Journal of Steroid Biochemistry, Vol. 10, pp. 267 to 275.
Pergamon Press Ltd. 1979. Printed in Great Britain

THE HYDROXYLAPATITE-COLUMN ASSAY OF ESTROGEN RECEPTORS: THE ROUTINE ANALYSES OF MANY SAMPLES AND THE CALCULATION OF THE EQUILIBRIUM ASSOCIATION CONSTANT

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(*Received* 12 *July* 1978)

SUMMARY

The hydroxylapatite-column assay of estrogen receptors, described previously [Erdos *et al. Analyt. Biochem.* 37 244 (1970)], has been modified for the routine analyses of many samples. Sixty assays can be performed in 4 h with the help of home-made filtering devices or even faster on commercial filter-racks. The method is at least as simple and rapid as its "batch" variant, it is particularly fitted for kinetic studies (the adsorption of protein and its elimination of hormone not bound to the receptor being very rapid) and allows for working with samples of large volume and low protein content (because protein is quantitatively and rapidly adsorbed even from very dilute solutions). The equilibrium association constant of the receptor can be calculated from data of experiments carried out by the hydroxylapatite-column method by the use of a correction factor, or directly, if the extract is diluted to contain less than 0.25 mg protein/ml.

INTRODUCTION

The observation of Jensen and Jacobson in 1960 on the specific retention of estradiol (E) in target organs [1] was soon followed by the demonstration that E forms a very stable complex with a cytoplasmic protein, called "receptor" (R), and that the complex penetrates into the nucleus, where it gets firmly bound to the chromatin. A burst of activity followed these early discoveries and similar findings were reported for all steroid hormones [for recent reviews see 2, 3, 4] but the field still remains wide open: the molecular parameters of the receptors, the mechanisms of the receptor–hormone interaction and the penetration of the complex into the nucleus, the nature of the "acceptor" sites on the chromatine and the supposed regulatory action of the receptor-hormone complex on transcription, are problems all far from being settled. Important international effort is still centered in this field and improvements of the receptor assays remain a necessity.

All routinely used R-assays are based upon the fact that the R–E complex is extremely stable, therefore unbound E can be eliminated at 0–4°C without significant dissociation of the complex. We described previously [5] a rapid and simple method based upon this principle: after equilibrating the R with tritiated E ([^{3}H]-E), the complex was adsorbed on small hydroxylapatite (HTP) columns, unbound E was eliminated by rinsing, and radioactivity retained was measured directly by mixing the HTP into the scintillation cocktail. The use of the HTP-column method has been extended by Truong *et al.* [6] to estimate R concentration by exchanging non radioactive E with [^{3}H]-E. The method, in a modified form ("batch method") proved to be "a rapid, reliable and sensitive assay for the routine analyses of many samples", in the hands of Williams and Gorski [7]. Recently, through the combination of equilibrium and non equilibrium conditions, Pavlik and Coulson [8] further extended the field of application of the HTP-"batch" method.

The HTP method seems to be superior in many respects over other R-assays and after eight years of experience, we think that its "column" form is more versatile and not less rapid than its "batch" variant. We want to present evidence here to support this statement, describing the method in detail as it is used presently in our laboratory for the study of R–E interaction.

The first section describes the technique itself. The second section treats the equilibrium situation in a crude extract and the calculation of the equilibrium association constant and serves as a basis for the considerations on the assay of the receptor and the "background" of the HTP-method discussed in the third section.

Abbreviations—R: uterine estradiol receptor; X: other estradiol-binding macromolecules than R in a crude uterine extract; E: estradiol; [^{3}H]-E: tritiated estradiol; E_t: total E in a system; E_f: free E; E_{bR}: E bound to R; E_{bX}: E bound to X; E_b: E bound to macromolecules under equilibrium conditions ($E_b = E_{bR} + E_{bX}$); E_u: E unbound to R ($E_u = E_f + E_{bX}$); $E_{bnonspec}$: E bound "non specifically" to the HTP column; HTP: hydroxylapatite; EDIAL: equilibrium dialysis; α: the value of the ratio E_{bX}/E_f, when the latter is constant under equilibrium conditions. K_A: equilibrium constant of association.

MATERIALS AND METHODS

Materials. Hydroxylapatite (Bio-Gel HTP, Bio-Rad); tritiated estradiol 99 Ci/mmol, Amersham; GF/C glass-fiber paper (Whatman); Omnifluor (NEN Chemical GMBH).

Preparation of the extract. The crude extract used throughout the experiments was prepared from total uteri of immature sheep by homogenising 1 g tissue in 3 ml buffer (0.04 M Tris–HCl buffer pH 7.2, containing 0.0015 M EDTA and 0.014 M 2-mercaptoethanol) in the Waring-blendor. The homogenate was centrifuged for 60 min at 120,000 *g*. All manipulations were carried out at 0–4°C. The supernatant, containing 15 mg protein/ml was rapidly frozen in small batches in liquid air and stored at −60°C without significant loss of activity at least for 3 months.

Equilibration of the extract with [^{3}H]-E before the HTP-assay. The extract and the buffer, containing [^{3}H]-E, were rapidly mixed (for instance 0.5 ml 3× diluted extract with 0.5 ml buffer to yield a 6× diluted extract) and kept overnight at 4°C. In some experiments an ethanolic solution of [^{3}H]-E was added directly to the extracts. Final concentration of ethanol did not exceed 1%. Before the assay total concentration of [^{3}H]-E (E_t) was measured on 50–200 μl aliquots. The assay itself is described in Section (1), estimation of non specific binding in Section (3).

Equilibrium dialysis was carried out according to Myer and Shellman [9]. 300 μl extract was equilibrated against 300 μl buffer containing [^{3}H]-E for 24 h at 4°C. The experiment was carried out in duplicates and radioactivity was measured on 100 μl–100 μl aliquots in duplicate.

EXPERIMENTAL

(1) *The HTP-column method*

(a) *The home made device.* The system as it is used presently in our Laboratory is shown in Fig. 1(a and b). The columns are prepared as described previously [5]: 5 ml plastic syringes (Jintan Terumo Co. Ltd, Tokio, Japan) are cut at the 0.2 mark. Into the separated rubber plunger of the piston a hole of 5 mm diameter is punched. The plunger is placed into the lower part of the syringe to hold a stainless steel grid disc, covered with a Whatman GF/C glass-fiber paper disc. The columns are assembled on the filter rack and filled with the HPT slurry to yield 0.5 ml settled bed under the mild suction of the water-pump. The semi wet HTP cake is covered with a second glass-fiber disc. The columns can be stored at least for a week in the cold room before use, as a routine they are prepared in advance in spare time. All manipulations are carried out at 0–4°C. Aliquots of the R-preparations, previously equilibrated with [^{3}H]-E, are pipetted onto the HTP columns, with stopcocks open. The ratio mg HTP in the column/mg protein in the sample, should not be less than 100. As the 0.5 ml columns contain 130 ± 10 mg HTP, the samples we

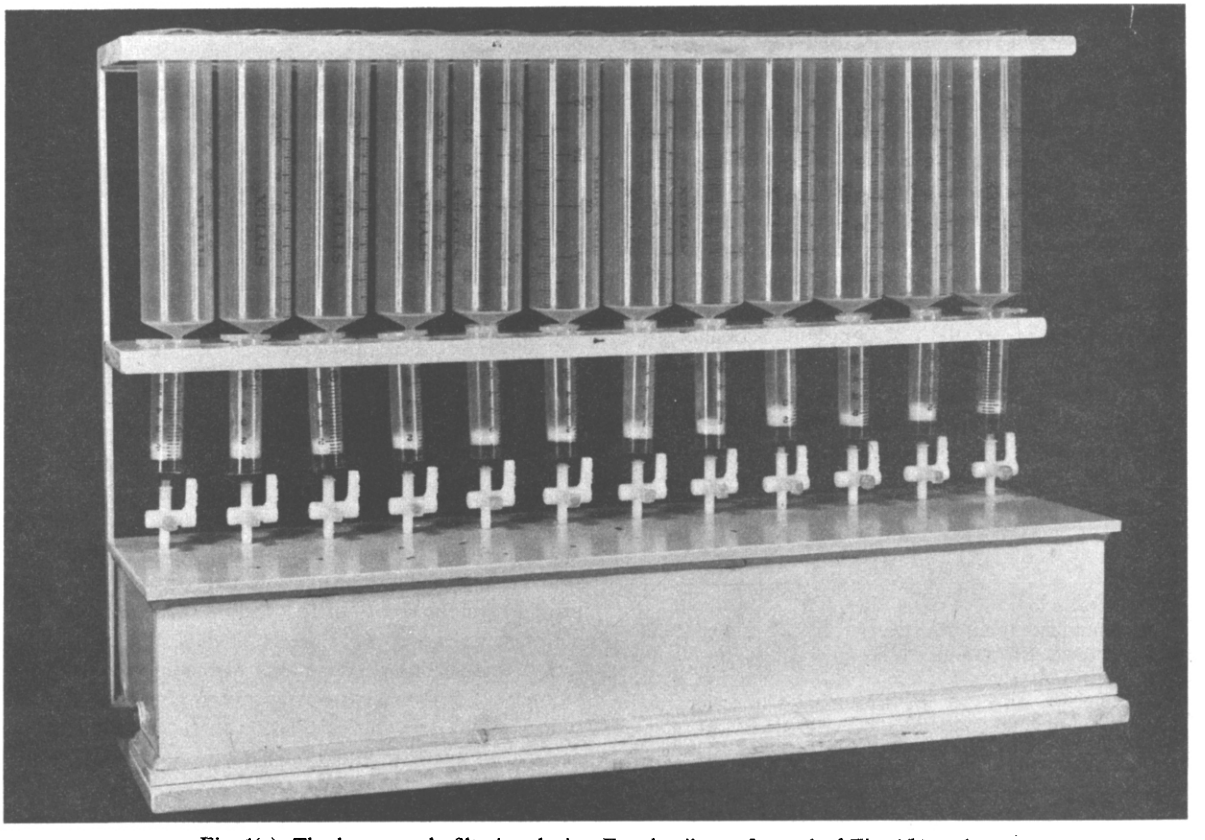

Fig. 1(a). The home-made filtering device. For details see Legend of Fig. 1(b) and text.

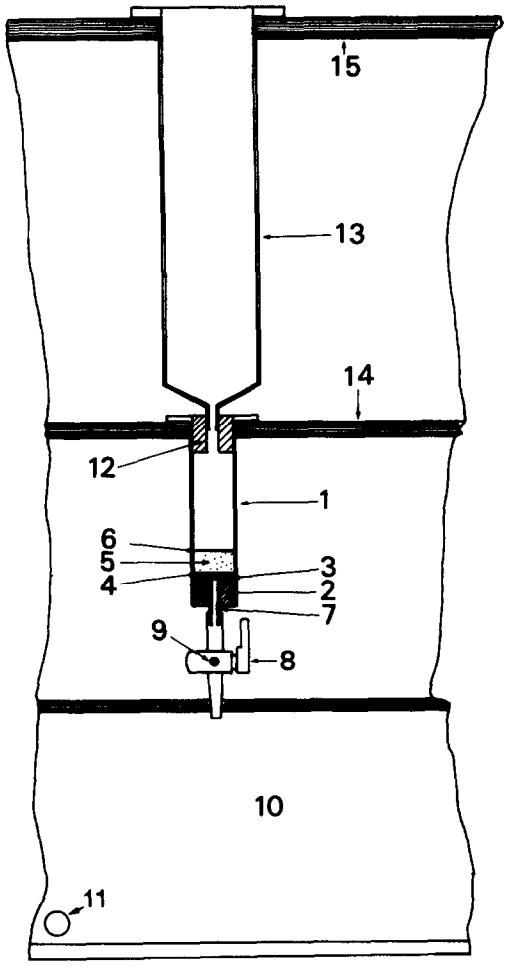

Fig. 1(b). Diagram showing a single unit of the home-made filtering device. (1) 5 ml plastic disposable syringe cut at the 0.2 ml mark. (2) Perforated rubber plunger of the original piston. (3) Stainless steel grid. (4) and (6) glass fiber paper discs. (5) Hydroxylapatite. (7) Connecting tube. (8) 3-way plastic stopcock. (9) 3rd outlet of stopcock, sealed permanently. (10) Plastic box connected to the water pump through tube (11). (12) Perforated rubber stopper. (13) 50 ml disposable plastic syringe. (14) and (15) Plastic holder for the 5 and 50 ml syringes respectively.

deposit on the column do not contain as a rule more than 1 mg protein.

Volumes, up to 250 μl, are rapidly taken up by the semi-wet HTP cake, for higher volumes gentle suction is applied. Then the columns are filled with buffer up to the 4 ml mark and each column is connected tightly with a 50 ml disposable plastic syringe. 30–40 ml buffer is poured in every 50 ml syringe and the water-pump is put to maximal power until all buffer has drained off. If the volume of the samples is higher than the small column can hold, the rest is put into the 50 ml syringe. The latter is now rinsed with 3 × 5 ml buffer under suction, before filling up with 30–40 ml buffer for the final wash. These manipulations are carried out in the cold room (4°C).

The columns and the 50 ml syringes are then disconnected (the latter will be reused without cleaning) and the columns taken off from the rack. Introducing the original piston of the 5 ml syringe into the column, the perforated rubber plunger and the metal grid is pushed from the column and saved. Pushing the piston further, the semi-wet HTP cake, enclosed between the two glass filter discs, is plunged directly into a plastic counting vial. As a routine, Bray's scintillation mixture is added. Occasionally, to obtain higher counting yields, the HTP cake is dried in glass counting vials, to allow the use of a toluene–Omnifluor mixture. The stoppered vials are vigorously shaken with a Vortex mixer for 10–20 s (as the R–E complex dissociates completely in all scintillation coktails, no other extraction procedure is needed) and centrifuged at 3000 rev./min in a swinging bucket head for 1 min and the radioactivity is counted. The presence of sedimented HTP and filters does not effect the counting yield. The semi-wet HTP cake and the filters retain about 200 μl buffer which in 13 ml Bray's solution reduces counting yield by only 5%.

The time needed for 60 assays (including the assembly of 5 × 12 columns, but not counting the time needed for equilibration of the samples with the hormone and measurement of radioactivity) is about 4 h.

(b) *The use of commercial filter holders.* A "sandwich" of HTP enclosed between two Whatman GF/C glass fiber paper discs of 25 mm diameter is prepared on a commercial filter holder.

After depositing the samples, rinsing is carried out by filling the tower of the filter 3 times successively with ice-cold buffer, manually. When all buffer has drained off, the "sandwich" is transferred into the counting vial. We observed that with certain filter-holders the results of parallel assays were not satisfactory. This was due to inadequate rinsing of the edges of the two glass-filter discs clamped between the filter holder and the tower. In these cases, without interrupting the suction, the filter towers were taken off and the sandwich was rapidly sprayed with ice cold buffer. As the whole procedure does not last more than a few minutes it is possible to work in the laboratory (instead of the cold room) if the filtering device is precooled with ice cold buffer.

In spite of the evident advantages of the commercial filter holders, their cost became prohibitory when several workers analysed many samples simultaneously. Therefore routine experiments were carried out with the help of the home-made device.

(2). *The estimation of the equilibrium association constant,* K_A

In order to calculate K_A of the R, the concentration of E bound to R (E_{bR}) and that of free E (E_f) in equilibrium have to be known. As pointed out previously [5, 10, 11] when a crude extract is equilibrated with E, E_f will be in equilibrium with E_{bR} and with E_{bX}, X signifying other E-binding macromecules than the R. Therefore neither equilibrium dialysis (EDIAL) nor the HTP-method furnish directly the information necessary to calculate K_A: EDIAL yields E_f and E bound to macromecules (E_b), but not E_{bR}. The HTP

Table 1. Binding of E to macromolecules in a crude uterine extract measured by equilibrium dialysis. Calculation of E bound to R

Measured values			Calculated values		
$E_b = E_{bR} + E_{bX}$	E_f	E_b/E_f	$E_{bX} = E_f \times \alpha$	$E_{bR} = E_b - E_{bX}$	E_{bR}/E_f
0.36	0.10	3.6	0.10	0.26	2.6
0.63	0.21	3.0	0.21	0.42	2.0
0.90	0.33	2.75	0.33	0.57	1.73
1.11	0.47	2.36	0.47	0.64	1.36
1.33	0.62	2.15	0.62	0.71	1.14
1.54	0.77	2.0	0.77	0.77	1.0
1.90	1.07	1.77	1.07	0.83	0.78
2.26	1.39	1.62	1.39	0.87	0.62
3.14	2.23	1.41	2.23	0.91	0.41
4.01	3.11	1.29	3.11		
7.50	6.50	1.15	6.50		
10.1	9.18	1.10	9.18		
16.5	16.1	1.02	16.1	*	
26.6	25.4	1.05	25.4		
52	53	0.98	53		
98	96	1.02	96		

The 6 fold diluted crude extract was dialysed against buffer containing increasing concentrations of [^{3}H]-E (0.5–300 × 10^{-9} M), E_b and E_f were measured as described under Methods. The last four points of the Table (see also Fig. 2) indicate that $\alpha = 1$ (for the definition of α, see Text). Knowing the value of α, E_{bX}, E_{bR} and the ratio E_{bR}/E_f has been calculated. All concentrations are expressed as the multiples of 10^{-9} M.

* Under the conditions of this experiment the calculation of E_{bR} is not possible without excessive error if $E_b > 4 \times 10^{-9}$ M.

method yields E_{bR} and E unbound to R (E_u), but not E_f. ($E_b = E_{bR} + E_{bX}$; $E_u = E_f + E_{bX}$). We want to describe here a simple method which allows to calculate K_A from data obtained from both types of experiments.

Table 1 and Figs 2 and 3 show the results of an EDIAL experiment carried out with a 6× diluted extract, containing 2.5 mg protein/ml. The curve of Fig. 2 and curve A of Fig. 3 were constructed from data of Table 1 by plotting the ratio E_b/E_f as a function of E_b, according to Scatchard [12].

The curve shown in Fig. 2 is typical for crude uterine extracts: the non-linear, steep part reflects the binding of E to R, the horizontal part of the binding of E to X. The observation that the curve has an

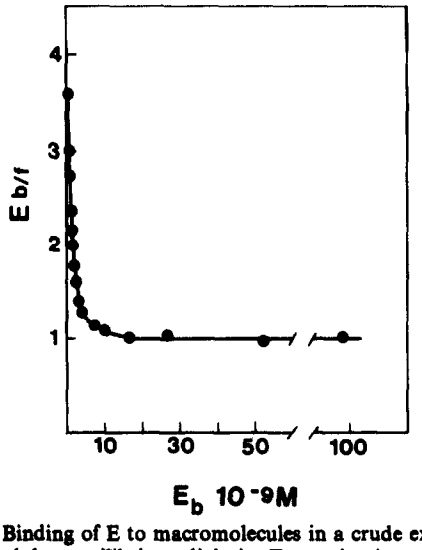

Fig. 2. Binding of E to macromolecules in a crude extract, measured by equilibrium dialysis. Determination of the constant α. The curve was constructed from data shown in Table 1 by plotting the ratio E_b/E_f as a function of E_b according to Scatchard. The scale was chosen to demonstrate the apparently horizontal part of the curve, where the ratio b/f = 1. For the definition of the constant α, see Text.

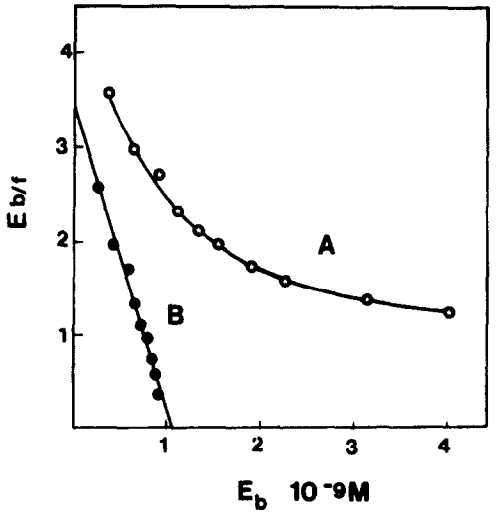

Fig. 3. Binding of E to macromolecules in a crude extract, as measured by equilibrium dialysis. Determination of K_A and of the concentration of R. Same experiments as shown in Fig. 2, but drawn on an enlarged scale. The curves were constructed from data shown in Table 1, curve A from the "measured" values by plotting E_b/E_f as a function of E_b and curve B from the "calculated" values by plotting E_{bR}/E_f as a function of E_{bR}. Linear regression analyses of data of curve B indicates [R] = 1.06 ± 0.06 × 10^{-9} M and K_A = 3.25 ± 0.11 × 10^9 M^{-1}.

apparently horizontal portion, where E_b/E_f remains constant, indicates that in this region the contribution of E_{bR} to the binding can be neglected and E_b corresponds for all practical purposes to E_{bX} in equilibrium with E_f, therefore $E_b/E_f = E_{bX}/E_f$. The constant E_{bX}/E_f will be called "α". Under the conditions of the experiment $\alpha = 1$. Knowing the value of α, it is possible to calculate E_{bX} in equilibrium with E_f, and consequently E_{bR} and the ratio E_{bR}/E_f. ($E_{bX} = E_f . \alpha$, $E_{bR} = E_b - E_{bX}$). These calculated values are shown in Table 1. In order to determine K_A and the total concentration of E-binding sites of the R (i.e. the "concentration" of the R ([R]), ratio E_{bR}/E_f was plotted as a function of E_{bR} in Fig. 3 (curve B). The curve is linear, by extrapolation we find the values [R] = 1.062×10^{-9} M and $K_A = 3.25 \times 10^9\ M^{-1}$. (Fig. 3 curve A reproduces on an enlarged scale the steep non linear part of the binding curve already shown in Fig. 2).

Table 2 and Fig. 4 show the results of experiments where binding of E to R was measured by the HTP column method. Experiment A has been carried out with the same 6× diluted extract as the EDIAL experiment. The total concentration of E present in the equilibrated system (E_t) and E_{bR} have been measured, E_u and E_{bR}/E_u calculated. (For details see Legend of Table 2). Curve A in Fig. 4 was obtained by plotting E_{bR}/E_u as a function of E_{bR}. The curve is linear, by extrapolation we find [R] = 1.065×10^{-9} M and $K_A = 1.61 \times 10^9\ M^{-1}$. Comparing these values with those calculated from data obtained by the EDIAL

Table 2. Binding of E to R in a crude uterine extract, measured by the HTP-column method

	E_t	E_{bR}	$E_u = E_t - E_{bR}$	E_{bR}/E_u
A	0.515	0.285	0.230	1.24
	0.748	0.400	0.348	1.15
	0.898	0.435	0.463	0.94
	1.205	0.550	0.655	0.84
	1.910	0.702	1.210	0.58
	2.396	0.788	1.608	0.49
	3.950	0.864	3.086	0.28
	4.957	0.927	4.030	0.23
B	0.0435	0.00952	0.0340	0.28
	0.0628	0.0145	0.0483	0.30
	0.0915	0.0183	0.0732	0.25
	0.133	0.0265	0.107	0.25
	0.213	0.0370	0.176	0.21
	0.343	0.0523	0.290	0.18
	0.531	0.0611	0.470	0.13
	0.787	0.0703	0.717	0.098

The crude extract was equilibrated overnight with increasing concentrations of [^{3}H]-E. E_{bR} was measured by the HTP-column method, as described under Section (1), nonspecific binding as described under Section (3). All concentrations are expressed as the multiples of 10^{-9} M. *Exp. A* = Protein concentration of the crude extract 2.5 mg/ml (6 times diluted extract). E_t and E_{bR} were measured on 200 μl aliquots. *Exp. B* = Protein concentration 0.25 mg/ml (60 times diluted extract). 200 μl aliquots were used to measure E_t, 2 ml aliquots were applied to the columns to measure E_{bR}.

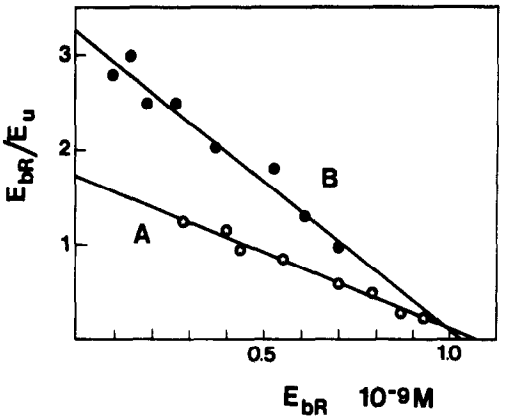

Fig. 4. Binding of E to R in a crude extract, measured by the HTP-column method. Determination of K_A and the concentration of R. The curves were constructed from data of Table 2 by plotting the ratio E_{bR}/E_u as a function of E_{bR}. Curve A was constructed from data corresponding to Exp. A (6× diluted extract). Exp. B was carried out with a 60× diluted extract, data obtained for E_{bR} and E_{bR}/E_u were multiplied by 10 and represented on the same scale as Exp. A. According to results of linear regression analysis, data of curve A yield [R] = $1.06 \pm 0.09 \times 10^{-9}$ M; $K_A = 1.61 \pm 0.08 \times 10^9\ M^{-1}$ and those of curve B: [R] = 1.05 ± 0.05; $K_A = 3.08 \pm 0.25 \times 10^9\ M^{-1}$.

method we find that [R] is practically identical in both experiments while K_A is smaller when calculated directly from data of the HTP experiment. This observation is easily explained because in Fig. 3(b) the ratio of E_{bR}/E_f, in Fig. 4(a) the ratio of E_{bR}/E_U is plotted as a function of E_{bR}. As $E_U > E_f$ (because $E_U = E_f + E_{bX}$) $E_{bR}/E_u < E_{bR}/E_f$; the slope of the curve is less steep in Fig. 4 as compared to Fig. 3. Evidently the correct value can be found by multiplying K_A by the ratio of E_U/E_f. As $E_{bX}/E_f = \alpha$ has been determined already by the EDIAL experiment, the value of the ratio E_U/E_f can be calculated: $E_u/E_f = \alpha + 1$. Under the given experimental conditions $\alpha = 1$, therefore the corrected value of K_A will be $1.61 \times 2 = 3.22$, practically identical with that calculated from the data of the EDIAL experiment. On the other hand, the finding that the [R] values are similar i.e. that both curves in Figs 3 and 4 extrapolate to the same value on the abscissa, is obligatory because at this point E_f is infinite and the competition of X with R for E becomes null.

For a given preparation α is a characteristic value and is a linear function of protein concentration (results not shown). For crude extracts prepared from lamb uteri, as described under Methods, $\alpha = 0.4$/mg protein/ml. (In the 6× diluted extract used for the above experiment protein concentration was 2.5 mg/ml and $\alpha = 1$.) To determine the value of α it is sufficient to perform a single EDIAL experiment. As routine, we adopted the following procedure: crude extracts are diluted to contain 2–4 mg protein/ml, EDIAL is performed against a buffer, containing 10^{-8} M hot + 2×10^{-6} M cold E. The ratio of c.p.m. bound/c.p.m. free yields directly the value of α. For

subsequent experiments with similar type of preparations only protein concentration is measured.

As α is proportional to protein concentration its value will decrease with the dilution of the extract, at the concentration 0.25 mg protein/ml $\alpha = 0.1$. Under this condition the value of K_A, calculated without correction from data of the HTP experiment, should be only 10% less than the theoretical value.

To test this prediction, binding of E to R was measured by the HTP method using a 60× diluted extract (0.25 mg protein/ml). In order to obtain the same precision as in the former experiment, 2 ml samples were deposited on the columns, i.e. 10× more than in the former experiment. The results are summarised in Table 2(b). In order to represent the results on the same scale as the former experiment, E_{bR} and E_{bR}/E_U were multiplied by 10 (Fig. 4 curve B). By extrapolation we obtain $[R] = 1.05 \times 10^{-9}$ M, $K_A = 3.09 \times 10^9\ M^{-1}$ values very close to those calculated by the use of the constant α, from the former EDIAL and HTP experiments.

In conclusion, the correct value of K_A can be calculated from data obtained by the HTP-column method by the use of the constant α. K_A = "K_A observed". $(\alpha + 1)$. If the uterine extract contains less than 0.25 mg protein/ml, K_A can be obtained directly with good approximation, even without the use of the constant α.

(3). *The assay of the R and the "background" of the HTP-column method*

The strategy of the R-assay by the HTP-column method does not differ from that of the other non-equilibrium methods. The binding of E to R is measured either in presence of near-saturating [^{3}H]-E concentrations or, for higher precision, binding is measured as a function of E-concentration and R-concentration is calculated from Scatchard plots. Like other non-equilibrium methods the HTP method has a certain "background": radioactivity retained in the column corresponds not only to E bound to R (E_{bR}) because a fraction of E bound to other binders than R (X) and some free E (E_f) is retained as well. If [^{3}H]-E is diluted substantially with non radioactive E, E_{bR} can be neglected and radioactivity retained on the column will correspond to the background of the method, generally called "non specific binding" ($E_{b\ nonspec}$). [For a recent review see Ref. 13].

We found that the ratio "c.p.m. bound to HTP/c.p.m. unbound to HTP" (x) remained practically constant, 0.0222 ± 0.0024, if the undiluted crude extract (15 mg protein/ml; $[R] = 6.42 \times 10^{-9}$ M, $K_A = 3.25 \times 10^9\ M^{-1}$, see preceeding section) has been equilibrated with 10^{-8} M [^{3}H]-E, diluted with 3×10^{-6}–10^{-5} M non radioactive E. Knowing the value of the constant x, $E_{b\ nonspec}$ corresponding to any E concentration can be calculated. In the samples equilibrated with [^{3}H]-E alone, $E_{b\ nonspec}$ = c.p.m. unbound to HTP. x. ($E_{b\ nonspec}$ is proportional to "E unbound" rather than to "total E" in the system: the more E is bound to R the less remains available to give rise to $E_{b\ nonspec}$). An example: the crude extract has been equilibrated with 4×10^{-8} M [^{3}H]-E, S.A. = 99 Ci/mmol. E_t and E bound to HTP were measured on 50 µl aliquots with 25% efficiency. E_t = 110,333 c.p.m. E bound to HTP = 18,567 c.p.m. E unbound to HTP (E_t − E bound to HTP) = 91,766 c.p.m. $E_{b\ nonspec}$ = 91,766 × 0.0222 = 2037 c.p.m. E_{bR} (E bound to HTP − $E_{b\ nonspec}$) = 16,530 c.p.m.

It is generally admitted that the background of a non equilibrium method ($E_{b\ nonspec}$) originates from E_f not eliminated, plus a fraction of E_{bX} which did not dissociate under the conditions of the assay. We found that the retention of E_f* on the column is a linear function of E_f in the sample (data not given). To quantify the participation of E_f and E_{bX} in $E_{b\ nonspec}$ and to see whether $E_{b\ nonspec}$ is proportional to protein concentration, we equilibrated extracts of different protein concentration with the constant concentration of 4×10^{-8} M [^{3}H]-E diluted with 10^{-5} M non radioactive E. c.p.m. retained on the column was assayed on 50 µl aliquots, containing 110,333 ± 500 c.p.m. each. Retention of E_f alone was assayed on a similar aliquot not containing extract. $E_{b\ nonspec}$ was plotted as a function of protein concen-

* In our early experiments (5) E_t retained on the column (as measured in the absence of extract) was about 0.1% of E_f in the sample. Later we observed occasionally values up to 1%. We attributed first this finding to variation of adsorption of E_f to a different HTP and glassfilter paper batches. An accidental observation furnished an another explanation. We observed that about 50–60% of E_f was always adsorbed on the upper glassfilter of the glassfilter–HTP "sandwich", 30–40% on the HTP cake itself and only 10% on the lower glass filter, whatever was the concentration or specific activity of the [^{3}H]-E. This observation suggested that [^{3}H]-E is not homogeneous and that only a fraction of it sticks preferentially to the column. To test this supposition [^{3}H]-E was filtered sequentially through 3 glass fiber filters or through 3 glass fiber filter–HTP "sandwiches". The [^{3}H]-E concentration of the through-flow was measured and the filters or sandwiches were extensively washed with buffer. Whatever was the percentage of [^{3}H]-E retained on the *first* filter or *first* sandwich (0.2–1% of the sample, depending upon the [^{3}H]-E batch used), the second and third filter or sandwich each retained only 0.15 ± 0.07% of [^{3}H]-E in the sample.

The results were identical using [^{3}H]-E of high specific activity only (55–99 Ci/mmol) or diluting [^{3}H]-E with 10^{-6}–10^{-5} non radioactive E. We concluded therefore that only 0.15 ± 0.07% of [^{3}H]-E adsorbed strongly to the "sandwich" corresponds to E in the preparation, and that higher adsorption is due to a contaminating radioactive compound. High adsorption was observed usually with [^{3}H]-E stored for several months and was probably due to radiolysis, but the nature of the supposed product has not been investigated. The practical consequence of this finding is that high "background" can be avoided to prefiltration through a HTP column of [^{3}H]-E, dissolved in buffer. If background due to E_f is low, the dimensions of the column can be increased if necessary or commercial filter-holders (using obligatory filters of larger diameter than the small home made columns) can be used without difficulty. (Evidently $E_{b\ nonspec}$ due to non dissociated E_{bX} is independent of the size of the column).

Table 3. The "nonspecific binding" of E to the HTP columns as a function of protein concentration: Experimental results (see Fig. 5) and the calculation of E_f and E_{bX} retained on the column as a function of their concentration under equilibrium conditions

Protein conc. (mg/ml)	Measured	Under equilibrium conditions			Calculated		
	$E_{b\ nonspec}$	α	E_f	E_{bX}	$E_{f\ retained}$	$E_{bX\ retained}$	$\frac{E_{bX\ retained}}{E_{bX}}$
15	2409	6	15,761	94,571	35	2374	0.0251
15/2	2113	3	27,583	82,749	61	2052	0.0248
15/4	1831	1.5	44,133	66,200	97	1734	0.0262
15/8	1278	0.75	63,047	47,285	139	1139	0.0241
15/16	937	0.375	80,242	30,091	176	761	0.0253
15/32	674	0.1875	92,912	17,421	204	470	0.0270
0	243	0	110,333	0	243	0	

All values represent c.p.m./50 μl. E_t = 110,333 c.p.m. in all samples. The theoretical concentration of E_f and E_{bX} under equilibrium conditions was calculated with the help of the constant α. In the undiluted crude extract, containing 15 mg protein/ml, α = 6 (see section 2). E_f retained at 0 protein concentration 243 c.p.m. if E_f = 110,333 c.p.m. in the system. Consequently E_f retained in general = E_f under equilibrium conditions × 234/110,333 = 0.0022. $E_{bX\ retained}$ = $E_{b\ nonspec}$ (measured) − E_f retained (calculated).

tration in Fig. 5, the calculated values of E_f and E_{bX} in the equilibrated system and those of E_f and E_{bX} retained on the column are shown in Table 3. Fig. 5 shows that $E_{b\ nonspec}$ does not increase linearly with protein concentration. Though the non-linearity of the curve might be misleading at first sight, it becomes evident knowing that E_{bX} depends not only upon the concentration of protein but upon that of E_f in the equilibrated system as well. As in the present experiment E_t was kept constant, relatively less E_f becomes available for binding to X with increasing protein concentration, therefore E_{bX} will not increase linearly. Data of Table 3 show that the ratio E_{bX} retained on the column/E_{bX} at equilibrium is constant (= 0.025 ± 0.001), i.e. whatever is its concentration in the system about 2.5% of E_{bX} will be retained on the column.

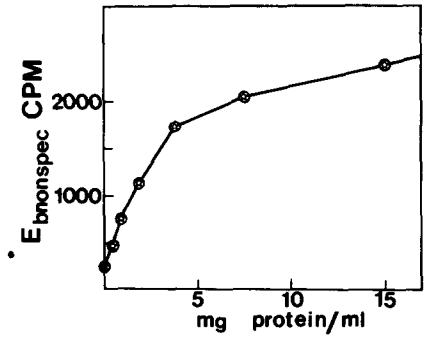

Fig. 5. Non specific binding of E on the HTP column as a function of protein concentration. A crude uterine extract containing 15 mg protein/ml was diluted 1–32×. To 2.5 ml samples 25 μl 4 × 10^{-6} M [^{3}H]-E (99 Ci/mmol) diluted with 10^{-3} M non radioactive E in ethanol was added to yield with the final concentration of 4 × 10^{-8} M [^{3}H]-E and 10^{-5} M E respectively. E_t was measured on 50 μl aliquots. Only samples containing 110,333 ± 500 c.p.m. were retained for the experiment. After equilibrating overnight at 4°C, c.p.m. retained on the HTP columns was measured on 50 μl aliquots. Retention of E_f alone was assayed on a similar aliquot not containing the extract. Radioactivity was measured in Bray's solution with 25% efficiency.

Experiments reported in this paper were carried out with a crude extract from lamb uteri. Crude extract prepared from calf uteri yield essentially the same results (data not given).

DISCUSSION

Elaborating a rapid assay for the R [5], HTP was adopted as adsorbant because it retained only 0.1% of free E after extensive rinsing. Considering that in general, adsorption and washing procedures are faster and more efficient when the adsorbent is in the form of a column rather than in suspension, the former solution was adopted. In addition, the small columns were prepared in a way that HTP could be quantitatively and directly transferred to counting vials with a single move. As described in the present report, this technique has been adapted for the routine analyses of many samples. We consider that the method is more versatile and not less rapid than its "batch" variant [7, 8]: (1) Proteins are quasi instantaneously adsorbed on the column, while it takes considerable time until quantitative adsorption is obtained when HTP is in the form of a suspension. (2) The volume of the sample is not critical if HTP is in the form of a column. (3) Rinsing and direct transfer of the HTP–glassfiber "sandwich" to counting vials is simpler, faster and more quantitative than repeated centrifugation of HTP in suspension, extraction of [^{3}H]-E with ethanol, transfer and evaporation, as used in the "batch" method.

In a crude uterine extract E is bound not only to the R but to other macromolecules (X) as well, therefore neither equilibrium nor non-equilibrium methods yield directly the value of K_A. Results of EDIAL experiments usually allow to calculate the equilibrium between E_f, E_{bR} and E_{bX}, and consequently K_A of R. Several methods are available for this purpose: the graphical method of Rosenthal [14], the logarithmic plot of Baulieu and Raynaud [15], the methods

of parameter fitting of Feldman [16], methods proposed by Bondeau and Robel [17] or the very elaborated New Fortran IV-G Program "Scatfit" of Faden and Rodbard [18], to mention only a few.

Recently Pavlik and Coulson [8], combining equilibrium and non equilibrium conditions, proposed to calculate K_A from data obtained by the HTP-batch method alone. This method offers the attractive possibility to actually measure E_f in equilibrium with E_{bR} by the HTP method itself. Our only objection to this method is that dilution of the equilibrated system by the adjonction of the HTP slurry will obligatory disturb equilibrium. This could be probably avoided by adding the HTP in the dry state. In a previous work [11] we already combined data of EDIAL and HTP-column experiments in order to calculate K_A, in the present report we describe an important simplification of this approach. The method takes advantage of the observation that for all practical purposes there are only two classes of binding sites in a uterine extract: R and the binders "X", and that under proper conditions, E_{bX} in equilibrium with E_f can be measured without the interference of E_{bR}. It is possible therefore to determine with help of an EDIAL experiment, carried out in the presence of a *single* adequate concentration of E, the constant $E_{bX}/E_f = \alpha$. Knowing α, the missing parameters, and finally K_A can be easily calculated from data of both types of experiments.

Further considerations suggest that if an extract is diluted to such an extent that E_{bX} becomes negligible, it should be possible to obtain K_A of the R without correction from data of both EDIAL and HTP experiments. While usually it is difficult to obtain the desired precision by the EDIAL technique working with highly diluted extracts, the HTP method does not limit the volume of the extract to be adsorbed on a small column, therefore precision will not decrease with dilution. Working with a 60× diluted extract we found indeed that K_A calculated without correction from data obtained by the HTP-column method was very close to the value considered as correct. We suggest that it is safe to accept the value of K_A obtained without any correction if the system contains less than 0.25 mg protein/ml.

We find the use of the constant α very convenient to quantify E_{bX} and also $E_{b\,nonspec}$ due to undissociated E_{bX}. This latter corresponds to 2.5% of E_{bX} in a crude extract, it is an intrinsic property of the system and is probably a minimal value under the conditions described. Indeed, we observed previously that a fraction of the complex binder X-[^{3}H]-E does not dissociate even under conditions as drastic as TCA precipitation [19]. On the other hand we found that the occasionally observed excessive retention of radioactivity, originally attributed to free [^{3}H]-E, is due an undefined radioactive compound (probably a product of radiolysis), which can be eliminated by filtering [^{3}H]-E dissolved in buffer through a small HTP column. After this treatment $E_{b\,nonspec}$, due to E_f retained on the column will be $\leqslant 0.2\%$ of E_f in the sample.

The "background" of a non equilibrium method is usually estimated by isotopic dilution. We followed the same way but instead of measuring $E_{b\,nonspec}$ corresponding to every experimental point, it has been measured only in the presence of a single adequate concentration (and specific activity) of E, and $E_{b\,nonspec}$ corresponding to the individual experimental points, has been calculated. This procedure is not only simpler but avoids a small systematical over-estimation of $E_{b\,nonspec}$. Very recently H. Richard-Foy *et al.* [20] developed a new method to measure $E_{b\,nonspec}$ in a non equilibrium system: they calculate this value from data obtained in presence of two different saturating concentrations of [^{3}H]-E. This method is particularly useful if the extract already contains E or [^{3}H]-E before the assay. We have no experience yet with this method.

Our present report describes the HTP-column method adapted for the routine analyses of many samples and the use of a simple method to calculate K_A. We think that the methods and concepts developed might be useful not only to study the E–R interaction but also to analyse the interaction of any macromolecule with its respective ligand, the only condition being that the macromolecule be adsorbable onto HTP and that the complex studied be stable under the conditions of the assay. The simple method proposed to calculate K_A will be valid for systems containing similar classes of binding sites as found for E in a uterine extract.

Acknowledgements—We wish to thank Drs H. Richard-Foy and D. Rodbard who made results available to us before publication. This investigation was supported by the following organisations: Délégation Générale à la Recherche Scientifique et Technique, Commissariat à l'Energie Atomique, Fondation pour la Recherche Médicale Française and the North Atlantic Treaty Organisation (Research Grant No. 826).

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