Drug-Protein Conjugates: Preparation of Triamcinolone-Acetonide Containing Bovine Serum Albumin/Keyhole Limpet Hemocyanin-Conjugates and Polyclonal Antibodies

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Summary

A radioimmunoassay has been developed for the quantitation of triamcinolone-acetonide (TAAc) at the picogram level. For use of TAAc as an antigenic epitope, first the drug was hemisuccinoylated at C-21 as confirmed by ^{13}C-NMR- and mass spectroscopy after derivatization. This hapten was conjugated to the carrier-protein bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) by different amide-bond generating methods (imidazolide-, carbodiimide-, carbodiimide/sulfo-*N*-hydroxysuccinimde-, mixed anhydride-method) yielding antigens of quite different conjugation number, solubility and usefulness. The mixed anhydride-method yielded most useful soluble conjugates bearing 0.3–31.5 mol TAAc per mol carrier-protein. Coupling by the carbodiimide-method yielded insoluble conjugates, inappropriate for antigen synthesis in hapten immunoassays because of formation of coupling agent modified residues and crosslinking of the carrier-protein. Specificity of the antisera obtained by immunization with TAAc-BSA and TAAc-KLH was assessed by isolation of the soluble hapten-antibody complex and a RIA protocol was developed providing a detection limit of 200 pg (0.46 pmol) TAAc/ml sample.

Introduction

The glucocorticoid Triamcinolone-acetonide (TAAc, Fig. 1) exhibits immunosuppressive effects and is widely used as a topical agent for the treatment of inflammatory and also of itching skin diseases. There is little information about the rate of liberation of TAAc from formulations such as creams and ointments applied to the skin. For assaying HPLC methods [1,2] including post column-derivatization and a radioimmunoassay[3] have hitherto been published, both providing a detection limit of 100 ng (222.3 pmol) TAAc/ml sample. Since study of the rate of liberation and absorption of TAAc from topical formulations *in vitro* requires a detection limit at the picogram level, we tried to generate antibodies appropriate for highly sensitive determination of TAAc.

The main problem and prerequisite for production of specific antibodies recognizing low molecular weight compounds such as drugs is the synthesis of reactive derivatives of the drug, appropriate for conjugation to a high molecular weight carrier protein. The structure of the molecule may only be altered in such a way that the desired epitope is exposed from the conjugation site on the carrier protein. A polyclonal antiserum obtained by immunization with such a hapten-protein conjugate not only contains protein-specific, but also hapten recognizing antibodies. The antigen binding site of an antibody corresponds to 1 kDa or to a volume of 1.5–2 nm length, 0.5–1.2 nm depth and 1.5 nm width and thus the hapten represents an independent epitope.

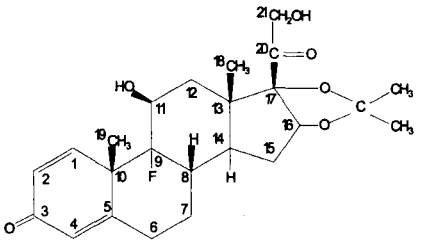

Figure 1: Triamcinolone-acetonide.

To obtain epitope specific polyclonal antibodies as a tool for sensitive determination of TAAc, we investigated various pathways for the synthesis of immunogens by different amide bond generating methods. Considering the extent of TAAc-incorporation, exposure of the hapten, kind of carrier protein, and solubility of the antigen, we selected the most appropriate immunogens for immunization of rabbits and generation of polyclonal antibodies.

Results and Discussion

Synthesis of Hapten

Production of hapten-specific antibodies as a tool for quantitative and specific determination of drugs requires an antigen, composed of an immunogenic carrier and the non-immunogenic, low molecular drug covalently bound. Concerning the structure of the molecule (Fig.1), the hydroxyls at C-11 and C-21, but also the semiquinone moiety of the molecule would be appropriate for linkage to the carrier protein. Carboxymethylation of TAAc using *O*-carboxymethyl-hydroxylamine yielded a more polar product, as could be seen from TLC ($R_{fproduct}$ = 0.17, R_{fTAAc} = 0.62; methylene chloride/dioxane/water, 2+1+1 (*v/v/v*), lower layer), but the fragmentation pattern in the mass spectrum of the product differed from that of a 3-(*O*-carboxymethyl)-oxime.

TAAc also was treated with carbonyldiimidazole to prepare a reactive imidazolide[4–7] for conjugation to the carrier protein via C-11 since the α-ketol side chain is stabilized by

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keto-enol tautomerism. Acylation of the product (R_f = 0.07; cyclohexane/ethyl acetate/water, 25+75+1 [*v/v/v*]) using ε-amino caproic acid gave only small amounts of a tetrazolium blue reducing product showing a R_f = 0.13.

On the other hand, treatment of TAAc with succinic anhydride yielded large amounts of a product with R_f = 0.10 (cyclohexane/ethyl acetate/water, 25+75+1 (*v/v/v*). Compared to TAAc the IR spectrum of the product exhibited an additional signal at 1730 cm^{-1} (C=Ov). Upon hemisuccinoylation of TAAc by ^{13}C-NMR-spectroscopy four additional signals were recorded resembling succinic acid (171.9 ppm C-1', 28.7 ppm C-2', 28.8 ppm C-3', 176.1 ppm C-4'[8]). The chemical shifts of C-11 and C-21 were not altered, only the C-20 carbon (TAAc 210.5 ppm, hemisuccinoyl-TAAc 203.5 ppm) was shifted to higher field by esterification due to slightly deshielding. This effect arises from prevention of H-bond formation within the α-ketol side chain and refers to acylation at C-21[9]. Thus synthesis of 21-hemisuccinoyl-TAAc was assumed. The molecular weight also was confirmed by FAB mass spectroscopy (*m/z* (%): 535 (5) [M^+]), but fragmentation patterns were recorded indicating esterification both, in C-11 and C-21.

Elucidation of the acylation site is a prerequisite for use of the TAAc-derivative as a hapten, since in rodents immunosuppression and lymphocytolysis was induced by the glucocorticoid[10]. Therefore it was of interest to find out whether hemisuccinoylation occurred at C-11 or C-21, since only C-21 derivatives provide generation of antibodies. The fragmentation patterns in mass spectroscopy of TAAc and hemisuccinoyl-TAAc after acetylation of their hydroxyl groups were compared to find out the acylation site. Catalyzed acetylation of TAAc (R_f = 0.50) yielded 11,21-diacetyl-TAAc (mp = 232 °C, R_f = 0.78, *m/z* (%): 518 (84) [M^+]) exclusively. In contrast, acetylation without catalysis led to formation of both monoacetyl-TAAc (mp = 260 °C, R_f = 0.69, *m/z* (%): 476 (49) [M^+]) in high yield and only minor amounts of diacetyl-TAAc. After purification by preparative TLC, monoacetyl-TAAc exhibited fragment ions with high intensities derived from 21-acetyl-TAAc, but also fragment ions of low intensities related to 11-acetyl-TAAc (Table 1). Acetylation of hemisuccinoyl-TAAc (R_f = 0.24) resulted in formation of monoacetyl-TAAc and diacetyl-TAAc. Additionally a third compound was isolated (mp = 205 °C, R_f = 0.43). The molecular ion was observed at *m/z* (%): 576 (4)[M^+]) corresponding to acetyl-hemisuccinoyl-TAAc, the fragmentation pattern and its base peak at *m/z*: 417 indicate acetylation at C-11. Therefore hemisuccinoylation occurred at C-21. Additional formation of monoacetyl-TAAc and diacetyl-TAAc would be caused by hydrolysis during purification procedure replacing the aprotic solvent by a protic one.

Due to low signal intensity in the mass spectra of acetyl-hemisuccinoyl-TAAc, the apolar methyl ester was synthesized for structural analysis. Methylation yielded traces of monoacetyl-TAAc – possibly caused by overflow of traces of basic, water saturated diethyl ether – and high amounts of a compound, exhibiting mp = 158 °C and R_f = 0.70. By mass spectroscopy the molecular weight was estimated to be 591 Da, corresponding to the methyl ester of acetyl-hemisuccinoyl-TAAc. Fragment ions characteristic for TAAc were observed at *m/z* (%): 299 (100), 297 (100), 279 (100), 147 (26), and 122 (100). Indicating acetylation at C-11, fragment ions were recorded at *m/z*: 418 and *m/z*: 359 showing relative intensities of 100%, while the fragment ion at *m/z*: 317 – characteristic for acetylation at C-21 – had a relative intensity of only 46 % (Table 1). In conclusion, acetylation and methylation of hemisuccinoyl-TAAc mainly yielded 1'(11-acetyl-TAAc-21)-4'-methyl-succinate.

Since the glucocorticoid activity of TAAc is predominantly related to the α-ketol partial structure at C-21 and less to the semiquinone moiety and the hydroxyl group at C-11,21-hemisuccinoyl-TAAc as a hapten is expected to show no immunosuppression and to provoke formation of TAAc recognizing antibodies in rabbits.

Beyond that, by succinoylation, a 4-carbon spacer arm was introduced to expose the drug hapten from the surface of the carrier-protein, permitting free rotation of the desired epitope and therefore facilitating the production of TAAc-specific antibodies. The use of a spacer between the hapten and the protein has been shown previously to improve specificity[11] and recognition of the hapten[12].

Antigen Synthesis

The carboxyl containing TAAc derivative was coupled to free amino groups of the carrier-protein by the current amide bond generating methods. BSA was used as a carrier protein because of its high molecular weight (66 kDa) and immunogenic activity. Moreover, the protein exhibits a high number of free amino groups (59 lysyl residues and amino terminus) and solubility even in water miscible organic solvents as required for synthesis of antigens containing hydrophobic drugs[13]. Due to lack of cross-reactivity with BSA, conjugates using keyhole limpet hemocyanin (100 kDa at pH 8) as a carrier were prepared for specificity testing of the antisera.

Table 1: Fragment ions characteristic for acetylation of TAAc or hemisuccinoyl-TAAc at C-11 or C-21. The percentage is related to the relative intensity of the base peak (100%).

Compound	Acetylation at C-11 fragmention *m/z* =				Acetylation at C-21 fragmention *m/z* =		
	418	398	359	339	376	317	101
Monoacetyl-TAAc	100%	19%	56%	34%	16%	16%	5%
11,21-Diacetyl-TAAc	100%	24%	86%	41%	15%	44%	26%
11-Acetyl-21-hemisuccinoyl-TAAc-	100%	8%	7%	4%	0%	2%	4%
1'-(11-Acetyl-TAAc-21)-4'-methyl succinate	100%	16%	100%	28%	12%	46%	17%

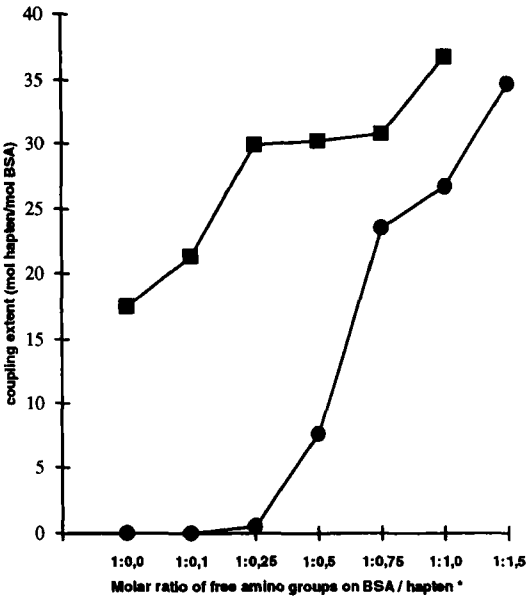

Figure 2: Coupling extent of hemisuccinoyl-TAAc-BSA conjugates. The curves show conjugates obtained by the mixed anhydride-method (●) and the carbodiimide-method (■). Coupling numbers were determined in duplicate by the dinitrofluorobenzene-test. The maximum number of derivatizable amino groups on BSA was 41 as determined by the same method.
*) 1:0 resembles BSA without hapten used for conjugation, whereas 1:1 is a mixture of 1 mol BSA (60 amino groups) and 60 mol hapten.

While antigen-synthesis using the imidazolide-method as described by Staab[14–16] failed, coupling by the carbodiimide-method yielded water-insoluble conjugates. The extent of coupling was estimated by the dinitrofluorobenzene (DNFB test, Fig. 2), showing an increase in the number of haptenated amino groups on BSA as the molar ratio of carrier-protein and drug increased. However, addition of only carbodiimide resulted in derivatization or crosslinking of about 50% of the accessible amino groups on the surface of the carrier-protein. Therefore the number of attached TAAc molecules had to be corrected by subtraction of the number of coupling agent modified amino groups of the carrier-protein alone from the number of modified amino groups of the conjugate. As a consequence, the true extent of coupling is reduced by about 17 mol compared to the number of modified amino groups of the carrier-protein. The TAAc conjugates, and also the carbodiimide-modified BSA, did not migrate in SDS-PAGE. As compared to conjugates prepared by the mixed anhydride-method, electrophoretic immobility of the conjugates is not due to derivatization by the hydrophobic steroid derivative, but derived from generation of coupling agent modified residues and intermolecular crosslinking of the carrier-protein by the coupling agent. These conjugates elicit antibodies recognizing the coupling agent modified residues but not the desired epitope[17].

Coupling by carbodiimide in a two step procedure according to Stein[18] led to formation of activated hemisuccinoyl-TAAc, but insoluble conjugates.

Using the carbodiimide/sulfo-NHS method according to Staros[19], KLH-TAAc-conjugates of low solubility were prepared. The coupling extent was 33 mol TAAc/mol KLH (assumed a molecular weight of 100 kDa at pH 8) as determined by the DNFB-test.

Table 2: Extent of coupling of hemisuccinoyl-TAAc-BSA-conjugates prepared by the mixed anhydride-method. Coupling numbers are related to the molar ratio of carrier protein/hapten and were determined by UV difference spectroscopy (UV-Diff), the dinitrofluorobenzene test (DNFB) or 7.5 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Molar ratio of free amino groups of BSA and hapten	UV-Diff	DNFB	SDS-PAGE	Percentage of haptenated amino residues on the surface of BSA
	(mol hapten /mol BSA)			
1 : 0.1	0.3	–	–	0 %
1 : 0.25	1.3	0.5	–	4 %
1 : 0.5	11.3	7.6	11	31 %
1 : 0.75	20.1	23.5	15	43 %
1 : 1.0	25.1	26.7	22	63 %
1 : 1.5	31.5	34.7	31	88 %

Antigen synthesis by the mixed anhydride-method using BSA as a carrier protein yielded water soluble conjugates without generation of coupling agent modified residues (Fig. 2). As the molar ratio of free carrier amino groups to hapten increased, the extent of coupling rose to 34 mol TAAc/mol BSA. A conjugation number of 34 mol TAAc corresponds to a derivatization of accessible lysyl amino residues on the surface of BSA to an extent of 88 % (58 % of the total number of amino groups). It was likely that maximum protein substitution was reached, since near saturating concentrations of the hapten in dioxane/water were used. As shown in Table 2, the results of the DNFB test are in accordance with those obtained by UV-difference spectroscopy of the soluble conjugates. Comparable coupling rates also were determined by SDS-PAGE (Fig. 3) considering the increase of molecular weight by substitution of BSA. Also intermolecular crosslinking of BSA by the derivatization reaction could be excluded.

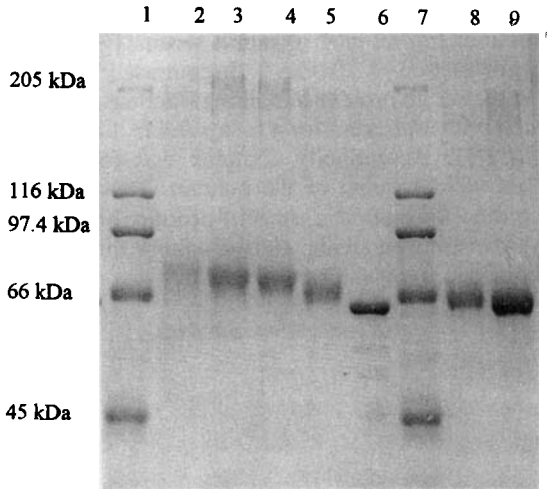

Figure 3: SDS-PAGE (7.5%) of TAAc-BSA conjugates. Molecular weight marker (myosin-subunit 205 kDa, β-galactosidase-subunit 116 kDa, phosphorylase B-subunit 97.4 kDa, BSA 66 kDa, ovalbumine 45 kDa): lane 1, 7; BSA: lane 6; TAAc-BSA conjugates (coupling number): lane 2 (31), lane 3 (22), lane 4 (15), lane 5 (11), lane 8 (1.3), lane 9 (0.3).

In a conjugate the haptens themselves serve as epitopes for binding to the antibodies on the B-cell surface and the carrier-protein provides the class II-T-cell receptor binding site. Therefore a minimum conjugation number of hapten, covalently attached to the surface of the carrier-protein should be reached. The minimum conjugation number of hapten ranges from 10 to 30 moles hapten per mol BSA [20,21]. In order to provoke both a cellular and a humoral immune response, the conjugate should possess at least partial water solubility.

Immunization

Using conjugates for immunization prepared by the carbodiimide-method not only provoked TAAc-specific antibodies, but also antibodies recognizing the carrier-protein and coupling agent modified residues. Thus a soluble BSA-conjugate, synthesized by the mixed anhydride-method, bearing 25 mol TAAc per mol BSA (Table 2) was used for immunization of two rabbits (antisera A and B). To gain TAAc recognizing antibodies appropriate for immunodiffusion experiments, another two rabbits were immunized (antisera C and D) using the KLH conjugate prepared by the method of Staros.

Specificity of the Antisera

For assessment of specificity of the antisera, an Ouchterlony double immunodiffusion assay [22] was performed. Precipitation lines were formed when BSA-containing antigen and antibodies of antiserum A or B combined, indicating an immune response related to the carrier-protein. By combination of the Ouchterlony double immunodiffusion assay and hapten inhibition techniques TAAc specific antibodies could not be detected, since formation of precipitin lines was not inhibited by addition of the drug. Also complex formation failed, when TAAc-BSA and antiserum C, D or KLH-TAAc and antiserum A, B were used in diffusion experiments (Fig. 4).

Because of the difference in molecular weight of IgG (160 kDa) and TAAc (436 Da), we tried to examine specificity by isolation of the soluble [^{3}H]TAAc-antibody complex using gel filtration. In presence of rabbit serum, [^{3}H]TAAc was detected in fraction 18-20 (Fig. 5). In contrast, [^{3}H]TAAc was found in the serum protein containing fractions 6 and 7 when incubated with antiserum for 4 h. A soluble, high molecular weight [^{3}H]TAAc-antibody complex was formed eluting nearer the void volume of the column. This effect is not attributed to nonspecific steroid-protein binding, since [^{3}H]TAAc exhibits a greater elution volume after incubation with rabbit serum for 4 h as well as 4 d.

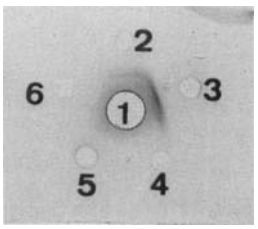

Figure 4: Ouchterlony double immunodiffusion assay of antiserum A. 1, antiserum A; 2, TAAc-BSA-conjuagte; 3, BSA; 4, KLH; 5, TAAc-KLH-conjugate; 6, phosphate buffered saline.

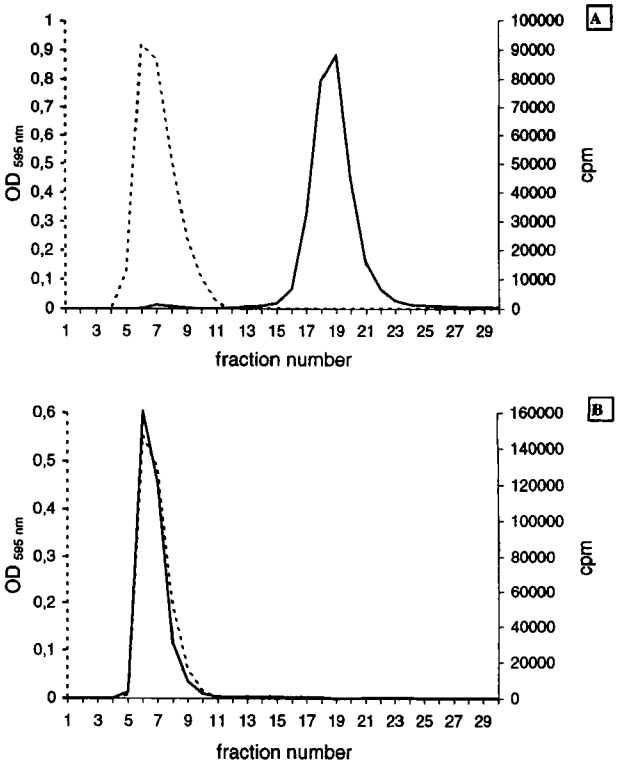

Figure 5: Column chromatography of a) 100 µl rabbit serum and 14.8 pg [^{3}H]TAAc after incubation at 4 °C for 4 h, b) 100 µl antiserum A and 14.8 pg [^{3}H]TAAc after incubation at 4 °C for 4 h. The protein content (– – –) was estimated by the Bradford assay, radioactivity (——) was determined by scintillation counting. The fraction size was 2 ml.

Radioimmunoassay

Using TAAc specific antibodies as a reagent in RIA for quantitative determination of the drug, there is a need of separation of free and antibody bound tracer. Precipitation of the TAAc-antibody complex by addition of saturated ammonium sulfate solution or goat anti rabbit-IgG was incomplete. Unbound TAAc could be removed quantitatively by nonspecific adsorption to dextran-coated charcoal, which forms a sticky pellet simply removable by centrifugation. As stated in preliminary assays (data not shown), for optimum results dilution of antiserum was 1:500, addition of 148 pg tracer was necessary using PBS for dilution and addition of 2 mg dextran-coated charcoal per sample is required.

Performing this RIA, upon addition of increasing amounts of TAAc the tracer was inhibited from binding to the antibody confirming TAAc-specificity of the antisera (Fig. 6). Reactivity of antisera obtained by immunization with a TAAc-BSA-conjugate was inhibited to 50 % (IC_{50}) by 291 ± 12 pg TAAc/100 µl (antiserum A, n = 3) and 729 ± 22 pg TAAc/100 µl (antiserum B, n = 3) respectively. Binding of the tracer to antisera derived from immunization with TAAc-KLH was also inhibited competitively by TAAc (antiserum C: IC_{50} = 221 ± 11 pg TAAc/100 µl, n = 3; antiserum D: IC_{50} = 354 ± 12 pg TAAc/100 µl, n = 3). According to the RIA-protocol, 20–1000 pg TAAc/100 µl specimen can be determined using antiserum A, C, or D 1:500, whereas upon the lower avidity of antiserum B, the lower detection limit is shifted to 250 pg TAAc. Exhibiting a standard error lower than ± 6 % (n = 3), the RIA method as described above allows

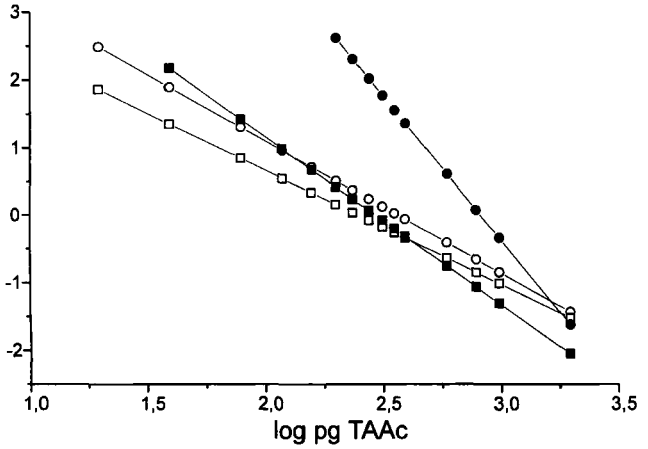

Figure 6: Inhibition of the binding of [^{3}H]TAAc to different antisera by addition of TAAc. RIA-procedures were as described in methods using a 1:500 dilution of antiserum A (■, $r = 0.995$, SD = 0.1165, $n = 14$), antiserum B (●, $r = 0.967$, SD = 0.3623, $n = 9$), antiserum C (❑, $r = 0.994$, SD = 0.098, $n = 15$) or antiserum D (○, $r = 0.996$, SD = 0.092, $n = 15$).

quantitation of TAAc in the range from 200 pg (0.459 pmol) TAAc to 10000 pg (22.9 pmol) TAAc/ml specimen. The lower detection limit of our assay is about 1000 times lower as compared to a published RIA-method[3] and would be sensitive enough for liberation studies of TAAc from ointments.

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Experimental Part

Chemicals

TAAc, BSA (99%, essentially globulin free), KLH (from Megathura crenulata), dinitrofluorobenzene, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride, complete and incomplete Freund's adjuvant were obtained from Sigma (St.Louis, MO, USA), [1,2,4,n-^{3}H]TAAc (2.83×10^7 cpm/mmol) from Amersham (Little Chalfont, U.K.), sulfo-*N*-hydroxysuccinimide from Pierce (Oud-Beijerland, Netherlands), solvents and buffer substances from Merck (Darmstadt, Germany) and Sephadex G-25 from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade and used as supplied.

Analytical Methods

TLC was performed on KGF_{254} sheets with methanol:water (4:1) as the mobile phase if not indicated otherwise. Detection of TAAc was performed by spraying with tetrazolium blue reagent[23]. (Just before use, equal volumes of 0.5 % tetrazolium blue in methanol and 8 % sodium hyroxide in methanol are mixed.). UV spectra were recorded on a DU 50 (Beckman), IR spectra on a Perkin-Elmer Model 257, ^{13}C-NMR on a Bruker WM 250 MHz. Mass spectra were recorded on a Shimadzu GC/MS-QP1000, DIMS; ion source: 70 eV, 250°C, 3×10^{-6} torr; scan: 50 – 700/2 s.

Synthesis of Hapten

TAAc (1 mmol, 435 mg) was converted to the corresponding hemisuccinate by refluxing with succinic anhydride (4 mmol, 400 mg) in 20 ml anhydrous pyridine in the dark for 4 h under water free conditions. After evaporation the oily residue was dissolved in chloroform and extracted with distilled water to remove excess of succinic acid. The dried organic layer was evaporated to dryness and the residue was crystallized from acetone. Recrystallization from acetone/*n*-hexane (2+1) gave 216 mg hemisuccinoyl-TAAc; mp = 219–221 °C, uncorrected; 40 % of theoretical yield.

To remove traces of TAAc, the product was purified by chromatography on 90 g silica 60 using chloroform/toluene/methanol, 5+2+1 (*v/v/v*).

Acetylation

A solution of 10 µmol steroid in 4 ml anhydrous pyridine and 4 ml acetic anhydride was stirred overnight in the dark at room temperature. The solution was poured into ice-water and the precipitate washed well with cold water. The residue was dissolved in chloroform and excess of acetic acid removed by washing with water. The dry residue was purified by preparative TLC (chloroform/methanol/water, 80+10+1 (*v/v/v*) and elution using chloroform/methanol (1+1, *v/v*) and methanol/water (1+1, *v/v*) respectively.

Methylation

Methylation was performed according to de Boer[24], which should assure methylation of only carboxylic acids and phenols. After evaporation of excess of diazomethane in diethyl ether, the residue was dissolved in chloroform and extracted with distilled water until neutral pH was reached. The dry residue was purified by preparative TLC as described above.

Preparation of Hapten-Protein Conjugates

In order to compare the coupling efficiency of all the batches, the total volumes of the different solutions used in the coupling procedures were held constant.

Coupling by the mixed anhydride-method was done as described by Erlanger [25] with slight modifications. In brief, 100 µmol (18.5 mg, 24 µl) tri-*N*-butylamine was added to a suspension of 100 µmol (53.5 mg) hemisuccinoyl-TAAc in 800 µl anhydrous dioxane to dissolve the steroid and the reaction mixture cooled to 10–13 °C. After addition of 100 µmol (13.65 mg, 13 µl) isobutylchloroformate the mixture was incubated at 12 °C for 30 min. The mixed anhydride of TAAc was poured into the solution of the carrier-protein of 4 °C, containing 1.6 µmol (105.6 mg) BSA in 6.5 ml dioxane/water (7+10; *v/v*). Immediately after initiation of the reaction 1M sodium hydroxide was added to raise the pH to 7.5–8.5 and the pH was kept constant for the first 2 h by readjustment with the same reagent. After incubation overnight at 4 °C, the conjugate was dialyzed against distilled water until no free drug was detectable.

Hemisuccinoyl-TAAc was also immobilized to BSA by the carbodiimide-method [18]. According to the molar ratio of hapten and BSA, a tenfold molar amount of an aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (pH 5.5) was added dropwise to the mixture of the solution of hapten in dioxane and 0.25 mM BSA (pH 5.5). The solution became more turbid as the molar ratio of carrier protein and hapten increased. After stirring overnight at room temperature, the conjugates were extensively dialyzed against distilled water.

Purity of the conjugates

For detection of remaining drug in the dialysis medium and possibly noncovalently bound hapten, the dialysis medium was evaporated to dryness after addition of n-propanol. The residue was dissolved in 100 µl chloroform and examined for TAAc after TLC (chloroform/methanol, 93+7 (*v/v*) by spraying with tetrazolium blue reagent.

After repeated sonification at intervals of 3 min. the liquid layer of an extraction step of about 10 mg conjugate in 3 ml distilled water and 3 ml acetone was evaporated to dryness and examined for unbound drug as described above.

Only conjugates, free of unbound drug were lyophilized for further analysis.

Determination of the Extent of Coupling

The coupling number of soluble and insoluble TAAc-protein-conjugates was determined by the dinitrofluorobenzene test[26] using *N*-ε-dinitrophenyl-lysin for calibration. Soluble conjugates were analyzed by UV difference spectroscopy too, calculating the number of haptens coupled per mol BSA from the absorption at 239 nm relatively to BSA. SDS-PAGE was used to

investigate gross structural changes of the carrier protein and calculation of the increase in molecular weight upon haptenation (Table 2)[27].

Immunization Procedure

TAAc-BSA (antiserum A, B) or TAAc-KLH (Antiserum C, D) was used for immunization of each of two outbred female rabbits (2.1–2.5 kg) using a solution of 1.0 mg TAAc-conjugate emulsified in a mixture of 1 ml sterile physiological saline and 1 ml complete Freund's adjuvant. Aliquots of 100 µl were injected intradermally into the back at six sites each. Twice, booster injections were given subcutaneously near the poplietal lymph nodes at intervals of four weeks using incomplete Freund's adjuvant and animals were bled 8 d later[28]. After clotting, serum was separated by centrifugation and stored in small aliquots at –20 °C.

Screening for Soluble [^{3}H]TAAc-Antibody Complex

Separation of [^{3}H]TAAc-antibody complex was carried out by gel filtration through a column of Sephadex G-25 (0.5 × 25 cm) using 50 mM phosphate buffered saline pH 7.4. The flow rate was controlled by a P2-pump (Pharmacia, Uppsala, Sweden) adjusted to 0.22 ml/min. The effluent was collected with a fraction collector in 2.0 ml fractions. Aliquots were analyzed by both, the Bradford assay[29] (200 µl aliquot and 2 ml Bradford reagent, OD at 595 nm after 30 min) to localize the protein peaks and by scintillation counting (1 ml sample and 4 ml PPO/POPOP-cocktail, 5 min) to localize the tritiated drug. The void volume was 12 ml and the range of separation was 74 ml, as determined by passing blue dextran or bromophenol blue respectively.

Radioimmunoassay Procedure

Phosphate buffer (50 mM, pH 7.4) containing 0.3 % BSA, 1 % Triton-X-100, and 1 mM EDTA was used as the diluent throughout. A standard curve was constructed by setting up duplicate Eppendorf vials containing 0–1950 pg of non-labeled TAAc (100 µl) and 148 pg [^{3}H]TAAc (100 µl). 100 µl of the antisera A, B, C, D 1:500 or pre-immune serum 1:500 (100 µl) were added and the mixture was incubated overnight at 4 °C. After incubation with 200 µl of 1 % dextran-coated charcoal (Norit A and dextran T70) for 15 min at room temperature, the bound and free steroids were separated by centrifugation at 4000 rpm. A 400 µl aliquot of each supernatant was transferred to a counting vial and 4 ml scintillation cocktail (1 g 2,5-diphenyloxazole and 50 mg 1,4-bis[5-phenyl-2-oxazolyl]-benzene in 200 ml toluene and 100 ml triton-X-100) were added. Radioactivity was quantitated in a Beckman LS 7000 liquid scintillation counter.

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