TRITIUM LABELING OF STEROIDS BY MICROWAVE DISCHARGE ACTIVATION OF TRITIUM GAS

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SUMMARY

Steroids with diazo and azido functional groups were labeled with tritium atoms by microwave discharge activation of tritium gas. The specific activity of the products was increased by maximizing the exposure surface using glass fiber paper for dispersion, reducing the amount of sample to be exposed, and increasing tritium pressure. A 500 fold increase in specific activity was achieved in dispersed sample as compared to the solid. Addition of tritium atoms to 4,5 double bond in Δ^4-3-keto steroid occurred, giving rise to a mixture of 5α- and 5β-isomers in about equal quantities.

Key Words: Steroids, Tritium Labeling, Microwave Discharge, Glass Fiber Paper, Addition, Substitution.

INTRODUCTION

Tritium labeling by microwave discharge activation of tritium gas was first reported by Ghanem and Westermark (1), later modified by Gosztonyi and Walde (2,3), and more recently utilized by Ehrenkaufer et al. (4). This method is rapid, convenient, and applicable to a broad range of organic molecules of biological interest, including peptides and proteins. The tritiated products that have been obtained have generally been of low specific

0362-4803/79/0416-0603$01.00

Received October 10, 1978

activity, and few reports on the application of this method for labeling steroids have appeared.

This report describes the labeling of steroids having diazo and azido functional groups by the microwave method and discusses the parameters affecting the specific activity of the tritiated products and the addition of tritium atoms to the double bond in Δ^4-3-keto steroid.

EXPERIMENTAL

a) Labeling Procedure

The apparatus that we used for labeling was essentially that described by Ehrenkaufer et al. (4), with a slight modification, the extention of the tritium inlet tube directly over the sample platform on the "cold finger" of the bent Dewar flask. The microwave generator was a 100-watt, 2.45 GHz, KIVA, Model MPG-2 (Opthos Instrument Co., Rockville, Maryland). The microwave cavity was of the Evenson type (5) which was finely tuned for minimum reflected power after the glow was initiated with a Telsa coil. Tritium pressure was measured with a Model AP-10 pressure transducer, range 0-20 Torr (Validyne Engineering Co., Northridge, California).

Carrier-free tritium gas was generated from uranium tritide by heating, just prior to use, and was carefully metered into the microwave system through a high vacuum valve. Steroids (0.15 to 2 mg) in 0.05 ml of deuterated chloroform or methyl alcohol were pipetted on to glass fiber paper (Gelman Type A- no binder), Millipore filter (Mitex filter LCWP 01300, Millipore Corp.), and micro cover glass which were all 8x10-mm in size. The dried sample was placed on the "cold finger", which had been cooled by liquid nitrogen. The system was evacuated to less than 0.003 Torr before tritium was admitted to a pressure of 5 Torr. The duration of exposure to tritium plasma was 20 minutes. The spent tritium gas was then pumped off. This was followed by a few flushes with

hydrogen gas to remove adsorbed tritium and the system was opened and the tritiated sample removed. Labeled materials were extracted from the solid support with ethyl alcohol, then the solution was evaporated to dryness under reduced pressure to remove any exchangeable tritium activity.

b) Materials

21-Diazo-21-deoxycorticosterone was prepared by the method previously described (6). 6-Azido-6-dehydrodexamethasone was obtained as a gift from Schering Corp.. Testosterone (Searle) was recrystallized from acetone, mp 154-155°C, λ_{max} 242 nm (ϵ 1.6×10^4). 5α-Dihydrotestosterone (Searle) and 5β-dihydrotestosterone (Sigma) were used without further purification. The solvents used in the purification of labeled steroids were redistilled or purified.

c) Purification

After removal of exchangeable tritium activity, the labeled steroids were repeatedly purified by thin layer chromatography (TLC) untill a constant specific activity was achieved. TLC was carried out on 5x20 cm glass plates having 0.25-mm layers of silica gel GF (E. Merck). These plates were prewahed by ascending chromatography in methyl alcohol and reactivated just before use by heating in an oven at 105°C for 15 minutes. Spots and bands on TLC plates were visualized by fluorescence quenching under UV (254 nm) illumination.

The UV spectra of the tritiated steroids were obtained on a Beckman DB-G Grating Spectrophotometer. An aliquot of the solution was then diluted for radioactivity measurement by the liquid scintillation counting using a Searle Mark III Liquid Scintillation System. The counting efficiency (56-60%) was determined by the internal standard method using ^{3}H-toluene (New England Nuclear). The specific activity of the labeled material was calculated from these measurements.

d) Photolysis

Photochemical reaction of the steroids were performed by irradiating solutions of steroids in methyl alcohol or 95% ethyl alcohol at concentration of 3.6×10^{-5} M or 6×10^{-5} M on a rotating stage of Rayonet Photochemical Reactor at 254 nm at room temperature. The extent of reaction was monitored with UV spectrophotometer and the maximum absorbance was determined at 235-250 nm.

RESULTS

Tritium labeled 21-diazo-21-deoxycorticosterone was purified by TLC using chloroform-ethyl acetate (1:3, v:v) as the developing solvent. Figure 1 shows the distribution of radioactivity of the crude labeled steroid before purification on a TLC plate. The labeled steroid was repeatedly purified by TLC until a constant specific activity was achieved. A radiochemical purity of greater than 96% was indicated on TLC in the solvent systems benzene-ethyl acetate (1:2, v:v) and benzene-ethyl alcohol (9:1, v:v). Specific activities of purified steroids on different supports are given in Table 1.

Identity of the purified ^{3}H-diazo steroid was confirmed in several ways: (i) the UV spectrum of the labeled steroid was identical to that of nonlabeled compound with λ_{max} at 247 nm and a shoulder at 280 nm; (ii) addition of hydrochloric acid caused a 30% decrease in UV absorbance in both labeled and non-labeled steroids because of the destruction of 21-diazo substituent, and the product of the reaction, 21-chloro steroid (Figure 2), from both labeled and non-labeled 21-diazo steroids, had identical R_f values on TLC as that of an authentic 21-chloro steroid (7); (iii) photolysis of labeled and non-labeled compounds in methyl alcohol at 254 nm gave the same initial rapid decrease in absorbance with irradiation time as shown in Figure 3, and the methyl ester

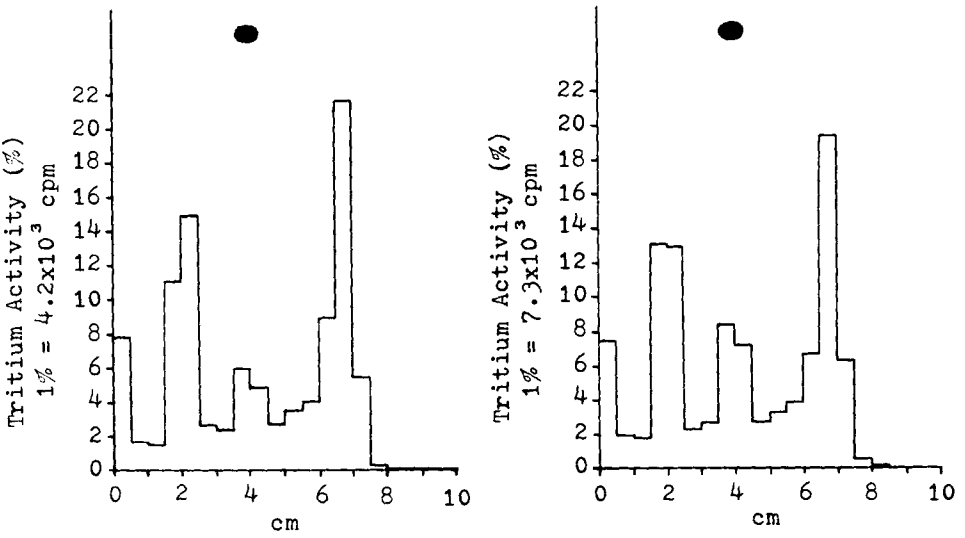

Figure 1. Radiochromatograms of crude tritiated 21-diazo-21-deoxycorticosterone labeled on glass fiber paper at 5 Torr (left) or 9 Torr (right). Dark spot represents pure 21-diazo-21-deoxycorticosterone chromatographed on the same TLC plate. Solvent system: chloroform-ethyl acetate (1:3, v:v).

Table 1. Labeling of diazo and azido steroids on solid supports with tritium atoms.

Steroids labeled	Amount labeled mg	Support materials	Specific activity mCi/mmol	Ratio of specific activity
21-Diazo-21-deoxy-	2	G[a]	2.0	1
corticosterone	0.15	P[b]	560	280
	0.15[c]	P	1000	500
6-Azido-6-dehydro-	0.9	G	4.5	1
dexamethasone	0.9	P	131	29

a. G = Micro cover glass.

b. P = Glass fiber paper.

c. At tritium pressure of 9 Torr.

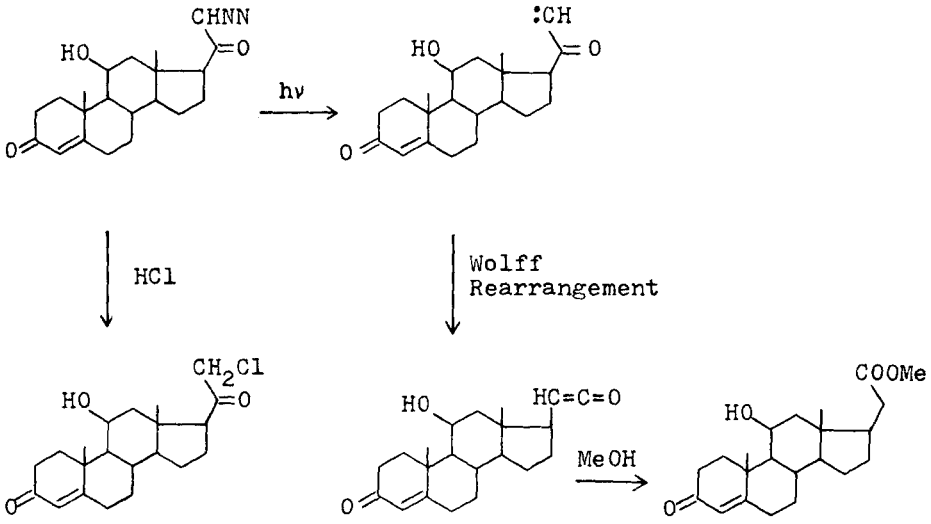

Figure 2. Photochemical reaction of 21-diazo-21-deoxycorticosterone.

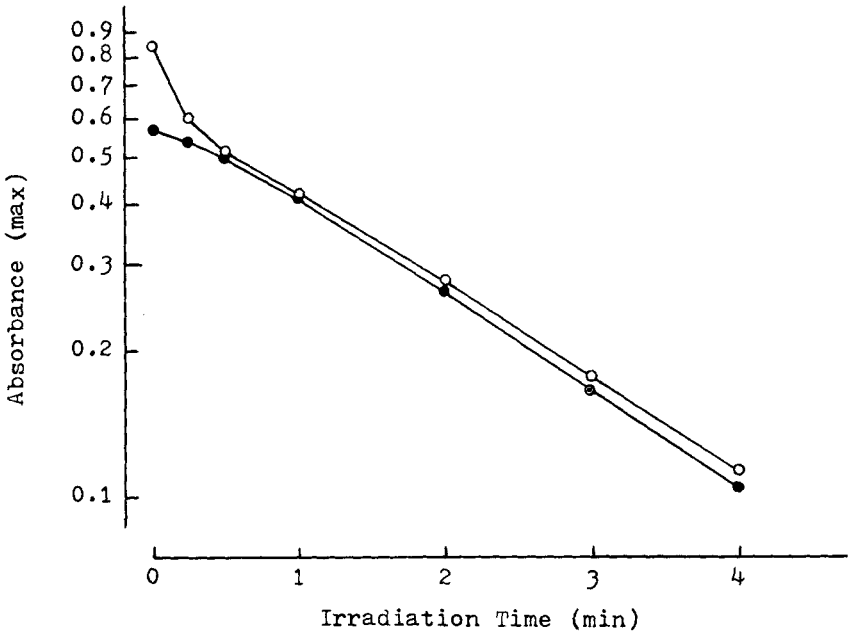

Figure 3. Photolysis of 21-diazo-21-deoxycorticosterone (o) and 21-deoxycorticosterone (•) in methyl alcohol (3.6×10^{-5} M) at 254 nm. Absorbance (max) was determined at 242-247 nm. Note the rapid (< 1 min) change in absorbance with the diazo steroid only.

(Figure 2) formed by photochemical Wolff Rearrangement of these compounds gave identical R_f values on TLC.

6-Azido-6-dehydrodexamethasone was similarly tritiated and purified to a constant specific activity by the method described above. The results are shown in Table 1. Identity of the purified ^{3}H-azido steroid was confirmed as follows: (i) the UV spectrum showed λ_{max}'s at 248 nm and 310 nm, and the ratio of absorbance at 248 nm to that at 310 nm was 2.2, identical to that obtained with authentic compound; (ii) photolysis of both labeled and non-labeled compounds at 254 nm gave the same initial rapid decrease in absorbance with irradiation time as shown in Figure 4.

Addition of tritium atoms to 4,5 double bond in steroid was studied with testosterone as the model compound because of the availability of the saturated isomers, 5α- and 5β-dihydrotestosterone (DHT), for comparison. Figure 5 shows the radiochromatograms of crude labeled testosterone before and after lyophilization in ethyl alcohol. Portions of the labeled preparation were added to carrier testosterone, 5α-DHT, and 5β-DHT, of $2x10^5$ fold excess. Testosterone was crystallized from acetone and 5α-DHT and 5β-DHT were crystallized from ethyl acetate. Tritium activity of these compounds were determined. The remaining crude testosterone was purified by TLC using benzene-ethyl acetate (1:1, v:v) as solvent system and the specific activity was determined. These results are giving in Table 2.

DISCUSSION

The importance of high-specific-activity diazo steroids in the purification and characterization of receptor proteins has been emphasized (8). Tritium labeled 21-diazo steroids have been prepared in this laboratory by catalytic tritiation of the corresponding 3-oxoandrosta-4,6-diene-17β-carboxylic acid to 3-oxo-

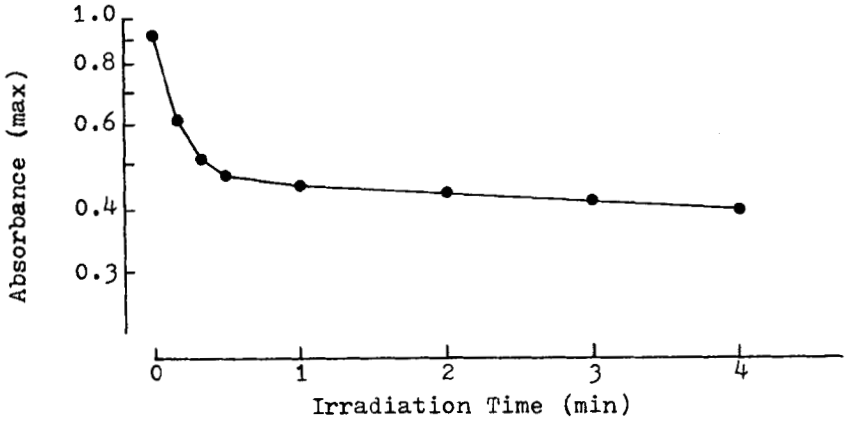

Figure 4. Photolysis of 6-azido-6-dehydrodexamethasone in 95% ethyl alcohol ($6x10^{-5}$ M) at 254 nm. Absorbance (max) was determined at 235-250 nm. Note the rapid (< 1 min) change in absorbance.

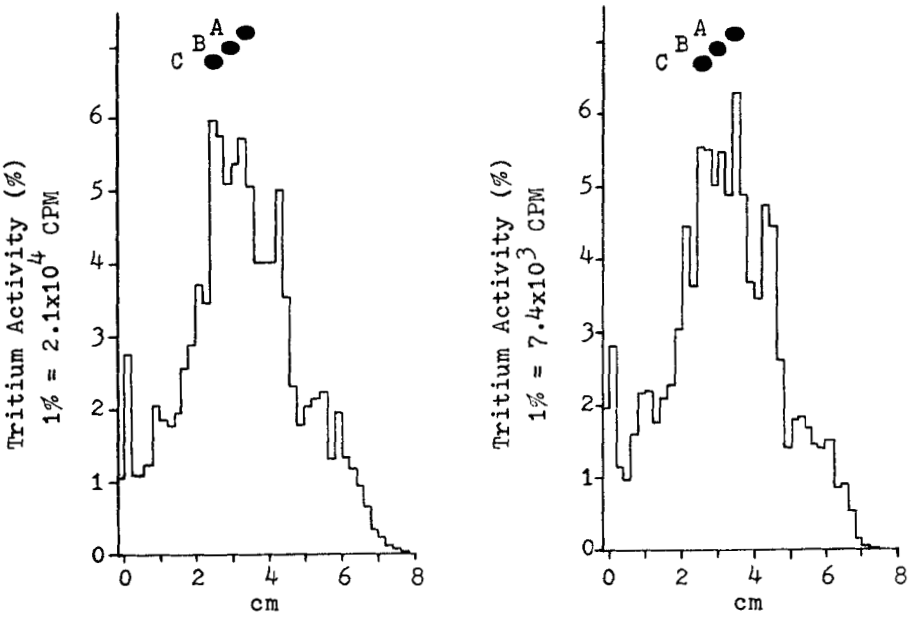

Figure 5. Radiochromatograms of crude tritiated testosterone labeled on glass fiber paper before (left) and after (right) lyophilization in ethyl alcohol. Dark spots represent testosterone (C) and its saturated derivatives, 5α-DHT (A) and 5β-DHT (B), chromatographed on the same TLC plate. Solvent system : benzene-ethyl acetate (1:1, v:v).

Table 2. Substitution versus addition in the labeling of testosterone by microwave method on solid supports.

Support materials	Micro cover glass	Mitex filter	Glass fiber paper
Amount labeled (mg)	1	1	1
Tritium activity (mCi)			
1) Crude preparation (A)	0.59 (1)[a]	3.12 (5)	16.0 (27)
2) Testosterone (B)	0.101 (1)	0.495 (5)	2.83 (28)
% radioactivity (B/A)	17.1%	15.9%	17.7%
Specific activity[b] (mCi/mmol) obtained by			
i) TLC purification	32	153	910
ii) crystallization	29 (1)	143 (5)	815 (28)
3) Addition products (C)			
i) 5α-DHT	0.082 (1)	0.411 (5)	2.46 (30)
ii) 5β-DHT	0.075 (1)	0.409 (6)	2.42 (32)
Total	0.157	0.820	4.88
% radioactivity (C/A)	26.6%	26.3%	30.5%
% yield by weight[c]	0.08% (1)	0.4% (5)	2.4% (30)
Addition to substitution ratio (C/B)	1.55	1.66	1.72

a. Figures in parentheses are ratios.
b. Initial specific activity.
c. Calculation based on the assumption that the addition was effected by tritium atoms only, with the specific activity of the saturated products approaching 58 Ci/mmol.

androst-4-ene-17β-carboxylic acid-6,7-$^3H_2$ followed by treatment with oxalyl chloride, then with diazomethane (6,8,9). The present study demonstrates that steroids with diazo and azido functional groups could be labeled by microwave discharge activation of tritium gas. The treated steroids showed no apparent degradation by UV absorption and TLC, but about 85 to 90% of the tritium activity in the crude preparations was associated with small quantities of highly radioactive impurities, as shown by radiochromatograms in Figures 1 and 5.

Tritium activity of the labeled steroids is dependent on tritium pressure, labeling surface area, and the amount of the sample to be labeled. As shown in Tables 1 and 2, at 5 Torr tritium pressure the glass fiber labeling produced a 30 fold increase in specific activity and the Mitex filter labeling produced a 5 fold increase compared to the cover glass reaction surface. Reducing the sample exposed from 2 mg to 0.15 mg increased the specific activity 280 fold. Increasing the tritium pressure from 5 Torr to 9 Torr caused an enhancement of the specific activity by 500 fold to the Ci/mmol level. However, it should be noted here that Hembree et al. reported a decrease in specific activity of the labeled material when tritium pressure was increased from 4 mm Hg to 12 mm Hg (Ref. 4, Table IV).

Since 1974 we have used glass fiber paper as a large labeling surface for the substrate for tritium labeling. This method not only yields high specific activity, but also simplifies sample handling. Very recently, Ehrenkaufer et al. reported a 10 to 300 fold increase in specific activity by using cellulose Millipore filter as labeling surface and reducing the amount of sample to be exposed (10). The results of our study indicate that glass fiber paper may be the preferred labeling surface because it provides larger reaction surface and contains few if any exchangeable hydrogens and may yield a labeled product of higher specific activity.

Saturation of double bond by tritium atoms occurs in Wilzbach labeling (11) as well as in microwave labeling (12). In our study we confirmed this using testosterone. The addition of tritium atom across 4,5 double bond in testosterone proceeded from both sides of the steroid molecule, giving rise to a mixture of 5α and 5β isomers (DHT) in about equal quantities. The tritium activity of

these saturated derivatives of testosterone (DHT) was approximately 26% of the initial tritium activity in the crude preparation. The addition to substitution ratio was about 1.6. The observed yield (by weight) of saturation of testosterone on cover glass, Mitex filter, and glass fiber paper was 0.08%, 0.4%, and 2.4% respectively, based on the assumption that the addition was effected by neat tritium atom only, with the specific activity of the saturated products approaching 58 Ci/mmol. The 30 fold increase in the yield of saturated steroids on glass fiber paper and the 5 fold increase on Mitex filter as compared to the cover glass paralleled the increase in specific activity of testosterone under same condition.

ACKNOWLEDGEMENTS

We are grateful to Dr. R. M. Lemon of Chemical Biodynamics Laboratory in Berkeley for permitting us to use the tritium containment facilities, and to Mr. Ray Aune of Lawrence Radiation Laboratory in Berkeley for his kind, generous cooperation.

This work was done during the tenure of a National Institute of Arthritis, Metabolism and Digestive Diseases Postdoctoral Research Fellowship, 1974-1976, to W.H.C..

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