

# Immunoadsorption as a Means for the Purification of Low Molecular Weight Compounds: Isolation of Ecdysteroids from Insects

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Two ecdysteroid-specific antisera, anti-ecdysone-albumin and anti-20-hydroxyecdysone-albumin, were induced in rabbits. The properties of both antisera were tested with a large number of ecdysteroids. The former antiserum was unique in its ability to discriminate between the moulting hormones, ecdysone and 20-hydroxyecdysone, whereas the latter had a broad specificity for ecdysone and several of its metabolites.

$\gamma$ -globulin fractions of both antisera were covalently linked to Sepharose 4B and used as immunoabsorbents. These were able to bind ecdysteroids from crude extracts of insects. Bound ecdysteroids could be eluted quantitatively from the adsorbents using 3 M sodium trichloroacetate. When a mixture of ecdysteroids which contained ecdysone and at least six different ecdysone metabolites were run over a column with anti ecdysone immunoabsorbent only ecdysone and its hydroxyderivatives 20- and 26-hydroxy ecdysone were bound to and subsequently eluted from the adsorbent. In contrast a column with anti-20-hydroxyecdysone immunoabsorbent retained most of the different ecdysteroids due to the broader specificity of the antibodies.

These immunoabsorbents provide the potential not only to purify ecdysteroids but also proteins crosslinked to ecdysteroids *via* photoaffinity labelling. It is well known that immunoabsorption is a powerful tool for the isolation of proteins. The results described here demonstrate that immunoabsorption may also be useful in the isolation of low molecular weight compounds of biological and medical interest.

## Introduction

The isolation of specific compounds from natural sources (e.g. homogenates and body fluids) is one of the major technical problems in biochemistry, pharmacology and related fields. For this purpose, affinity chromatography has gained increasing importance. This special type of adsorption chromatography is usually applied to the isolation and purification of macromolecules such as enzymes, plasma proteins, hormone receptors, polynucleotides etc. using small molecules as ligands immobilized to an insoluble support. Very little literature is as yet available on a procedure using this principle conversely, *i.e.* to isolate small molecules like nu-

cleotides, metabolites of pharmacological agents or steroids by their affinity to macromolecules [1, 2].

Steroid hormones occur in rather low concentrations compared to other biomolecules of low molecular weight. In insects the range is 0.01–5 nmol per g fresh weight [3]. In the course of our studies on the biochemistry of the steroid hormone ecdysone, it was our aim to isolate ecdysteroids (ecdysone plus its metabolites) from the blue blowfly, *Calliphora vicina*, for chemical analysis in order to corroborate results from radiotracer experiments [4]. Because of the limited amount of biological material available – one mature larva has a weight of about 80 mg – a purification method was needed which combined high selectivity with high efficiency. Since immunoabsorption could be expected to have such properties, this method was developed to isolate ecdysone and related compounds.

Immunoabsorption has several advantages over conventional isolation and purification methods. Because of this reason the method is widely used to purify macromolecules [5]. However, to our knowledge, immunoabsorption has not yet been used to isolate *low* molecular compounds. Therefore, the aim

**Abbreviations:** ecdysone-CMO, ecdysone-6-carboxymethoxime; HPLC, high performance liquid chromatography; LSC, liquid scintillation counting; NMR, nuclear magnetic resonance; RIA, radioimmuno assay; TLC, thin-layer chromatography.

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of the studies described here was to develop an immunoabsorption technique for the isolation of low molecular weight compounds using ecdysteroids as a representative and interesting example.

The following steps are described in detail:

- \* preparation of an antigen by linking the low molecular compound, to a protein and production of an antiserum against the hapten-protein conjugate,
- \* characterization of the binding specificity of the antiserum,
- \* coupling of the  $\gamma$ -globulin fraction of the antiserum to an insoluble support, and finally,
- \* evaluation of the conditions for binding and elution of the compound(s) of interest.

## Materials and Methods

### Reagents

All reagents were of analytical grade and were obtained from Merck (Darmstadt) except where otherwise stated. Unlabelled steroids were purchased from Simes s. p. a. (Milano, Italy) except 2-deoxyecdysone and 2-deoxy-20-hydroxyecdysone, which were generous gifts from Dr. Horn (Melbourne, Australia), 3-epi-20-hydroxyecdysone from Dr. Rees (Liverpool, England), 3-epiecdysone, 26-hydroxyecdysone and 20,26-dihydroxyecdysone from Drs. Weirich and Thompson (Beltsville, USA), 2,14,22,25-tetrahydroxyecdysone and 2,22,25-trisdeoxyecdysone from Dr. Hoffmann (Strasbourg, France). 3-Dehydroecdysone was prepared enzymatically as previously described [6]. [23,24- $^3\text{H}$ ]Ecdysone with a specific radioactivity of 68 Ci/mmol was obtained from Zoecon (Palo Alto, USA) and purified prior to use [7]. Radiochemical purity in TLC (see below) was 97%. Radiolabelled ecdysteroids were produced by incubation of tritiated ecdysone with isolated fat body of the blowfly, *Calliphora vicina*, *in vitro* (for the details see: [4]).

### Methods

*Preparation of ecdysone-6-carboxymethoxime (ecdysone-CMO).* — Preparation of ecdysone-CMO followed in general the procedure of Borst and O'Connor [8] and Maroy *et al.* [9]. Ecdysone was mixed with [ $^3\text{H}$ ]ecdysone to monitor the reaction process.

The ecdysone-derivative was characterized: by analytical TLC (silica gel plate 0.25 mm, type 60 F<sub>254</sub>,

solvent  $\text{CH}_3\text{OH}/\text{CHCl}_3$  40/60 (v/v)) and radioscaning: purity 98%; by ultraviolet spectroscopy; by infrared spectroscopy in KBr (infrared spectrometer type 577, Perkin Elmer, Überlingen): band at  $1600\text{ cm}^{-1}$  (C = N bond); and, by NMR spectroscopy in D<sub>5</sub>pyridine (spectrometer type XL-100, Varian, Darmstadt): signals at 0.726 ppm (singlet, C-18), 1.338/1.331 ppm (doublet, C-21) and 1.372 ppm (singlet, C26/C27).

*Antigen-formation.* — We used the method of Horn *et al.* [10], to couple the ecdysteroid derivative to bovine serum albumin.

The ratio of steroid to protein in the conjugate was calculated by the ratio of radioactivity (amount of ecdysone) to protein content (Folin's method).

*Preparation of test-antigen for the capillary test.* — Following the same protocol as in the antigen (= ecdysone-bovine serum albumin conjugate) formation test antigens (ecdysone-rabbit-immunoglobulin and 20-hydroxyecdysone-rabbit-immunoglobulin) were prepared. Ecdysteroid-CMO (5.3  $\mu\text{mol}$ , 0.14  $\mu\text{Ci } ^3\text{H}$ ), rabbit immunoglobulin (0.066  $\mu\text{mol}$ ) and carbodiimide derivative (263.7  $\mu\text{mol}$ ) were incubated in 1.0 ml phosphate-buffered saline (20 mM sodium phosphate, pH 7.3, 150 mM sodium chloride). A steroid to protein ratio of 6 was achieved with ecdysone and 4 with 20-hydroxyecdysone.

*Immunization of the rabbits and capillary test.* — Two rabbits for each antigen (ecdysone-bovine serum albumin conjugate and 20-hydroxyecdysone-bovine serum albumin conjugate) were subcutaneously immunized each with 2.0 mg ecdysteroid-bovine serum albumin conjugate in 1.0 ml phosphate-buffered saline mixed with 1.0 ml complete Freund's adjuvant. Thus, 0.2 ml portions were injected at different positions of the back of the animals. Three weeks later the animals were boosted in same way.

Sera were tested by semiquantitative quantitation using glass capillaries (dimension: 80 mm length, 1 mm i. d.). Batches with the highest anti-hapten antibody concentration were pooled and named DUL-1 and DUL-2 ("Dieter-Ursel-Lutz" after the initials of the investigators) for each rabbit. Antisera against 20-hydroxyecdysone-BSA conjugate were named DBL-1 and DBL-2 ("Dieter-Bettina-Lutz"). The antisera with the index 1 had the higher antibody titre and were used throughout this study.

*Characterization of the antisera by radioimmunoassay (RIA).* — The general method used was de-

scribed recently [7]. To separate bound from free hapten in the RIA two different methods were used, ammonium sulphate precipitation [7] or equilibrium dialysis (modification of the method used by De Reggi *et al.* [19]). RIA measurements were always done in triplicates which were averaged.

Dilution curves of the antisera were measured to obtain optimal dilution factors for further assays. The sera were diluted with borate buffer containing 6% normal rabbit serum (DUL-1 2500-fold, DBL-1 1500-fold). Standard response curves with different ecdysteroids were recorded by variation of the steroid concentration. From the standard response curves the concentrations were taken at which 50% of the radiolabelled ecdysone bound to antibodies was displaced. A comparison of these concentrations between the ecdysteroids tested and ecdysone gave the cross reaction factors. Dissociation constants of the ecdysone antiserum complex were derived from Scatchard-plots [12], which were drawn from standard response curves of ecdysone.

*Coupling of the  $\gamma$ -globulin fraction to Sepharose 4B.* — Sepharose 4B (Pharmacia, Freiburg) was activated with CNBr. The subsequent coupling of the  $\gamma$ -globulin fraction followed the method of March *et al.* [13].  $\gamma$ -globulin (5 mg) of DUL-1 (DUL-1-Sepharose 4B) and 9.4 mg  $\gamma$ -globulin of DBL-1 (DBL-1-Sepharose 4B) obtained by three-fold precipitation in 50% saturated  $(\text{NH}_4)_2\text{SO}_4$ , respectively, were coupled per ml packed activated gel. Coupling efficiency was 95%, with the remaining reactive groups blocked by treatment with ethanolamine. The adsorbents stored at 4°C in phosphate-buffered saline containing 0.1%  $\text{NaN}_3$  (w/v).

*Binding of ecdysone to the immunoabsorbent.* — A column (0.5 cm i.d.) was filled with 3.3 ml (DUL-1)-Sepharose 4B. Tritiated ecdysone (49 pmol, 3.33  $\mu\text{Ci}$ ) in 0.5 ml phosphate-buffered saline was passed through the column within 5 min, which was subsequently washed with 40 ml saline. Bound ecdysone was dissociated and eluted from the immunoabsorbent by washing the column with 10 ml 3.0 M sodium trichloroacetate. This eluate was desalted immediately by gel chromatography on Sephadex G-10 (Pharmacia, column 1.5 cm i.d., bed height 150 cm, solvent  $\text{CH}_3\text{OH}/\text{H}_2\text{O}$  50/50 (v/v)). Ecdysone concentrations in the pooled unbound and bound fractions were determined by LSC of the radioactivity. Chromatographic analysis of ecdy-

steroids were performed by TLC (see below) and by HPLC as described earlier [4].

The capacity of the immunoabsorbent was tested with a column (0.5 cm i.d.) packed with 13.7 ml DUL-1-Sepharose 4B. Tritiated ecdysone (1.17  $\mu\text{mol}$ , 1.45  $\mu\text{Ci}$ ) dissolved in 10 ml phosphate-buffered saline was applied to the column. Washing and elution of bound material was as described above. Ecdysone concentrations were determined both by LSC and by measurement of ultraviolet absorption ( $\epsilon_{242}$  in MeOH 12000, [14]).

*Binding of [ $^3\text{H}$ ]ecdysone metabolites to the immunoabsorbent.* — Tritium labelled ecdysone metabolites were obtained from *in vitro* experiments with larval fat body of the blowfly. Their preparation was described in detail, recently [4]. Prior to and following immunoabsorption the metabolites were analyzed by TLC on precoated silica gel plates (0.25 mm type 60 F<sub>254</sub>, Merck,  $\text{CHCl}_3/\text{CH}_3\text{OH}$  80/20 (v/v)).

For immunoabsorption of these ecdysteroids either 9.0 ml DUL-1 or 6.0 ml DBL-1 immunoabsorbent were used and treated as described above.

*Analysis of the stability of ecdysone in TCA.* — Radiolabelled ecdysone ( $10^{-9}$  mol, 1.0  $\mu\text{Ci}$ ) was dissolved in 3.0 M trichloroacetate and allowed to stand at room temperature. At fixed times aliquots were withdrawn, desalted by gel chromatography (see above) and analyzed by TLC and radioscanning (see above). The decomposition of ecdysone was indicated by the appearance of labelled substances with  $R_f$  values different from ecdysone.

## Results

*Preparation of ecdysteroid specific antibodies.* — The steroids ecdysone and 20-hydroxyecdysone are not immunogenic. Therefore, they were coupled to the carrier protein bovine serum albumin to obtain conjugates that could be used for immunisation of rabbits. Identical methods were used to prepare the protein conjugates of ecdysone and 20-hydroxyecdysone and to raise antibodies against these conjugates. The antisera obtained were named DUL (anti-ecdysone-albumin) and DBL (anti-20-hydroxyecdysone-albumin).

*Characterization of antisera DUL-1 and DBL-1 by radioimmunoassay.* — The titres, as well as, the steroid specificities of antisera DUL-1 and DBL-1 were analysed by radioimmuno assay [7].



The antisera could be diluted 2500 fold (DUL-1) and 1500 fold (DBL-1) to bind 50% of labelled steroid ( $[^3\text{H}]$ ecdysone). The specificity of the antisera against ecdysteroids were analysed by measuring the cross-reactivity of different ecdysteroids with ecdysone (Table I). Both antisera were specific for ecdysteroids, *i.e.* they bound ecdysone and ecdysone-related compounds. Ecdysone precursors like 2,14,22,25-tetradecyecdysone and 2,22,25-tridecyecdysone were virtually not bound. This was also true for certain ecdysteroids with definite structural differences to ecdysone and 20-hydroxyecdysone.

Both antisera had a specificity directed mainly against the side chain. However, the most obvious difference between the two antisera was the extent of specificity for the C-20 structure of ecdysone. 20-Hydroxyecdysone was discriminated with a factor of 47 by DUL-1, whereas this was only 3.0 with DBL-1. The  $K_{D,av}$  values for ecdysone were determined in Scatchard plots to be  $2.4 \times 10^{-9} \text{ M}$  ( $SD = \pm 0.6 \times 10^{-9}$ ;  $n = 3$ ) for DUL-1 and  $3.2 \times 10^{-9} \text{ M}$  ( $SD = 0.6 \times 10^{-9}$ ;  $n = 3$ ) for DBL-1.

**Preparation and characterization of immunoabsorbents.** —  $\gamma$ -Globulin fractions of the antisera were coupled to Sepharose 4 B by CNBr activation. Aliquots of the resulting immunoabsorbents were packed in small columns to analyse the binding characteristics of the material. In the following,

experiments with DUL-1 ( $\gamma$ -globulin)-Sepharose 4 B will be described in detail. The other immunoabsorbent DBL-1 ( $\gamma$ -globulin)-Sepharose 4 B exhibited similar binding characteristics, except in steroid specificity (see below).

Firstly, the ability of DUL-1 ( $\gamma$ -globulin)-Sepharose 4 B to bind ecdysone was tested. For this purpose a solution of tritiated ecdysone (49 pmol,  $3.33 \mu\text{Ci}$  for 3.3 ml immunoabsorbent) in phosphate-buffered saline was passed slowly through the column. Subsequently, the column was thoroughly washed with saline. In the flow-through and wash only 1.7% of the label was detected, which was unbound ecdysone. The bound ecdysone (98%) could be eluted completely with 3.0 M sodium trichloroacetate. Immediately after elution the immunoabsorbent had to be washed with phosphate-buffered saline to preserve its binding capacity.

Other common chaotropic reagents like NaCl and NaSCN [15] were also tested in various concentrations. However, they were ineffective in dissociating the steroid from the antibodies. The same was found with lower concentrations of trichloroacetate. In 3.0 M trichloroacetate ecdysone has a limited stability (half-life at room temperature 12 h). Therefore, after its elution from the immunoabsorbent, ecdysone was immediately separated from the salt by gel chromatography. Analysis of the resulting ecdysone by TLC and HPLC, as well as, by LSC

Table I. Specificities of antisera DUL-1 and DBL-1 detected as crossreaction factors of various ecdysteroids with ecdysone in radioimmuno assay. 50% binding of ecdysone was observed at a concentration of 13 nM (= 0.65 pmol in 50  $\mu\text{l}$ ; DUL-1) and 24 nM (= 1.2 pmol in 50  $\mu\text{l}$ ; DBL-1).

Ecdysteroid	Source	Concentration range tested [nM]	Cross reaction factor	
			DUL-1	DBL-1
ecdysone	Simes	$1 - 10^3$	= 1	= 1
2-deoxyecdysone	Melbourne	$1 - 10^3$	1.6	4.0
makisterone A	Simes	$1 - 10^3$	2.1	2.9
3-epiecdysone	Beltsville	$1 - 10^3$	3.5	1.8
3-dehydroecdysone	enzymatic preparation	$1 - 10^3$	3.8	17
20-hydroxyecdysone	Simes	$1 - 10^3$	47	2.8
3-epi-20-hydroxyecdysone	Liverpool	$10 - 10^4$	120	3.0
2-deoxy-20-hydroxyecdysone	Melbourne	$10 - 10^4$	140	30
26-hydroxyecdysone	Beltsville	$10 - 10^4$	360	6.8
polypodine B	Simes	$10 - 10^4$	200	45
20,26-dihydroxyecdysone	Beltsville	$10 - 10^4$	560	21
muristerone	Simes	$10 - 10^4$	620	350
inokosterone	Simes	$10 - 10^4$	1000	9.0
2,14,22,25-tetradecyecdysone	Strasbourg	$10^3 - 10^6$	8200	$\infty$
2,22,25-tridecyecdysone	Strasbourg	$10^3 - 10^6$	$\infty$	$\infty$
ponasterone A	Melbourne	$1 - 10^3$	$\infty$	3.3
cyasterone	Simes	$1 - 10^3$	$\infty$	11
poststerone	Simes	$1 - 10^4$	$\infty$	18

Table II. Metabolites of ecdysone from blowfly before and after adsorption to DUL-1 and DBL-1-sepharose 4B. The amount of each compound was expressed in percent of the initial amount of total radiolabelled ecdysteroids (= 100%). The table gives the quantitative evaluation of Fig. 1–5. The sum of each column (A, B or C) does not amount to 100% because minor peaks were omitted.

Ecdysteroids	peak	A [%]	B 1 [%]	B 2 [%]	C 1 [%]	C 2 [%]
high polarity compounds	I	29	16	0	6	21
20,26-dihydroxyecdysone	III	3	2	0	0	4
26-hydroxyecdysone	IV	2	0	5	0	2
peak V substances	V	11	6	0	0	11
20-hydroxyecdysone	VI	9	0	16	0	6
ecdysone	VII	20	0	29	0	18

A, Metabolic composition before immunoabsorption (Fig. 1); B 1, Metabolites not adsorbed by DUL-1-Sephacrose 4B (Fig. 2); B 2, Metabolites adsorbed by DUL-1-Sephacrose 4B and eluted with sodium trichloroacetate (Fig. 3); C 1, Metabolites not adsorbed by DBL-1-Sephacrose 4B (Fig. 4); C 2, Metabolites adsorbed by DBL-1-Sephacrose 4B and eluted with sodium trichloroacetate (Fig. 5).

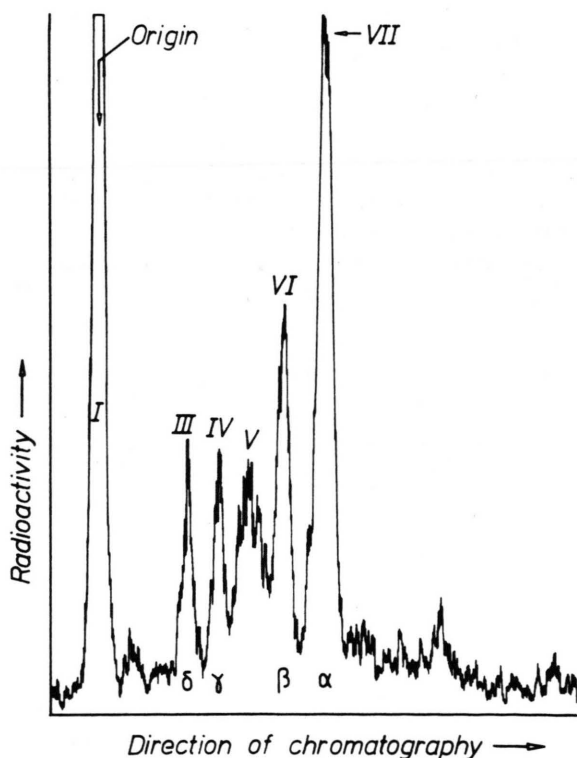


Fig. 1. TLC separation of radiolabelled ecdysteroids after incubation of tritiated ecdysone with blowfly fat bodies *in vitro*. The peaks in the order of increasing polarity consist of: I: high polarity compounds, III: 20,26-dihydroxyecdysone, IV: 26-hydroxyecdysone, V: inokosterone and other, VI: 20-hydroxyecdysone, VII: ecdysone [4]. The positions of unlabelled reference substances have been marked with  $\alpha$  (ecdysone),  $\beta$  (20-hydroxyecdysone),  $\gamma$  (26-hydroxyecdysone) and  $\delta$  (20,26-dihydroxyecdysone).

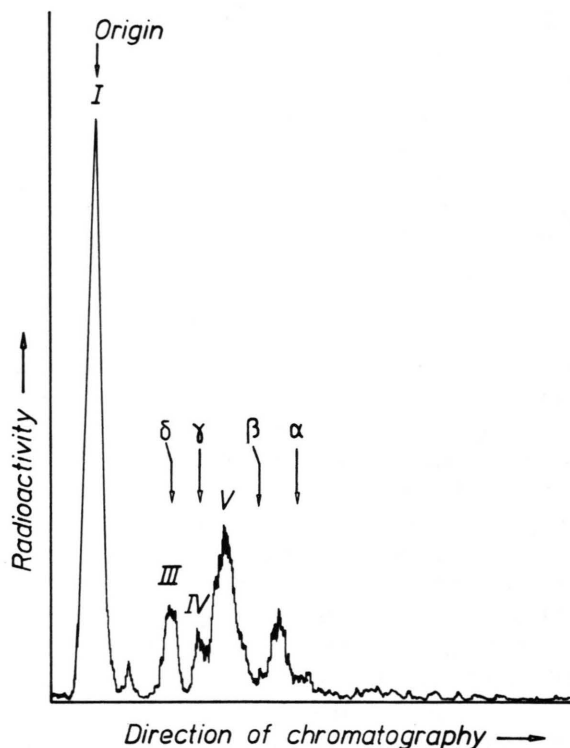


Fig. 2. TLC separation of the same radiolabelled ecdysteroids as in Fig. 1 after passage through a column filled with the immunoabsorbent DUL-1-Sephacrose 4B.

demonstrated that the steroid was unchanged and that the overall recovery in the immunoabsorption chromatography was greater than 95%.

In order to measure unspecific binding of ecdysone to the immunoabsorption material an analogous adsorbent was prepared with the  $\gamma$ -globulin fraction from serum of a non-immunized rabbit. Under identical conditions as in the preceding experiment the material bound less than 0.1% of labelled ecdysone.

The capacity of the immunoabsorbent was tested by offering an excess of ecdysone (1.17  $\mu$ mol) which was labelled with some radioactive ecdysone. The steroid was dissolved in phosphate-buffered saline and applied to a column packed with 13.7 ml immunoabsorbent. The amount of ecdysone in the flow-through and wash, as well as, the amount bound and eluted were measured using both parameters, ultraviolet absorption of ecdysone and its radioactivity, with 1.1 nmol steroid per ml packed volume of adsorbent bound and subsequently eluted.

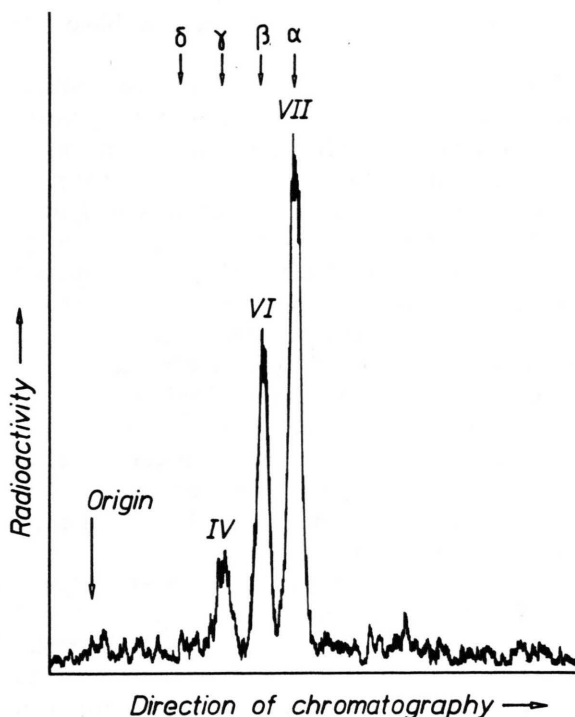


Fig. 3. TLC separation of the radiolabelled ecdysteroids shown in Fig. 1 which were bound by DUL-1 Sepharose 4B, subsequently eluted with 3.0 M sodium trichloroacetate and desalted by gel filtration.

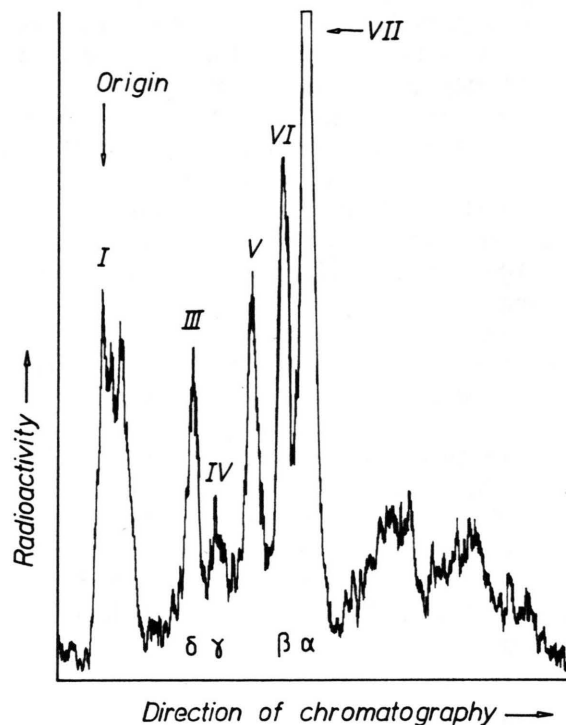


Fig. 5. TLC separation of the radiolabelled ecdysteroids shown in Fig. 1 which were bound by DBL-1-Sepharose 4B, subsequently eluted with 3.0 M sodium trichloroacetate and desalted by gel filtration.

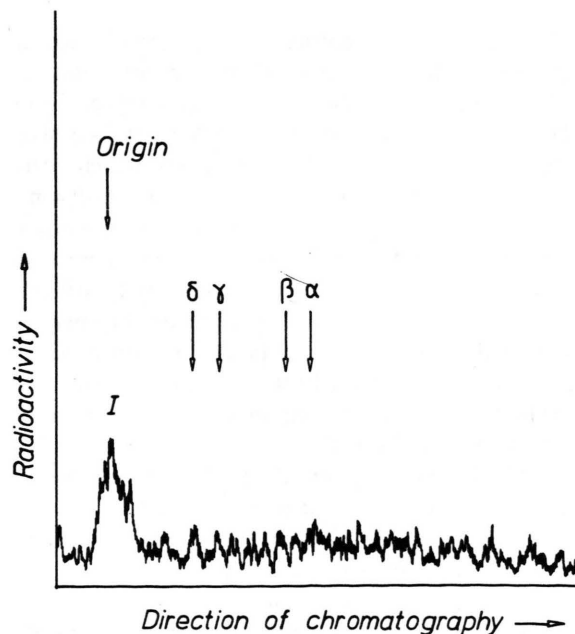


Fig. 4. TLC separation of the same radiolabelled ecdysteroids as in Fig. 1 after passage through a column filled with DBL-1-Sepharose 4B.

The specificity of the two immunoabsorbents towards different ecdysteroids was tested with radiolabelled metabolites of ecdysone. The metabolites were produced by *in vitro* incubation of fat body from blowflies with tritiated ecdysone [7] and included the ecdysteroids listed in Table II. An aliquot of the solution containing ecdysteroid in PBS was analysed by TLC and radioscanned (Fig. 1). The relative concentration of single ecdysteroids are given in Table II. Other aliquots of the solution with a total of 21 pmol ecdysteroids were passed in separate experiments through a column filled with DUL-1 or DBL-1-Sepharose 4B. Of the ecdysteroid applied 30% were not bound by DUL-1 and 6% not by DBL-1-Sepharose 4B. TLC analyses revealed that the unbound material from the DUL-1-Sepharose 4B consisted mainly of high polarity compounds, 20,26-dihydroxyecdysone and peak V substances (Fig. 2 and Table II). Unbound material from DBL-1-Sepharose 4B was due to high polarity compounds only (Fig. 4). No ecdysone and 20-hydroxyecdysone could

be seen in both fractions of unbound ecdysteroids. By elution with 3.0 sodium trichloroacetate the labelled ecdysteroids bound to the immunoabsorbents were recovered (DUL-1: 65% of the applied material, DBL-1: 87%). The ecdysteroids from DUL-1-Sepharose 4B as analysed by TLC and HPLC were mostly 26-hydroxyecdysone, 20-hydroxyecdysone and ecdysone (Fig. 3 and Table II). From DBL-1-Sepharose 4B all of the different metabolites applied were eluted (Fig. 5 and Table II).

This experiment demonstrated that the immunoabsorbents retained the specificities of the antibodies in solution detected by RIA. DUL-1 again was highly specific for ecdysone and bound few of its hydroxylated derivatives only. As in RIA, DBL-1 showed the broader specificity for the entire ecdysteroid family. Furthermore, it was obvious from this and subsequent experiments with the same materials that the immunoabsorbents retained their ability to bind ecdysteroids throughout the entire procedure.

## Discussion

Immunoabsorption has been widely employed for purification of antigens and specific antibodies [16]. The results presented here demonstrate that the method can be extended to the purification of small molecules which *per se* are not antigenic.

For the preparation of the specific antisera ecdysone and 20-hydroxyecdysone were chemically modified. Since ecdysteroids are sensitive to acidic, alkaline and other drastic conditions [17] we tried to avoid any of the above. Thus, it should be stressed that the ecdysteroid-specific antibodies are directed against pure compounds of known structure.

The antisera DUL-1 (anti-ecdysone-albumin) and DBL-1 (anti-20-hydroxyecdysone-albumin) exhibited properties known from similar antisera (for a comparison see: [7, 18]). The antibody concentration in the antisera, expressed as dilution factors (DUL-1 = 2500, DBL-1 = 1500), showed the expected values. The antibody specificities were tested with a larger number of ecdysteroids. Obviously both antisera were ecdysteroid specific (Table I). High cross-reaction factors of many ecdysteroids with DUL-1 indicated that this antiserum was ecdysone-specific. In fact, the cross reaction factor of 20-hydroxyecdysone with DUL-1 (= 47) was unique in its size [8]. This antiserum will be useful for the RIA deter-

minations of ecdysone concentrations in biological extracts of insects.

DBL-1 on the other hand exhibited broader specificity for ecdysone and several of its hydroxylated metabolites. This antiserum will be more convenient for the quantitation of ecdysteroid mixtures. However, the intended specificity of DBL-1 for the hapten used for its production (*i.e.* 20-hydroxyecdysone) was not achieved. In this regard our results confirm the findings of several other laboratories (see [18] and references cited therein). For the lack of specificity for 20-hydroxyecdysone no convincing explanation can be presently given.

Coupling of the antibodies to an insoluble support followed commonly used techniques. The resulting immunoabsorbents prepared from the  $\gamma$ -globulin fractions of antisera DUL-1 and DBL-1 had the following properties:

1) The adsorbents bound ecdysone with a capacity of 1.1 nmol/ml packed adsorbent (DUL-1) and 1.3 nmol/ml (DBL-1). On the basis of bound molecules per ml packed adsorbents the values appear to be low, however, they are similar to the capacity of immunoabsorbents for proteins [19].

2) Nonspecific binding is negligible. This was shown with the immunoabsorbent prepared from nonimmunized rabbit serum, which did not retain steroid.

3) The immunoabsorbents were specific for a definitive steroid structure. Only ecdysone and its hydroxyderivatives 20- and 26-hydroxyecdysone were retained when the DUL-1-Sepharose 4B was tested with a mixture of ecdysteroids which contained ecdysone and at least six different ecdysone metabolites. DBL-1 immunoabsorbent on the other hand had a broader specificity and retained most of the different ecdysteroids. The selectivity of the immunoabsorbents clearly depended on the specificities of the antisera used. For that reason binding properties could be fairly well predicted from the specificities of the antisera in RIA tests for cross reaction factors (Table I).

4) The bound ecdysteroid could be eluted quantitatively by use of a strong chaotropic reagent. When the ecdysteroid was separated from this eluant immediately after elution the structural integrity of the steroid was preserved.

5) The immunoabsorbent was stable and insoluble in the solvents. It could be used repeatedly without loss of affinity and capacity.

The immunoadsorbents for ecdysteroids were developed in order to isolate ecdysone metabolites from non-radiolabelled crude extracts of the blowfly. From the experiment with radiolabelled ecdysteroids it is obvious that DUL-1 immunoadsorbent is too specific for this purpose. DBL-1 immunoadsorbent with its broader specificity seems to be more suitable and will be used for the isolation of unlabelled ecdysteroids from insect material. This work will be reported in the future.

By photoactivation of ecdysteroids these hormones can be linked covalently to macromolecules with affinity for ecdysteroids [20, 21]. Such macromolecules include presumptive transport proteins in the insect haemolymph, intracellular hormone receptors in target organs, as well as, enzymes of ecdysteroid metabolism.

Surprisingly, these ecdysone-protein-complexes are still specifically adsorbed by the immunoadsorbent. They can be dissociated and eluted under appropriate conditions (unpublished results). By these means, for

instance, the isolation of sufficient amounts of ecdysteroid receptor will be possible to study its physical properties and to use it for the preparation of antireceptor antibodies.

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