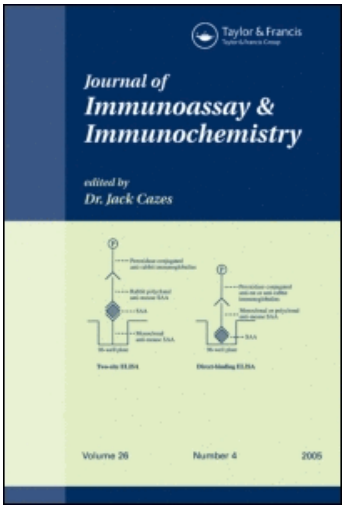

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"Custom" Synthesis of Radioligands for RIA through Activated Esters I. Testosterone

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"CUSTOM" SYNTHESIS OF RADIOLIGANDS FOR RIA THROUGH ACTIVATED ESTERS I. TESTOSTERONE

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ABSTRACT

The current method of labeling small molecules for radioimmunoassay by coupling iodohistamine to haptens through a mixed anhydride reaction is unacceptable to clinical laboratories. Therefore, we propose the use of a simple two-step procedure: treatment of ^{125}I-2-iodohistamine with the activated ester of a small molecule followed by thin layer chromatography to remove unlabeled ligand. Only one radioactive substance, ^{125}I-2-iodohistamine, need be stocked, and the availability of labeled ligands is limited only by the number of nonradioactive activated esters. This principle is illustrated by the use of testosterone. N-Hydroxysuccinimidyl esters of testosterone hemisuccinate and of testosterone-3-carboxymethyloxime were coupled to 2-iodohistamine, ^{125}I-2-iodohistamine or to ^{125}I-2,5-diiodohistamine. Optimum conditions required reaction of 20-50 fold molar excess of ester in 75µL of tetrahydrofuran with iodohistamine in 75µL of buffer at pH 8.5 for 30 min at 4°. The reaction mixture was applied directly to a pre-absorbent TLC plate coated with silica gel and run in the system, benzene:ethanol:acetic acid, 75:24:1 (v:v:v). The desired radioligand was eluted in 85% yield.

The performance of iodine-125 mono-and diiodinated histamine derivates of testosterone 3-carboxymethyloxime (T-3-CMO) and testosterone hemisuccinate were tested as model compounds using testosterone antisera directed against both ends of the molecule. Using non-radioactive testosterone as the displacing ligand, apparent association constants were not greatly different from those using ^{3}H-testosterone as the radioligand. When non-radioactive T-3-CMO was used as the displacing ligand in a homologous

system, however, the apparent association constant increased more than tenfold. The specificity of both antisera improved slightly using ^{125}I-2-iodohistaminyl ligands. More work will be required to determine the best combination for clinical usage; theory would favor the heterologous system.

INTRODUCTION

Ciiby et al. (1) suggested that iodoimidazoles might be more stable than iodophenols and, hence, more suitable for labeling ligands in radioimmunoassay procedures. More specifically, they suggested replacing iodotyrosine derivates with iodohistamines. In a previous paper, we published the synthesis of three iodohistamines as well as that of ^{125}I-2-iodo- and ^{125}I-2,5-diodohistamines. The latter was shown to be less stable than the former (2).

Coupling of iodohistamines to the assay ligand has always been done by means of a mixed anhydride reaction (3-7). This procedure, requiring several extractions as well as a thin layer chromatographic step, has presented a barrier to its adoption in the clinical chemistry laboratory. In this paper we present an alternative proposal which has the hallmark of universality.

N-Hydroxysuccinimide esters are currently widely used in biochemistry. As activated esters, they are less reactive than N-hydroxyphthalimidyl (8,9) and dinitrophenyl (10,11) esters (and, hence, more stable), but sufficiently reactive to be attacked by even a weak nucleophile. Their synthesis through a carbodiimide reaction is technically less demanding than the mixed anhydride reaction and, in our hands, provides higher yields. Such esters will react with any primary, unhindered amine, such as histamine or tyrosine esters, either free or iodinated, to form a stable ligand in a single step.

The radioactive ligands produced were compared with ^{3}H-T in binding to two anti-testosterone antisera. There was general agreement among the various ligands, indicating essential equivalence of ^{125}I-2-iodohistamine ligands with tritium labeled ligand.

We question, however, the suitability of 2,5-diiodohistamine ligands on several grounds.

EXPERIMENTAL

Materials

Testosterone-3-(O-carboxymethyl) oxime (T-3-CMO,I) (12) was synthesized by three methods: that of Brenner *et al.* (13) gave higher crude yields (80%) than did the strongly alkaline conditions of Erlanger *et al.* (14) (68%) or Chen *et al.* (15) (65%). The properties of the compound used are as follows: mp 179-181° (lit., 167-185° crude product (14)); λ_{max}^{EtOH}, 250 nm, ε_{250}^{EtOH}, 15,400. Testosterone hemisuccinate (T-17β-succ, IV) was synthesized in 85% yield by refluxing the steroid with succinic anhydride. Properties are as follows: mp 186-187°; λ_{max}^{EtOH}, 241 nm, ε_{241}^{EtOH}, 17,400. 2-Iodohistamine (decomposition point, 220°; λ_{211}^{EtOH}, 5,500) and ^{125}I-2-iodohistamine were prepared by us (2). Cyclohexylcarbodiimide, N-hydroxysuccinimide, histamine dihydrochloride and carboxymethoxylamine (O-(carboxymethyl) hydroxylamine) hemihydrochloride were purchased from Aldrich Chemical Co., Milwaukee, Wis.; steroids from Steraloids, Inc., Wilton, NH; alumina (Woelm neutral, activity grade 1) from Sigma Chemical Co., St. Louis, MO.; one antibody was produced in sheep against T-3-CMO-RSA (Research Plus Steroid Laboratories, Inc., Denville, NJ) and the other was produced by us in a goat against T-17β-succinyl-BSA in 1973; 1β,2β-^{3}H-testosterone (40 Ci/ mmole) was purchased from New England Nuclear Corp. (Boston, MA); and all other chemicals were purchased from Fisher Scientific Co., Rochester, NY.

Solvents

All solvents were grade A and were used without purification with the exception of ethers. Water-insoluble ethers were purified by shaking with a fresh solution of 10% potassium iodide

while water-soluble ethers were passed through a column of alumina (Woelm neutral, activity grade 1).

Equipment

Melting points, determined on a Fischer-Johns melting point apparatus, are uncorrected. Infrared spectra of nujol mulls were recorded on a Beckman IR-33 spectrophotometer, calibrated using the 1603 cm^{-1} band of a thin polystyrene film. Data are reported in reciprocal centemeters. Ultraviolet spectra were recorded on a Perkin-Elmer double beam spectrophotometer connected to a Perkin-Elmer recorder; data are reported in nanometers. Radioactivity was quantitated on a Packard model 1578 gamma scintillation spectrometer. Microanalyses were performed in the Department of Chemistry at this University or by Galbraith Laboratories, Knoxville, TN.

Buffers

Sodium phosphate buffer, 0.1 M, pH 6.5-8.0 and sodium borate buffer, 0.1 M, pH 8.5 and 9.0 were employed. The RIA assay buffer was phosphosaline-gelatin (PS-G) buffer, 0.05 M, pH 7.0, prepared by dissolving 6.89g of $NaH_2PO_4 \cdot H_2O$, 7.1g of Na_2HPO_4, 8.76g of NaCl, 1.00g of NaN_3 (practical) and 1.00g of unflavored gelatin in 1.00 L of solution.

Polyethylene glycol (PEG) solution

30g of Carbowax 6,000 and 0.1g of NaN_3 was dissolved in 100 mL of water.

Chromatography

Thin layer plates coated with silica gel containing a phosphor (Quantum LQDF preadsorbent plates, Pierce Chemical Co., IL) were used in the solvent system, toluene:ethanol:acetic acid, 75:24:1 (v:v:v).

Syntheses:

N-Hydroxysuccinimidyl ester of testosterone-3-(O-carboxymethyl) oxime (II)

(O-(2-oxo-3-(2', 5'-dioxypyrrol-1'-yl-3-oxapropanyl) N-17β-hydroxyandrost-4-en-3-ylidene hydroxylamine). N-Hydroxysuccinimide was recrystallized from ethyl acetate by addition of di-2-propyl ether. Then, 1.15g (10 mmoles) of this compound and 3.61g (10 mmoles) of testosterone-3-(O-carboxymethyl) oxime were dissolved in a minimum volume (15 mL) of purified pyridine. This mixture was cooled to less than -10° (the beaker containing the solution was placed in acetone-dry ice) at which point, 2.47g (12 mmoles) of dicyclohexyl carbodiimide was added. The solution was stirred at -10° for two hours, left at room temperature for ten hours and then treated with 0.12 mL of acetic acid to destroy excess dicyclohexyl carbodiimide. After one hour, the mixture was diluted with 5 mL of tetrahydrofuran; the precipitate consisting of dicyclohexyl urea was filtered off and washed with tetrahydrofuran. The combined filtrate and washings were evaporated to dryness under reduced pressure at $25-30^{\circ}$ to yield a dark, oily product which solidified upon addition of cold water. The product was dissolved in methanol and treated several times with neutral charcoal until a white solid was obtained. It was then crystallized twice from methanol and once from tetrahydrofuran/light petroleum ether. Yield of crude product, 82%. The pure compound had the following properties: mp, 157-159°; λ_{max}^{EtOH}, 244; ε_{244}^{EtOH}, 36,900; νmax, 1830 and 1790 (C = O of succinimide), 1740 (C = O ester), 1635 (N = C conj.); R_f, 0.55; calcd. for $C_{25}H_{34}N_2O_6$: C, 65.48, H, 7.47; exptl, C, 65.67, H, 7.50.

N-Hydroxysuccinimidyl ester of testosterone hemisuccinate (V) (3-oxoandrost-4-en-17β-yl N-2,5-dioxypyrrolyl succinate)

Testosterone hemisuccinate (3.9g or 10 mmoles) was esterified by the method described above, using 1.15g (10 mmoles) of N-hydroxysuccinimide and 2.4g (12 mmoles) dicyclohexyl carbodiimide.

Crude yield, 80%. Its properties are as follows: mp, 139-142; λ_{max}^{EtOH}, 242; ε_{max}^{EtOH}, 34,200; νmax 1815 and 1790 (C=O of succinimide) 1750 (C=O, ester), 1630 (C=O, ketone); R_f, 0.50; calcd. for $C_{27}H_{35}NO_7$; C, 66.78; H, 7.27; exptl. C, 66.80; H, 7.07.

0-(histaminylcarboxymethyl) testosterone oxime (O-(2-oxo-5-(imidazol-4'- yl)-3-azapentanyl) N-17β-hydroxyandrost-4-en-3-ylidene hydroxylamine) (III without iodine)

To a solution of 0.37g (2 mmoles) of histamine dihydrochloride in a minimum volume (5 mL) of borate buffer, pH 8.0, was added 0.925g (2 mmoles) of the N-hydroxysuccinimide ester of testosterone-3-O-carboxymethyloxime in 10 mL of tetrahydrofuran. The mixture was stirred at 4° for two hours after which it was poured into cold water. Attempts at crystallization were unsuccessful in producing the desired compound in the following solvents: methanol/water; 100% methanol; benzene/water or benzene/acetone. (A crystalline byproduct was identified as testosterone-3-O-carboxymethyloxime by its melting point and R_f). The mother liquor of the mixture, after filtering off the byproduct, was evaporated and the amorphous compound obtained was treated with charcoal until a white product was obtained; this compound crystallized from methanol. Crude yield, 59%. The properties of the compound are as follows: mp, 212-225; λ_{max}^{EtOH}, 200 and 248; ε_{200}^{EtOH}, 5,200 ; ε_{248}^{EtOH}, 14,600 , ν_{max},3340 (N-H + OH); 1630 (C=O, amide I); 1575 (N-H, amide II), 1310 (N-C, amide III); R_f, 0.60.

Oxoandrostenyl histaminyl succinamate (3-oxoandrost-4-en-17β-yl N-2-(imidazol-4'-yl)ethyl succinamate) (VI without iodine).

It was synthesized as above, using 0.46g (2.5 mmoles) of histamine dihydrochloride and 1.21g (2.5 mmoles) of N-hydroxysuccinimidyl ester of testosterone hemisuccinate. The same method of purification of the product was applied. Crude yield, 65%. Its properties are as follows: mp 218-221°, λ_{max}^{EtOH}, 202 and 242, ε_{202}^{EtOH}, 6,000; ε_{242}^{EtOH}, 16,100; ν_{max} , 3360 (N-H); 1630 (O-C-N, amide I); 1580 (N-H, amide II); 1320 (C-N, amide III); R_f, 0.62.

O-(2-iodohistaminylcarboxamidomethyl) testosterone oxime (III) {O-2-oxo-5(2'-iodoimidazol-4'-yl)-3-azapentanyl N-17β-hydroxy-androst-4-en-3-ylidene hydroxylamine}

This compound was synthesized in the same manner as the non-iodinated form using 0.62g (2 mmoles) of 2-iodohistamine and 0.92g (2 mmoles) of N-hydroxysuccinimidyl ester of T-3-CMO. The product was purified with charcoal as previously described. Crude yield, 60%. Its properties are as follows: mp, 202-205, λ_{max}^{EtOH}, 203 and 248; ε_{203}^{EtOH}, 5,500 ; ε_{248}^{EtOH}, 14,600; ν_{max},(similar to noniodinated form); R_f, 0.31.

Oxoandrostenyl-2-iodohistaminyl succinamate (V) {3-oxoandrost-4-en-17β-yl N-2-(2'-iodoimidazol-4'-yl)ethyl succinamate}

This compound was also synthesized in the same manner as the non-iodinated form; 0.62g (2 mmoles) of 2-iodohistamine and 0.98g (2 mmoles) of N-hydroxysuccinimidyl ester of T-17β-hemisuccinate were used. The same method of purification was applied. Crude yield, 57%. Its properties are as follows: mp, 190-192; ε_{202}^{EtOH}, 5,800 ; ε_{240}^{EtOH}, 15,900 ; ν_{max},(similar to non-iodinated form); R_f, 0.35.

Reaction of ^{125}I-2-iodohistamine with activated esters

Fifty nanomoles (10 µL of a 5.0 mM ethanolic solution) of N-hydroxysuccinimidyl ester of testosterone-3-(O-carboxymethyl) oxime or testosterone hemisuccinate was added to a crude iodination mixture (2) and stirred for 30 min at 4°; the mixture was then chromatographed on a thin layer plate. After air drying for one hour; the plates were directly applied to X-ray film (Kodak RP, rapid processing film) by inserting them between two boards and covering them with aluminum foil. After 30 min in the dark, the films were developed and the areas corresponding to the 2-iodohistamine derivatives were carefully scraped off and extracted with 10 mL methanol. The diiodohistamine derivatives were also located, scraped off and extracted with 10 mL methanol. These

derivatives were stored at these concentrations in the cold room and diluted as needed at the time of radio-immunoassay.

Radioimmunoassay

To 12 x 75 mm disposable polyethylene tubes was added in triplicate 100 µL PS-G buffer, 100 µL of nonspecific bovine gamma globulin, 100 µL of antiserum diluted with PS-G buffer to give 50-60% binding, 100µL of radioactive ligand (*ca*. 0.1 pmole of iodinated ligand or 0.2 pmole of ^{3}H-T) and 100 µL of steroid (10µg/mL of 95% ethanal diluted to 50-1,000 pg/100 µL with the assay buffer) or plasma extract. The "zero" tube contained no nonradioactive steroid. After standing overnight at room temperature, the tubes were placed in an ice-water bath for 30 min; then 400 µL of PEG solution was added except for three "zero" tubes to which 400 µL of assay buffer was added in order to determine total radioactivity. The tubes were vortexed, incubated a further 15 min and centrifuged. The supernatant, which contains the unbound fraction, was decanted into counting vials to which 5 mL of liquid scintillation fluid was added. When a gamma counter was used, the bound radioactivity in the precipitate was counted directly.

Binding constants were derived by Scatchard analysis using the method of Walker (16) in which $Y = \frac{Y' - \beta}{\alpha - \beta}$ where α and β are the upper and lower limits of Y', the fraction bound when α and β are not taken into account. In each case, α was determined by using a concentration of antibody 10-100 times that used for the standard curve while β was the nonspecific binding found using 1µg of nonradioactive steroid. Values are given in Table 3.

Relative binding activity (RBA) used the relationship proposed by Rodbard and Lewald (17) in which

$$RBA = \frac{\frac{f}{b} + 1}{\frac{K_t}{K_s} \times \frac{f}{b} + 1}$$

where K_t and K_s are association constants of testosterone and cross-reacting steroid, respectively. This method has the advantage of using points in the region of highest cross-reaction at low concentrations of antigens.

RESULTS AND DISCUSSION

N-hydroxysuccinimidyl ester of testosterone-3-(O-carboxymethyl) oxime (II)

This compound was obtained in good yield by the method of Rudinger and Ruegg (18), using equimolar amounts of testosterone carboxymethyloxime and N-hydroxysuccinimide with 20% excess dicyclohexyl carbodiimide. The presence of the succinimidyl group considerably increased the molar extinction coefficient of the steroid derivative (ca. twice that of testosterone). This was also noticed on TLC with N-hydroxysuccinimidyl esters showing a more intense purple color under ultraviolet light (254 nm) than did those of testosterone. The infrared spectrum showed characteristic bands of an ester derivative; i.e., strong ester carbonyl stretching band at 1740 cm^{-1}, strong ester C-C and C-O coupled stretching at 1220 cm^{-1}. Convincing proof of the assigned structure was the elemental analysis which was in good agreement with the calculated values for carbon and hydrogen.

N-hydroxysuccinimidyl ester of testosterone hemisuccinate (V)

Physical properties similar to those above were observed. The infrared spectrum showed a characteristic band at 1750 cm^{-1} (carboxylic ester carbonyl stretching); the C-C and C-O coupled stretching were present at 1220 cm^{-1}; the C-3 ketone carbonyl group was still present at 1670 cm^{-1}. Elemental analysis provided further proof of the assigned structure.

Stability of the activated esters

N-Hydroxysuccinimidyl ester of testosterone carboxymethyloxime

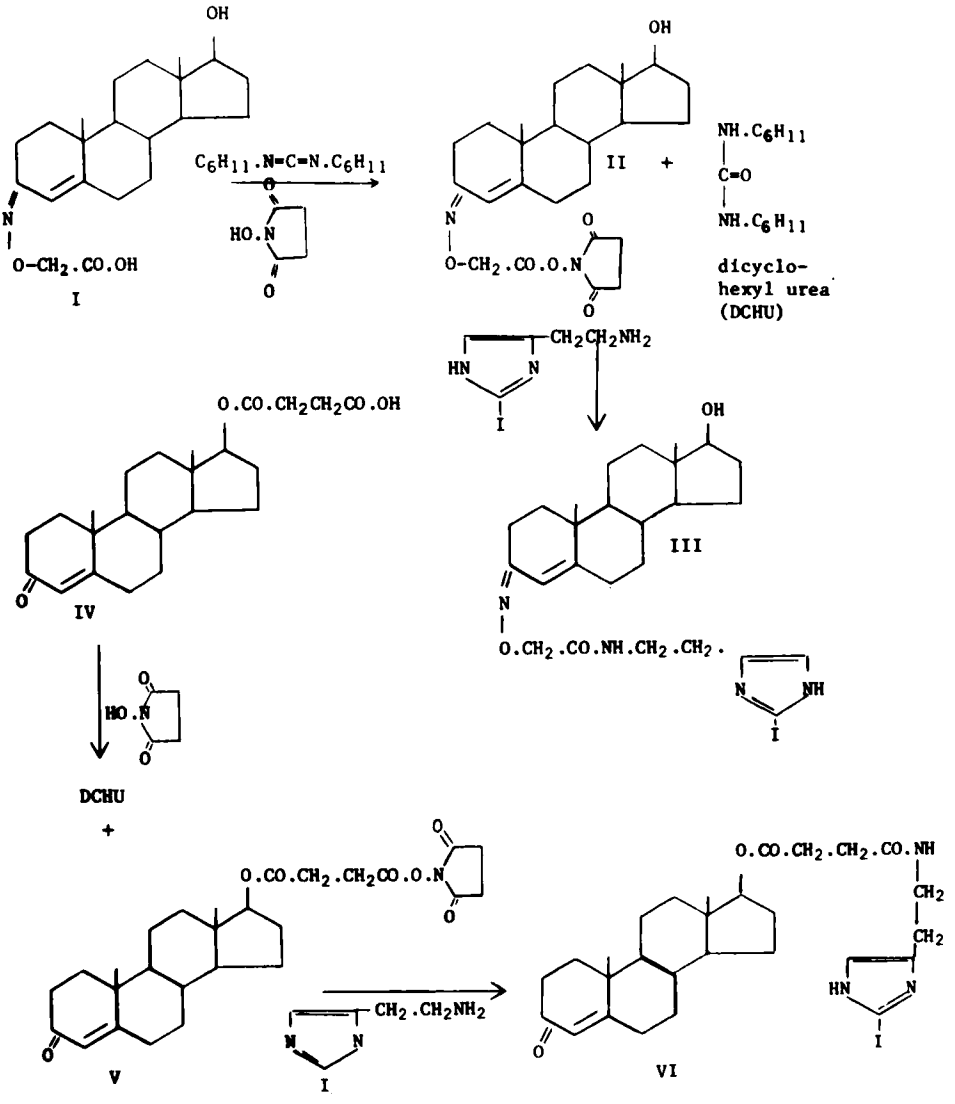

FIGURE 1. Reaction Scheme

was taken as an example for this study. Even though N-hydroxy-succinimidyl esters are not as reactive as other activated esters, they are reactive enough to be very quickly attacked by a nucleophile (even a weak one), and we anticipated some hydrolysis in aqueous media. At pH 6.5, only 9% of the ester was hydrolyzed

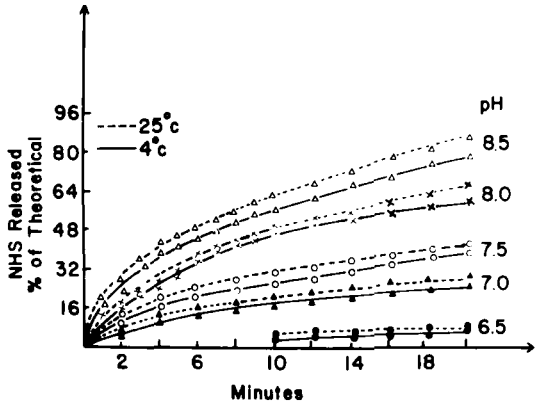

FIGURE 2

Stability study of N-hydroxysuccinimidyl ester. Two hundred and fifty micromoles of N-hydroxysuccinimidyl ester of testosterone carboxymethyloxime was dissolved in dioxane:buffer (1.5 ml:1 ml) at the pH indicated. At the times indicated aliquots were tested for the release of N-hydroxysuccinimide (NHS) by a modified Folin method (19), %NHS released = (concn. NHS in aliquot/initial concn) 100.

after 20 min, whereas at pH 8.5, 86% of the ester was hydrolyzed (Fig 2). The hydrolytic process was only slightly slowed by decreasing the temperature. From this information we recommend that the activated ester should be stored in an anhydrous condition in plastic containers at low temperature.

Reaction of iodohistamine with activated esters

The coupling reaction was attempted at different pH's (Table 1); the optimum yield was obtained at pH 8.5. This result, which may seem peculiar at first glance because it appears contradictory to the fact that there is rapid hydrolysis of the ester at this pH can be interpreted as follows. There are two amino groups in histamine having different pK's. Coupling requires the presence of an unprotonated amino group to act as a nucleophile. The

pK of the imidazolyl amino group is around 5.87 (20) and at low pH, coupling presumably involves only this amino group. N^{im}-Acyl imidazolyl derivatives, however, are unstable. Boyer (21) reported the impossibility of synthesizing N^{im}-acetyl imidazole in an aqueous medium and N^{im}-acyl histidine was similarly reported to be unstable (22). The net result of the two-step reaction is an acceleration of the hydrolysis of the activated ester. The pK of the side chain amino group is 9.70 (22); at pH 8.5, 6.3% are dissociated. Reaction favors the primary amino group rather than the ring secondary amino group and the amide formed is stable. Therefore, as one increases the pH, there is an increase in the amount of stable product, but also more hydrolysis of activated ester. Thus, the outcome is the "resultant" of two opposing forces: as the pH increases, there is (1) increasing dissociation of the primary amine favoring increased acylation to give the stable

TABLE 1

Effect of pH on the Yield* of the Coupling Reaction**

	pH			
Expt.	7.0	8.0	8.5	9.0
#1	38	52	60	58
#2	40	51	65	55

*The yields determined gravimetrically are expressed as percent of iodohistamine incorporated and are corrected for procedural losses determined as follows: 200 mg of compound III (Fig 1) was dissolved in methanol; the solvent was evaporated, the precipitate washed with water, collected and dried. About 177 mg was obtained, corresponding to a recovery of about 85%.

**Reaction between compound II and 2-iodohistamine (Fig 1). The latter compound was dissolved in 2.5 ml borate buffer in experiment #2 and in 2.5 ml phosphate buffer in experiment #1 at the indicated pH; the ester was dissolved in *ca.* 4 ml of tetrahydrofuran. The reaction was carried out at 4° for 30 min with a twofold molar excess of ester (0.46 g of ester and 0.15 g of 2-iodohistamine).

TABLE 2

Effect of Time on Coupling*

	Time (min)						
	10	20	30	40	60	90	120
Yield**	28	46	59	60	61	60	59

*Reaction was carried out at 4° using 0.46g (1 mmole) of N-hydroxysuccinimidyl ester of T-3-CHO in ca. 4 ml tetrahydrofuran with 0.15 g of 2-iodohistamine (0.5 mmole) dissolved in 2.5 ml 0.1 M borate buffer, pH 8.5 for the length of time indicated.

**yields corrected for procedural losses are based on iodohistamine.

product and (2) increased hydrolysis of activated ester lowering the availability of substrate.

A maximal yield was attained by 30 min when a twofold excess of ester was employed (Table 2). This yield was increased to 75-80% when a 50 fold molar excess of either reactant was used (data not shown).

The purity of the non-radioactive ligands obtained was established through the sharpness of the melting point and the constancy of the molar extinction coefficient in the ultraviolet. The presence of characteristic amide absorption bands in the infrared confirmed the identity of the products. Adducts with 2,5-diiodohistamine were also prepared even though 2,5-diiodohistamine is less stable than 2-iodohistamine (2).

Coupling on a microscale using labeled iodohistamines gave products identical to those of the authentic standards. When 5 nmoles of histamine was iodinated with 0.25 nmoles of iodide-125 (500 μCi) and the crude reaction mixture then reacted with 50 nmoles of activated ester, we isolated after TLC 345 μCi (69% yield) of 2-iodohistaminyl adduct and 22 μCi (4% yield) of 2,5-diiodohistaminyl adduct.

We did not isolate ^{125}I-2-iodohistamine prior to coupling as this would have entailed an additional TLC. Calculation shows,

however, that if we had done so the yield of the coupling reaction would have been 85-90% (after TLC). It is anticipated that ^{125}I-2-iodohistamine will be commercially available in the near future.

The ligands labeled with iodine-125 were compared with the traditional tritiated ligand. No consistent trends are seen in the association constants of the 2-iodohistaminyl ligands, the association constants remaining mostly in the range of 0.33-3.0 times the association constant of ^{3}H-T (Table 3) except perhaps in the case of the homologous ligands.

Gilby and Jeffcoate (23) have pointed out that in the homologous system both the ligand and the antigen contain the same bridge and the antibody "sees" the "bridged" ligand as more like

TABLE 3

Properties of the Two Antibodies for Each of Five Labelled Testosterone Preparations

Radioligand	α	β	T-3-CMO-RSA Ab titer	$K_a(4^o)nM^{-1}$
^{3}H-T	85	5	1:800†	1.25 ± 0.3*
T-3-CMO-2-I	48	1	1:1,100	0.3 ± 0.02
T-3-CMO-2,5-I	80	5	1:300	1.0 ± 0.1
T-17β-succ-2-I	90	3	1:700	3.0 ± 1.0
T-17β-succ-2,5-I	50	2		0.3 ± 0.01
			T-17β-succinyl-BSA Ab	
^{3}H-T	92	10	1:5,000†	0.6 ± 0.1*
T-3-CMO-2-I	60	1	1:6,000	0.1 ± 0.05
T-3-CMO-2,5-I	72	3		0.2 ± 0.02
T-17β-succ-2-I	88	7	1:18,000	0.3 ± 0.07
T-17β-succ-2,5-I	48	1	1:4,000	0.9 ± 0.01

†titers producing 50% binding with ca. 0.1 pmole of iodinated ligand and 0.2 pmole of ^{3}H-T

*Values represent the mean ± S.D. of interassay points (n=3) after correction for α and β (see Methods for detail); note that the radioligand designated was displaced by nonradioactive testosterone.

itself and binds it more avidly than it does tritiated testosterone. We tested this by using T-3-CMO instead of non-radioactive testosterone in an assay with anti-T-3-CMO-RSA antiserum and T-3-CMO-2-I as the radioligand. The association constant was 12 ± 3.4nM^{-1} (n = 4), confirming previous observations (23) that the bridge does have a positive effect on binding.

Since assay sensitivity is directly related to the association constant (or the minimal amount detectable is inversely related to K_{ass}) (16), one could improve the assay sensitivity by at least an order of magnitude by derivitizing the ligand before assay. This is, of course, the basis of a vastly improved assay for cAMP (24,25). In the case of steroids, it will be profitable to attempt a similar course only in the case of those steroids present in very low concentrations, for example, estradiol and aldosterone.

The 2,5-diiodohistaminyl ligands were not fully investigated for reasons not apparent here. Diiodination of histamine does not occur readily at neutral pH's and by the time pH 10 is reached, triiodination has become prominent (2). Therefore, preparation of ^{125}I-2,5-diiodohistamine is economically unattractive. Furthermore, 2,5-diiodohistamine is somewhat unstable, decomposing with a half-life of approximately 180 days, whereas 2-iodohistamine is quite stable (2). The data shown (Table 3) indicates that in 3 out of 4 cases the diiodinated derivate binds as well as or better than the monoiodinated ligand.

The other very important parameter in RIA is specificity. We checked this for each homologous system (Table 4). In all but one case, the 2-iodohistaminyl ligand was slightly more specific than the tritiated testosterone in contrast to a previous report (7).

Thus, the two major criteria of RIA, sensitivity and specificity, are satisfied. It is, of course, obvious - as it has been for years - that the anti-T-3-CMO-RSA antiserum should be used in practical applications.

We have not addressed the next question of whether to use a

TABLE 4

e Association Constants and Relative Binding Activity (RBA) of Various Steroids Using
ed Labeled Steroids and Non-Radioactive Steroid

	T-3-CMO-RSA Ab				T-17β-succ-BSA Ab			
	K_a		RBA		K_a		RBA	
d	^{3}H-T	T-3-CMO-2-I	^{3}H-T	T-3-CMO-2-I	^{3}H-T	T-17β-succ-2-I	^{3}H-T	T-17β-
one	1	3	1.00	1.00	0.2	0.56	1.00	1
	0.2	0.11	0.56	0.29	0.15	0.12	0.97	0
one	0.0005	0.0005	0.001	0.001	0.12	0.10	0.78	0
corti-								
one	0.012	0.005	0.006	0.001	0.18	0.07	0.51	0
nedione	0.005	0.003	0.03	0.01	0.07	0.05	0.32	0
nediol	0.015	0.01	0.04	0.02	0.03	0.01	0.29	0
e	—	—	—	—	0.06	0.03	0.30	0

homologous or heterologous radioligand in practice. Theory predicts (16) the inadvisability of making K* > K. Since we did not have analytically pure (as determined by elemental analysis) 2-iodohistaminyl ligands, K*, the association constant of the radioligand, was never determined. T-3-CMO, however, had an association constant more than tenfold greater than that of testosterone, indicating that the association constant of T-3-CMO-^{125}I-2-iodohistamine would probably be much greater than that of testosterone when the anti-T-3-CMO-RSA antiserum was employed. Conversely, one might predict that T-17β-succinyl-^{125}I-2-iodohistamine would have an association constant to the same antiserum slightly less than that of testosterone. If so, this radioligand would produce a steeper standard curve and a more reproducible assay.

In summary, the advantages of the activated ester method are the ease, simplicity and rapidity of preparation of radioligands, making "custom" synthesis feasible. Reaction takes 30 min, TLC 1-2 hr and detection and elution 0.25 - 1 hr. Thus, pure labeled ligand can be obtained in 2-4 hr. It is stable and can be counted in a gamma counter. The only limitations presently are the availability of activated esters and the decision in regard to the use of a homologous or heterologous radioligands.

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12. The abbreviations used are as follows: T, testosterone, 17β-hydroxyandrost-4-en-3-one; T-3-CMO, testosterone-3-O-carboxymethyloxime (I); T-3-CMO-2-I, the primary amide formed from 2-iodohistamine and T-3-CMO (III); T-3-CMO-2,5-I, the 2,5-diiodohistamine analog of (III); T-17β-succ, testosterone hemisuccinate (IV); T-17β-succ-2-I, the primary amide formed from T-17β-succ and 2-iodohistamine (VI); T-17β-succ-2,5-I, the 2,5-diiodohistamine analog of (VI); 5α-DHT, 17β-hydroxy-5α-androstan-3-one; androstenedione, androst-4-ene-3,17-dione; androstenediol, androst-5-ene-3β,17β-diol; RSA, rabbit serum albumin; HSA, human serum albumin.

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