agar Biolife medium at 37 °C until the late logarithmic phase of growth. Various combinations of aldehyde fixatives, *tris*, 1-aziridynil phosphine oxide, Polysciences (TAPO) and osmium tetroxide were coupled with lanthanum nitrate, based on slight modifications of the procedures described elsewhere[3–5], the following one

[1] A. M. GLAUERT and M. J. THORNLEY, A. Rev. Microbiol. *23*, 159 (1969).
[2] M. T. SILVA and J. C. F. SOUSA, J. Bact. *113*, 953 (1973).
[3] O. BEHNKE, J. Ultrastruct. Res. *24*, 51 (1968).
[4] S. M. SHEA, J. Cell Biol. *51*, 611 (1971).
[5] W. DJACZENKO and A. CASSONE, J. Cell Biol. *52*, 186 (1972).