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The Preparation and Properties of Some Tritiated Diazonium Salts and Related Compounds*

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ABSTRACT: Several tritiated diazonium salts of high specific activity and radiochemical purity have been investigated. The methods used in the preparation and characterization of these compounds and their azo derivatives are presented in detail. These compounds have been effective in the specific labeling of antibody active sites, and should be of general use in connection with protein structure studies.

In the course of several years' studies from this laboratory on the affinity labeling of antibody active sites, several diazonium salts and their derivatives, both nonradioactive and tritiated to high specific activities, have been prepared and their properties investigated. Diazonium compounds are ordinarily quite unstable and, for most biochemical applications, have been prepared *in situ* from their corresponding amines and then used immediately. For our purposes, these conventional procedures were not satisfactory; the preparation of stable highly radioactive diazonium compounds was required to obtain quantitative and reproducible labeling results. Fluoroborate salts of diazonium compounds have commonly been used as stable reagents in organic chemistry, and two of these in nonradioactive form have been employed in our earlier studies (Wofsy *et al.*, 1962; Metzger *et al.*, 1963). In this paper, our experience with three tritiated diazonium fluoroborates and their derivatives is described not only as a reference for our own investigations (*cf.* Good *et al.*, 1967), but also in the hope that these

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compounds and the methods we have utilized may be of general interest in studies of active site labeling and protein modification.

Materials

Reagents. *N*-Chloroacetyl-L-tyrosine (NCAT)[1] from Mann Research Laboratories was recrystallized twice from water to yield white needles melting at 155–156°. *p*-Cresol was recrystallized from hexane, mp 34–35°. 2,2-Dimethoxypropane was a practical grade from Matheson Coleman and Bell. *p*-Dimethylaminoacetanilide was prepared by acetylation of *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride in a tenfold excess of acetic anhydride containing a few drops of concentrated sulfuric acid. The crude product was recrystallized twice from water to yield white needles melting at 131.5–132° (lit. (Hodgson and Crook, 1932) mp 130°). Ethyl thioltrifluoroacetate was prepared by a published procedure (Hauptschein *et al.*, 1952). Fluoroboric acid (48–50%) was a purified grade from J. T. Baker. Methyl *p*-toluenesulfonate was recrystallized from toluene–hexane, mp 28–30°. *m*-Nitroacetanilide was recrystallized from toluene to yield very pale yellow glistening leaflets melting at 151–151.5°. *p*-Nitroacetanilide was recrystallized from methanol as extremely pale yellow fluffy needles, mp 213.5–214°. All other chemicals were reagent grade, used as received.

Radioactive Diazonium Fluoroborates. The tritiated nitroanilines desired as precursors for the diazonium salts were obtained by acid-catalyzed exchange of aromatic hydrogens on their respective acetanilides, followed by deacetylation. Acetylation of the amines prior to tritiation provides stabilization to oxidation and facilitates ring exchange by converting the deactivating ammonium group to an activating acetamido group.[2]

[^3H]*m-Nitroaniline.* *m*-Nitroacetanilide was sent to New England Nuclear Corp. where it was exchanged with tritium. The reaction mixture, containing 100 mg (0.555 mmole) of *m*-nitroacetanilide, 0.3 ml of trifluoroacetic acid, 0.025 ml of trifluoroacetic anhydride, 10 c of tritiated water, and 25 mg of prereduced platinum catalyst, was heated, with stirring, at 70° for 3 days. (If 50 mg of the catalyst was used, excessive decomposition of the compound resulted.) After removal of labile tritium with 10 ml of methanol, it was shipped back in a solution of methanol.

The tritiated material was hydrolyzed without prior isolation by refluxing 6 ml of a methanolic solution, containing 0.5 M hydrochloric acid and 3 M water, for 4 hr. The reaction mixture was then diluted with hot water and concentrated ammonium hydroxide was added until the pH rose to 8. Methanol was removed by flash distillation. The residual hot aqueous mixture was filtered to remove dark brown insoluble material. When the filtrate was cooled, the product crystallized out. Successive recrystallizations from carbon tetrachloride and toluene–isooctane yielded 35.6 mg (46% of theory) of bright yellow needles, with a constant specific activity of $1.56 \pm 0.04 \times 10^5$ dpm/μmole.

[^3H]*MNBDF.* To a solution of 34.3 mg of [^{3}H]*m*-nitroaniline and 30.9 mg of carrier *m*-nitroaniline (total of 0.472 mmole) in 0.5 ml of 50% fluoroboric acid–0.25 ml of water, cooled to 0–5°, was added dropwise 0.17 ml of an aqueous solution containing 38.2 mg (0.553 mmole) of sodium nitrite. The reaction mixture was stirred magnetically during this addition and for 1 hr thereafter. The crude product, which precipitated as a cream solid, was collected by filtration and successively washed with ice-cold 25% fluoroboric acid, anhydrous ethanol, and anhydrous ether. Two recrystallizations from acetone–ether, utilizing a maximum temperature of 50°, yielded 39.3 mg (35% of theory) of glistening off-white needles having a specific activity of $7.72 \pm 0.16 \times 10^7$ dpm/μmole. In three separate preparations the yields ranged from 25 to 48%. The compound was dissolved in 0.01 M hydrochloric acid at a concentration of $1.5–2 \times 10^{-3}$ M and stored at −20°.

[^3H]*p-Nitroaniline.* The procedures used to prepare this compound followed closely those employed for the *meta* isomer. *p*-Nitroacetanilide was tritiated as described above. It was diluted with 150 mg of carrier *p*-nitroacetanilide before hydrolysis and subsequent work-up. The recrystallized [^{3}H]*p*-nitroaniline had a constant specific activity of $2.16 \pm 0.05 \times 10^8$ dpm/μmole. The over-all yield was 76%.

[^3H]*PNBDF.* To a solution of 80 mg (0.58 mmole) of [^{3}H]*p*-nitroaniline in 0.5 ml of 50% fluoroboric acid–0.4 ml of water, cooled to 0–5°, was added dropwise 0.1 ml of an aqueous solution containing 46 mg (0.67 mmole) of sodium nitrite. The reaction mixture was stirred magnetically during this addition and for 45 min thereafter. The crude product which precipitated as a pale yellow solid was collected by filtration and successively washed with ice-cold 25% fluoroboric acid, anhydrous ethanol, and anhydrous ether. Recrystallization from methanol yielded 49 mg (36% of theory) of very pale yellow needles, having a specific activity of $2.15 \pm 0.06 \times 10^8$ dpm/μmole. The compound was dissolved in 0.01 N hydrochloric acid and stored at −20°.

[^3H]*PTBDF.* To prepare this compound, *p*-dimethylaminoacetanilide was first converted to trimethyl(*p*-aminophenyl) ammonium chloride hydrochloride by a more satisfactory procedure than that previously published (Pressman *et al.*, 1946); the latter compound was then tritiated and subsequently converted to the diazonium salt.

Trimethyl(p-acetamidophenyl)ammonium p-Toluenesulfonate. A solution of 14 g (79 mmoles) of *p*-dimethyl-

[1] Abbreviations used: NCAT, *N*-chloroacetyl-L-tyrosine; MNBDF, *m*-nitrobenzenediazonium fluoroborate; PNBDF, *p*-nitrobenzenediazonium fluoroborate; PTBDF, *p*-(trimethylammonium)benzenediazonium difluoroborate.

[2] In one experiment where the direct tritiation of *p*-nitroaniline was carried out (V. T. Maddaiah and S. J. Singer, unpublished results), a poor recovery of the pure compound of low specific activity was obtained.

aminoacetanilide and 16.2 g (87 mmoles) of methyl *p*-toluenesulfonate in 100 ml of acetone was refluxed, with stirring, for 3 hr. The product, which crystallized out of the reacting solution, mp 217–218° dec, was obtained in 85% yield. *Anal.* Calcd for $C_{18}H_{24}N_2O_4S$: C, 59.32; H, 6.64; N, 7.69; S, 8.80. Found: C, 59.17; H, 6.69; N, 7.70; S, 9.11.

Trimethyl(p-aminophenyl)ammonium Chloride Hydrochloride. A solution of 2.50 g (6.86 mmoles) of trimethyl(*p*-acetamidophenyl)ammonium *p*-toluenesulfonate in 20 ml of 50% (v/v) concentrated hydrochloric acid–ethanol was refluxed for 2 hr. Acetone was added to the cloud point and the solution allowed to cool. Glistening white crystals, melting at 235.5–236° dec (lit. mp 219 dec (Reilly and Drumm, 1935) and 220° (Pressman *et al.*, 1946)), were obtained in 80% yield. *Anal.* Calcd for $C_9H_{16}Cl_2N_2$: C, 48.44; H, 7.23; Cl, 31.78; N, 12.55. Found: C, 48.62; H, 7.20; Cl, 31.62; N, 12.37.

[^{3}H]Trimethyl(p-aminophenyl)ammonium Chloride Hydrochloride. Trimethyl(*p*-aminophenyl)ammonium chloride hydrochloride (95 mg, 0.426 mmole) was sent to New England Nuclear Corp. where it was exchanged by heating in 0.2 ml of trifluoroacetic acid containing 10 c of tritiated water and 25 mg of platinum catalyst for 3 days at 70°. After removal of labile tritium it was shipped back in a solution of methanol. The compound had a very high specific activity, presumably due to heavy labeling of the methyl hydrogens. Accordingly, to about 10 ml of methanolic solution was added 400 mg of unlabeled carrier compound. Bubbling anhydrous hydrochloric acid through the solution caused the compound to crystallize out. The crystals, collected by filtration of the cooled mixture, were wine colored. An attempt to decolorize by addition of Darco G-60 charcoal to a hot methanolic solution and recrystallization by addition of acetone was unsuccessful.

Decolorization was achieved by the following procedure. A saturated aqueous solution of the compound was titrated with 1 N sodium hydroxide to pH 7. After evaporation of water, the residue was boiled in about 200 ml of isopropyl alcohol, which dissolved the desired compound. Darco G-60 charcoal was added and the mixture boiled for 10 min. Filtration of the hot mixture removed insoluble sodium chloride with the charcoal and produced a water-white filtrate, which was then concentrated to about 80 ml. Addition of gaseous hydrochloric acid produced very fine white needles which were collected by filtration of the cooled mixture. The specific activity of this material was $4.02 \pm 0.24 \times 10^8$ dpm/μmole. The compound was recrystallized by addition of gaseous hydrochloric acid to a hot methanolic solution. Glistening white plates, collected by filtration of the cooled mixture, melted at 235–236° dec (sealed tube) and had a specific activity of $4.03 \pm 0.25 \times 10^8$ dpm/μmole. The over-all yield was 25%.

The radioactive compound appears to be unstable. At −20°, either as the solid, in an evacuated desiccator over phosphorus pentoxide, or in solution in methanol, the originally colorless material developed a pink coloration within 24 hr.

Nonradioactive PTBDF. This compound has not been described previously. To a suspension of 100 mg (0.448 mmole) of trimethyl(*p*-aminophenyl)ammonium chloride hydrochloride in 1.2 ml (9 mmoles) of 50% fluoroboric acid, cooled to 0–5°, was added dropwise 0.25 ml of an aqueous solution containing 35.5 mg (0.515 mmole) of sodium nitrite. The reaction mixture was stirred magnetically during this addition and for 15 min thereafter. It was then transferred to a freezer at −20° for 3 hr. The product, which precipitated out of the reaction mixture, was collected by filtration and washed successively with ice-cold 50% fluoroboric acid, anhydrous ethanol, 2,2-dimethoxypropane, and anhydrous ether. There was obtained 110 mg of white crystals. The product could be recrystallized by dissolving in a minimal volume of 25% fluoroboric acid, followed by addition of methanol, to yield fluffy white crystals, which first smoked, then degassed at 170–171°, but did not completely melt.

The elemental analyses on both the initial product and recrystallized material were consistent with the cocrystallization of 1 mole of sodium tetrafluoroborate/mole of diazonium compound. *Anal.* Calcd for $C_9H_{13}B_2F_8N_3 + NaBF_4$: C, 24.20; H, 2.93; N, 9.41; F, 51.04; mol wt, 446.63. Found (initial product): C, 24.16; H, 2.82; N, 9.53. Found (recrystallization product): C, 24.60; H, 2.95; N, 9.98; F, 50.80. Therefore, in calculating the molarity of solutions of [^{3}H]-PTBDF (which were not sent out for analyses) a molecular weight of 446.6 was assumed.

To prepare [^{3}H]PTBDF, [^{3}H]trimethyl(*p*-aminophenyl)ammonium chloride hydrochloride (50.20 mg) was mixed with 49.97 mg of carrier and diazotized exactly as described above for the nonradioactive compound. The initial product was not recrystallized in this case, but was vacuum dried over P_2O_5 at room temperature for 2 hr. There was obtained 91.44 mg (46% of theory) of white crystals: which were immediately dissolved in 0.01 M hydrochloric acid to make 100 ml of solution. The solution was stored and handled as described in the preparation of [^{3}H]MNBDF.

Model Compounds and Azo Derivatives

N-Chloroacetyl-3-(m-nitrophenylazo)tyrosine. For spectral assays of the formation of *m*-nitrophenylazotyrosine upon the reaction of MNBDF with proteins, this model compound was prepared and isolated. A solution of 237 mg (1.0 mmole) of nonradioactive MNBDF in 30 ml of 0.01 N hydrochloric acid was added dropwise to a solution of 309 mg (1.2 mmoles) of NCAT in 25 ml of 0.2 M sodium phosphate buffer at pH 6.2, with stirring at 0°. Aqueous sodium hydroxide was added as needed to keep the reaction mixture at pH 6.2. Three hours after completion of addition, stirring was stopped and the reaction mixture was allowed to stand at room temperature overnight. The reaction mixture, which had formed a gel, was acidified with concentrated hydrochloric acid to pH 2, whereupon the product precipitated out. The crude product was recrystallized three times from methanol, each time

yielding a mixture of orange and yellow crystals. It was further recrystallized twice from aqueous acetone, which afforded uniform bright yellow-orange fine crystals melting at 214–215° dec. *Anal.* Calcd for $C_{17}H_{15}ClN_4O_6$: C, 50.19; H, 3.72; Cl, 8.72; N, 13.77. Found: C, 50.08; H, 4.03; Cl, 8.65; N, 13.68.

[3-^{3}H](m-Nitrophenylazo)tyrosine Hydrochloride. In connection with studies of proteins labeled with MNBDF, authentic *m*-nitrophenylazotyrosine, both cold and radioactive, was required as a reference compound. The α-amino group in tyrosine was first blocked by trifluoroacetylation (Schallenberg and Calvin, 1955; Goldberger and Anfinsen, 1962) to prevent modification of the amino group during the coupling reaction (Zahn *et al.*, 1953).

N-Trifluoracetyltyrosine. To a saturated aqueous solution of 3.62 g (0.02 mole) of L-tyrosine at pH 10.0 was added 22.5 ml (0.175 mole) of ethyl thioltrifluoroacetate. The reaction mixture was stirred vigorously and 5 N sodium hydroxide was added dropwise as needed to keep the pH between 9 and 10. When the reaction ceased, trifluoroacetic acid was added to pH 2 and the solution was concentrated to about 50 ml on a rotary evaporator. The crude product which precipitated was collected by filtration and extracted with 75 ml of ether to separate it from insoluble, unreacted tyrosine. The ether extract was evaporated and the residue was recrystallized from toluene containing a small amount of acetone. The yield of fluffy, white needles melting at 196–197° [(lit. mp 192.5–193.5 (Shine and Niemann, 1952) and 192–193° (Weygand and Geiger, 1956)] was 3.88 g (70% of theory).

N-Trifluoroacetyl-3-(m-nitrophenylazo)tyrosine. A solution of 474 mg (2 mmoles) of MNBDF in 50 ml of 0.01 N hydrochloric acid was added dropwise to a solution of 665 mg (2.4 mmoles) of *N*-trifluoroacetyltyrosine in 35 ml of 0.200 M disodium phosphate stirring at 0°. One hour after completion of addition, the reaction mixture was allowed to warm to room temperature and was stirred further for 21 hr. The reaction mixture, which had formed a gel, was heated to 70° and acidified with concentrated hydrochloric acid to pH 2, whereupon the product precipitated out. The mixture was stirred at 80–90° to transform the initial gelatinous precipitate to a filterable solid. The crude product was collected by filtration of the hot reaction mixture, resuspended in 100 ml of hot water, and refiltered. There was obtained 0.83 g of a yellow-orange solid. Attempts to recrystallize this material inevitably led to formation of a gel; the product was, therefore, deacetylated without further purification.

3-(m-Nitrophenylazo)tyrosine Hydrochloride. N-Trifluoroacetyl-2-(*m*-nitrophenylazo)tyrosine (0.70 g, 2.1 mmoles) was dissolved in 10 ml of 1 M piperidine. The resultant blood red solution was allowed to stand at room temperature for 24 hr, then diluted to about 50 ml with water. Addition of concentrated hydrochloric acid to pH 0 precipitated the crude product. Recrystallization from 1 N hydrochloric acid yielded fine yellow-orange crystals, melting at 250° dec. *Anal.* Calcd for $C_{15}H_{15}ClN_4O_6$: C, 49.12; H, 4.12; N, 15.28. Found: C, 48.96; H, 4.32; N, 15.36.

The titriated form of this compound was prepared from 0.17 mmole of a sample of [^{3}H]MNBDF having a specific activity of 3.00 ± 0.06 × 10^6 dpm/μmole, and 56.4 mg (0.203 mmole) of *N*-trifluoracetyltyrosine, followed by deacetylation of the coupled product. The procedure described above was followed. Yield of product having a specific activity of 3.08 ± 0.04 × 10^6 dpm/μmole was 70%.

2-(p-Trimethylammoniumphenylazo)-4-methylphenol Fluoroborate. A model compound was required as a basis for the spectral assay of the formation of azotyrosyl linkages upon the reaction of PTBDF with proteins. Attempts to isolate the corresponding azo derivative of NCAT were unsuccessful. Instead, the azo derivative of *p*-cresol was made. A solution of 0.447 g (1.0 mmole) of PTBDF in 5 ml of 0.01 N fluoroboric acid was added dropwise to a solution of 1.08 g (10 mmoles) of *p*-cresol in 20 ml of 50% (v/v) aqueous methanol, stirring on a magnetic stirrer. Aqeuous 1 N sodium hydroxide was added in drops to keep the pH between 7 and 8.5. The crude product precipitated out of the reacting solution. After standing at room temperature for 4 hr, the reaction mixture was acidified to pH 3 with 25% fluoroboric acid and cooled to 0°. The product was collected by filtration and washed copiously with ether to remove adhering *p*-cresol. There was obtained 330 mg (92% of theory) of orange crystals, melting at about 265° dec. Two sets of successive recrystallizations from methanol and from water yielded orange needles melting at 292° dec. *Anal.* Calcd for $C_{16}H_{20}BF_4N_3O$: C, 53.81; H, 5.64; F, 21.28; N, 11.77. Found: C, 54.07; H, 5.83; F, 20.89; N, 11.57.

Methods and Results

Melting points were determined on the Thomas-Hoover capillary melting point apparatus and are uncorrected. Microanalyses were by Schwartzkopf Microanalytical Laboratories, Woodside, N. Y.

Radiochemical Assay. Radiochemical counting was conducted in Wheaton vials with a Nuclear-Chicago liquid scintillation system (720 series). The scintillation solution was prepared by appropriate dilution of Liquifluor (Pilot Chemicals) with toluene. Hyamine hydroxide, 1 M in methanol, was from Packard Instrument Co. Counting efficiency was determined by addition of standard [^{3}H]toluene from New England Nuclear Corp.

Quadruplicates of each sample, containing at least 35,000 cpm/vial, were counted twice. The counts registered by the machine were reproducible to within 2%. The range in specific activities here reported represents the maximum deviation of quadruplicate samples from the average value.

For counting of the radioactive diazonium salts, these were first treated with aniline. To 0.200 ml of each solution, in the neighborhood of 2 × 10^{-3} M in 0.01 N hydrochloric acid,[3] was added 1000-fold molar excess of aniline in 1 ml of methanol. An in-

TABLE I: Spectral Properties of Monoazo Coupling Products.

Diazonium Salt	Aromatic Compd	Method	pH 5.6–6.2[d] λ_{max} (mμ)	pH 5.6–6.2[d] ϵ	pH 12.7–13.2[d] λ_{max} (mμ)	pH 12.7–13.2[d] ϵ
MNBDF	NCAT	*a*	320	19,530	325	12,710
					490	10,020
	N-Acetylhistidine	*b*	367	20,000	415	22,000
PNBDF	NCAT	*a*[c]	340	20,700	345	13,400
					520	12,800
	N-Acetylhistidine	*b*	392	23,000	472	27,500
PTBDF	*p*-Cresol	*a*	322.5	19,890	328	12,540
					493	10,800
	NCAT	*b*	322.5	19,900	328	12,600
					490	10,400
	N-Acetylhistidine	*b*	369	20,700	420	22,600

[a] From spectrum of pure isolated product. [b] From spectra of solutions after reaction of diazonium salts with 100-fold molar excess of aromatic compound. Exact concentration of diazonium salts determined spectrophotometrically by simultaneous reaction with NCAT in case of MNBDF and PNBDF, and with *p*-cresol in case of PTBDF. [c] Determined by Metzger *et al.* (1963). [d] ϵ's were constant at these pH ranges.

stantaneous bright yellow color was formed. The reacting solution was allowed to stand for 1–2 hr, after which it was diluted to 100 ml with methanol. This diluted solution (1 ml) was combined with 14 ml of scintillation solution. Other radioactive compounds were dissolved in methanol or Hyamine and 1.0 ml of solution was diluted with 14 ml of scintillation solution for counting.

Spectrophotometric Methods. DETERMINATION OF SPECTRA AND EXTINCTION COEFFICIENTS. Over-all spectra were determined on the Cary 14 recording spectrophotometer. Measurements of absorbances at λ_{max}, used in the calculation of extinction coefficients (ϵ), were made on the Zeiss PMQ II spectrophotometer.

The spectra and ϵ_{max} of *N*-chloroacetyl-3-(*m*-nitrophenylazo)tyrosine and of 2-(trimethylammoniumphenylazo)-4-methylphenol tetrafluoroborate were determined by dissolving the pure compounds in 0.10 M sodium phosphate buffer at pH 6.2, to make up a solution in the neighborhood of 2×10^{-5} M. They were then redetermined after addition of sufficient 5 N sodium hydroxide to raise the pH to 12.7. The spectral parameters are given in Table I.

Since the product of the reaction of PTBDF and NCAT was not isolated in pure form, its spectra and ϵ_{max} values were determined indirectly as follows (Tabachnick and Sobotka, 1959). PTBDF at a concentration of 8×10^{-5} M was essentially completely converted to the monoazo product by reaction with a 100-fold excess of NCAT in 0.20 M sodium acetate buffer at pH 5.0 after 2.5 hr at room temperature. The reaction mixture was diluted 1:4 with 0.20 M sodium phosphate buffer, pH 6.2; the spectra were determined; the solutions were brought to pH ~13 by the addition of 5 N NaOH; and the spectra were redetermined. The values of ϵ_{max} were calculated from the initial concentration of PTBDF determined separately as described in the next section. The close agreement (Table I) between corresponding spectral parameters for the two monoazo compounds of PTBDF determined by the direct and indirect procedures described provides strong evidence that the indirect method is quantitatively satisfactory for monoazotyrosine derivatives.

The spectra of the products of the reactions of the diazonium compounds with *N*-acetylhistidine were also determined by the indirect method, using a 100-fold excess of the latter compound in a 0.10 M borate buffer at pH 9.0 (Table I). The values of ϵ_{max} and, in the case of PNBDF,[4] the shape of the alkaline spectrum, were affected by the pH at which the coupling reaction was conducted. The reaction was exceedingly slow at pH 5. At pH 6.2, there was appreciable reaction but, after completion, the ϵ_{max} values

[3] The diazonium solutions (0.200 ml) were also directly diluted to 100 ml with methanol. Samples for counting were prepared by adding 1 ml of this solution to (1) 14 ml of scintillation solution, (2) 0.5 ml of Hyamine, followed by 13.5 ml of scintillation solution. The specific activities determined by either of these methods tended to be erratic and usually lower than those obtained after reaction with aniline.

[4] This is probably why the spectrum of the reaction product of PNBDF and *N*-acetylhistidine reproduced in Figure 1a of Metzger *et al.* (1964) differs from that described in this paper.

TABLE II: Radiochemical Assays.

Compound	Purity[b] (%)	Sp Act. (dpm/μmole)	mc/mmole
[^{3}H]*m*-Nitroaniline		$8.21 \pm 0.21 \times 10^{7}$ [c]	37.0
[^{3}H]*m*-Nitrobenzenediazonium fluoroborate	97.4	$7.72 \pm 0.16 \times 10^{7}$ [d]	34.8
		$7.93 \pm 0.16 \times 10^{7}$ [e]	35.7
[^{3}H]*p*-Nitroaniline		$2.16 \pm 0.05 \times 10^{8}$	97.3
[^{3}H]*p*-Nitrobenzenediazonium fluoroborate	97.7	$2.15 \pm 0.06 \times 10^{8}$ [d]	96.8
		$2.20 \pm 0.06 \times 10^{8}$ [e]	99.1
[^{3}H]Trimethyl(*p*-aminophenyl)ammonium chloride hydrochloride[a]		$2.02 \pm 0.13 \times 10^{8}$ [c]	91.0
[^{3}H]*p*-(Trimethylammonium)benzenediazonium difluoroborate sodium fluoroborate	98.3[f]	$2.04 \pm 0.09 \times 10^{8}$ [d]	91.9
		$2.08 \pm 0.09 \times 10^{8}$ [e]	93.7

[a] Ring and methyl labeled. [b] By spectrophotometric assay. [c] Calculated for diluted material. [d] Based on weight. [e] Based on spectral purity, assuming residual nonradioactive contaminant. [f] Assayed with *p*-cresol instead of NCAT.

were lower than after reaction at pH 9.0. The spectral parameters shown in Table I are closely similar to those obtained by Tabachnick and Sobotka (1959) for the products of the reactions of *p*-arsono-, *p*-sulfo-, and *p*-aminobenzenediazonium salts with *N*-acetylhistidine carried out at pH 9.3–9.5.

Spectrophotometric Assay. The purity of each preparation of diazonium salts was determined by reaction with NCAT (Table I). The following procedure was typical. NCAT (0.400 ml of 0.100 M) and a freshly prepared solution of [^{3}H]MNBDF (0.200 ml) in 0.01 M hydrochloric acid (calculated by weight to be 1.657×10^{-3} M) were diluted to 5.00 ml with 0.20 M sodium acetate buffer at pH 5.0. The solution was allowed to stand at room temperature for 2–4 hr, corresponding to approximately 10–20 half-lives of reaction. After suitable dilution into 0.10 M sodium phosphate buffer at pH 6.2, absorbances were measured at 320 mμ. The solution was then brought up to pH 12.7 by addition of 0.100 ml of 5 N sodium hydroxide and absorbances were remeasured at 325 and 490 mμ. A solution containing all components except the diazonium salt was carried through all operations as a spectral blank. Duplicate measurements usually agreed to within 1%. The purity was then calculated by comparison with ϵ_{max} of isolated *N*-acetyl-3-(*m*-nitrophenylazo)tyrosine at each of these wavelengths.

Radiochemical Purity of the Diazonium Fluoroborates. A summary of radiochemical assays of these diazonium salts is shown in Table II. In every instance, the direct precursor amine was recrystallized, from at least two different solvent systems, to constant specific activity. The invariance of specific activity, within an experimental uncertainty of about 4%, between the isolated diazonium fluoroborates and precursor amine is considered to be good evidence of their radiochemical purity.

In the case of [^{3}H]MNBDF, further evidence was obtained upon its reaction with *N*-trifluoroacetyltyrosine and subsequent deacetylation. Purified tritiated 3-(*m*-nitrophenylazo)tyrosine, obtained from [^{3}H]-MNBDF having a specific activity of $3.00 \pm 0.06 \times 10^{6}$ dpm/mmole, had an activity of $3.08 \pm 0.04 \times 10^{6}$ dpm/mmole.

Discussion

The results described in this paper demonstrate that several tritiated diazonium salts can readily be prepared in relatively stable form with high specific activities and radiochemical purity. For each of these diazonium salts, azo derivatives have been prepared with tyrosine and histidine which can be employed to investigate the reactions of the salts with proteins. The methods used to prepare and characterize these tritiated compounds and their derivatives should be applicable to a wide variety of other diazonium compounds as well. These compounds should have a number of important applications in biochemistry. They have already been employed in studies of the affinity labeling of the active sites of antibody molecules (*cf.* Good *et al.*, 1967). They may also be useful in enzyme structure studies. For example, PTBDF has recently been shown to affinity label the enzyme bovine erythrocyte acetylcholinesterase (D. Michaeli and L. Wofsy, personal communication) because of its specific affinity for the enzyme active site. Similarly, the affinity labeling of specific proteins of membranes and other ultrastructural elements may be an important, if not essential, step in their isolation (Singer, 1967). Here again, the reagent PTBDF may be effective in a particular instance. It has been proposed (*cf.* Podleski and Nachmansohn, 1966) that excitable membranes contain a protein component, acetylcholine receptor, the binding of acetylcholine to which triggers off the processes of nerve conduction. Tritiated PTBDF may serve to label and identify this receptor.

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Chemistry and Metabolism of Sphingolipids. On the Biosynthesis of Phytosphingosine by Yeast*

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ABSTRACT: A gas chromatographic method was developed for the determination of extracellular tetraacetylphytosphingosine produced by *Hansenula ciferrii* grown aerobically in shake culture. Studies on the origin of the oxygen atoms in this product were carried out with ^{18}O-labeled water and molecular oxygen, using combined gas chromatography–mass spectrometry for the differential determination of isotopic abundance in various oxygen atoms in the molecule. *None* of the oxygen atoms of tetraacetylphytosphingosine was derived from molecular oxygen. All but one of the oxygen atoms were derived from, or exchanged with, oxygen of water molecules in the medium.

The hydroxyl group on C-4 of phytosphingosine was very slightly labeled with ^{18}O from water; it has been concluded, however, that this hydroxyl group is derived from some unknown hydroxyl donor in the medium.

The yeast *Hansenula ciferrii* has been found by Stodola and co-workers to produce fully and partially acetylated sphingolipid bases in relatively large amounts (Stodola and Wickerham, 1960; Stodola *et al.*, 1962). The principal sphingolipid products, which were found in abundance in the cells and in the culture medium, were tetraacetylphytosphingosine (TAPS)[1] and triacetyldihydrosphingosine. The production of TAPS by this organism was shown to occur during aerobic growth and to closely parallel glucose dissimilation (Maister *et al.*, 1962). In later studies on the biosynthesis of phytosphingosine by *H. ciferrii*, Greene *et al.* (1965) found that [3-^{14}C]serine and [9,10-^{3}H]-palmitic acid were incorporated equally well into extracellular TAPS, suggesting an enzymatic mechanism not unlike that previously proposed for the synthesis of sphingosine and dihydrosphingosine in animals (see, for example, Zabin, 1957; Brady *et al.*, 1958; Fujino and Zabin, 1962). Although a pathway involving the condensation of a C_{16} chain with serine is clear from the studies of Greene *et al.* (1965), the source of oxygen for the secondary hydroxyl group on C-4 in phytosphingosine, and the exact step in the sequence when this OH group is introduced, are two points which remain to be determined. Of several plausible mechanisms, the following three reactions appeared to be the most likely possibilities.[2] Incorporation of α-

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[1] Abbreviations used: TAPS, tetraacetylphytosphingosine; $OSiMe_3$ in chemical structures, *O*-trimethylsilyl.

[2] These schemes ignore the fact that both phytosphingosine and dihydrosphingosine are isolated as the acetylated derivatives; thus, precursors shown above may or may not be fully or partially acetylated.