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The preparation of tritium labeled N-nitrosamines *

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SUMMARY

Dimethylnitrosamine, nitrosomorpholine, nitrosopyrrolidine, nitrosopiperidine, nitrosoazetidine, hexamethylenenitrosamine, nitrosomethylcyclohexylamine, nitrosomethylaniline and dinitrosopiperazine have been prepared labeled with tritium by nitrosation of the corresponding amines which had been labeled by exposure to tritium gas. The nitrosamines were purified by distillation in aqueous solution and by extraction with ether or chloroform. The specific activities varied from 17 μc/mg for nitrosomorpholine to 540 μc/mg for nitrosomethylaniline.

INTRODUCTION

In order to study the interaction of N-nitrosamines with various constituents of the cell, including nucleic acids and proteins, it was necessary to obtain these compounds radioactively labeled. The difficulty in preparing the more complex of these compounds labeled with carbon-14 led to consideration of the much easier and cheaper labeling with tritium. Because of the risk of partial reduction of the nitrosamine by exposure to tritium gas in the Wilzbach procedure [1], it was decided to label the amine by exposure to tritium gas and to prepare the labeled nitrosamine from the base. The first such preparation made, that of dimethylnitrosamine [2], was successful and the product had fairly high specific activity. Furthermore, it was shown in the biochemical experiments carried out with this material that the labeling was as satisfactory as with carbon-14; that is, there was little or no tendency for the tritium atoms attached to carbon atoms to exchange with hydrogen in solution.

Consequently, a series of bases (purified by fractional distillation using a high efficiency column) were labeled with tritium by exposure for 2 weeks to

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15 curies of tritium gas (carried out by New England Nuclear Corporation, Boston, Mass.), either as the free amine (for liquids) or as a salt (for very volatile or solid amines). The labeled free amines were rendered non-volatile by addition of hydrochloric acid. Labile tritium was removed by evaporation with methanol under reduced pressure.

The dry salt of the base was transferred with 15 ml of water to a weighed vial. All subsequent operations were carried out with weighed aliquots of the solutions, thus avoiding contamination of expensive calibrated pipettes.

Assays of radioactivity were made by weighing 2 or 3 drops (approximately 50 mg) of solution into a flask, adding 10 ml (or other suitable volume) of ethanol and mixing 25 µl of this solution with 15 ml of scintillation « cocktail » (PPO + POPOP in toluene). These solutions were counted in Packard Tri-Carb Liquid Scintillation Spectrometer ; the efficiency for tritium was established using a standard solution.

The nitrosamines were prepared following the general method of Dutton and Heath [3]. An aliquot of the solution of the amine salt (containing 0.2 to 0.5 g of base) was dissolved in 5 to 6 ml of glacial acetic, 10 ml of a 30% solution of sodium nitrite was added, together with 10 ml of water, and the reaction allowed to proceed at 25 °C for 1 hour. Preliminary experiments using unlabeled amine showed that these conditions gave almost quantitative yields of nitrosamine. At the end of this time 20 ml of 35% sodium hydroxide solution was added, followed by 20 ml of water and the solution distilled to dryness into weighed flasks. Depending on the volatility in steam of the nitrosamine the first distillate was large or small and the subsequent distillate was the converse. The remainder of the aqueous solution was collected in a third flask. Weighed aliquots of each solution were assayed for radioactivity in the same way as the solutions of the amine salts. The concentration of nitrosamine in each distillate was determined by UV spectrometry ; the absorptivities of all of the nitrosamines were determined using the freshly prepared and purified unlabeled compounds.

Since each of the distillates containing the nitrosamines also contained tritiated water (which might or might not interfere with subsequent biological experiments) together with possible traces of impurities (including unreacted amine), it was desirable to isolate the nitrosamine, even though this might lower the yield. Such purification was possible because of the high partition coefficient of most of the nitrosamines in ether or chloroform versus water. These partition coefficients (at 25 °C) were determined using UV spectrometry and are given in Table 1, together with the spectral data.

Among the nitrosamines reported here, dimethylnitrosamine was the only one which could not be isolated from the tritiated water in the distillate. Neither was it possible to purify it completely. However, by comparing the results obtained in biochemical experiments with those using highly purified carbon-14 labeled dimethylnitrosamine, no effect attributable to the presence of an impurity could be observed. With this nitrosamine all of the compound was in the

first 50 ml of distillate. The subsequent distillate was tritiated water, the radioactivity due to which was subtracted from the total activity of the nitrosamine solution to give the activity due to the nitrosamine itself. The preparation of tritiated dimethylnitrosamine has been described previously [2].

TABLE I. UV Absorption and partition characteristics of nitrosamines

Compound	λ Max (mμ)	a_M	Conc. in Ether / Conc. in Water	Conc. in Chloroform / Conc. in Water
Dimethylnitrosamine	335	109	1 : 5.5	1 : 1.7
Nitrosoazetidine	332	95	1 : 5.6	3 : 1
Nitrosopyrrolidine	333	112		9 : 1
Nitrosopiperidine	335	87	2.4 : 1	42 : 1
Hexamethylenenitrosamine	334	93	6 : 1	49 : 1
Nitrosomorpholine	340	93		7 : 1
Dinitrosopiperazine	341	178	1 : 4.3	3 : 1
Methylcyclohexylnitrosamine	332	79	23 : 1	40 : 1
Nitrosomethylaniline	370	210		
	270	7500		

EXPERIMENTAL PART

Nitrosoazetidine (*Trimethylenenitrosamine*) — 515 mg azetidine-(bi)sulfate (prepared by the method of Marckwald [4]) after nitrosation, adding alkali and distillation gave (1) 20 g distillate containing 2 mg/g, (2) 53 g distillate containing 4.2 mg/g and, (3) 6 g distillate containing 3.4 mg/g (282 mg total or 100% yield). The 53 g distillate was acidified with a few drops of dilute sulfuric acid (to convert any free base to the salt) and extracted 4 times with approximately 30 ml chloroform. The combined chloroform solutions were washed twice with 15 ml water and the chloroform evaporated in a stream of nitrogen at room temperature. The residual pale yellow oil was dissolved in 35 ml water and the solution assayed for radioactivity. The nitroso compound was estimated spectrometrically. A similar extraction of the 6 g distillate with a larger proportion of chloroform removed virtually all of the nitroso compound, enabling the specific activity of the tritiated water produced in the nitrosation to be determined. The yield of pure nitrosoazetidine and its specific activity are given in Table 2. Apparently a considerable proportion of the nitrosoazetidine was lost by evaporation. The total activity of nitroso compound and that of the tritiated water were calculated and the ratio of activity of carbon-bound tritium to nitrogen-bound tritium in the original salt determined. This is given in table 2.

TABLE 2

PREPARATION AND PROPERTIES OF TRITIATED NITROSAMINES

	AZETIDINE	PYRROLIDINE	PIPERIDINE	HEXAMETHYLENEIMINE	MORPHOLINE	PIPERAZINE	METHYLCYCLOHEXYLAMINE	METHYLANILINE	DIMETHYLAMINE		
									Wilzbach	Wilzbach (3 yrs. standing)	Electric Arc
Wt. of labeled amine salt (mg)	515	590	470	820	460	930	800	815	1180	360	757
Specific activity (μc/mg)	203	116	72	200	55	140	187	680	153	131	36
Total activity (mc)	105	69	33	276	25	130	150	555	181	47	27
DISTILLATES											
1) Conc. of nitrosamine (mg/g)	2.0	18.5	9.5	11.8	13.4	0	21.8		18.9	5.2	11.5
Weight (g)	19.7	5.8	45.7	12.4	4.5	10.6	9.0	58.2	50	43.7	56.4
Total activity (d/s)	47×10^7	220×10^7	144×10^7	590×10^7	18×10^7	54×10^7	340×10^7		620×10^7	66×10^7	98×10^7
2) Conc. of nitrosamine (mg/g)	4.2	9.7	0.9	5.6	7.6	0.3	4.7	0	1.3	0.1	0.7
Weight (g)	53.4	40.9	8.8	65.1	49.9	16.1	56.0	16.8	6.9	10.9	19.2
Total activity (d/s)	122×10^7	60×10^7	4×10^7	273×10^7	74×10^7	110×10^7	105×10^7		19×10^7	6×10^7	8×10^7
3) Conc. of nitrosamine (mg/g)	3.4	0	0	0	0		0			0	0
Weight (g)	6.1	7.4	12.9	12.0	9.4		19.0			13.3	16.0
Total activity (d/s)	16×10^7	4×10^7	6×10^7	34×10^7	11×10^7		23×10^7			7×10^7	7×10^7
Yield of nitrosamine (mg)	282(99%)	502(91%)	443(100%)	515(67%)	438(100%)		456(60%)		955(89%)	228(70%)	664(97%)
Recovery of radioactivity in distillates (d/s)	175×10^7	284×10^7	154×10^7	897×10^7	103×10^7		468×10^7		639×10^7	79×10^7	113×10^7
PURIFIED NITROSAMINES											
Weight (mg)	148	384	405	277	137	712	230	633	945	227	650
Yield (%)	52	70	92	36	32	83	30	82	88	70	95
Conc. in solution (mg/ml)	3.5	8.1	13.0	5.9	5.3	solid	4.9	18.1	18.9	5.2	11.5
Specific activity (μc/mg)	135	17.5	56	146	17	22	82	540	138	50	30
Total activity in nitrosamine (d/s)	141×10^7	32.5×10^7	107×10^7	278×10^7	37×10^7	59×10^7	138×10^7	1270×10^7	490×10^7	43×10^7	74×10^7
Total activity in water (d/s)	57×10^7	31×10^7	21×10^7	125×10^7	77×10^7	550×10^7	51×10^7	264×10^7	160×10^7	34×10^7	41×10^7
ORIGINAL AMINE											
Activity per C-bound hydrogen / Activity per N-bound hydrogen	$\frac{1}{2.4}$	$\frac{1}{7.7}$	$\frac{1}{2.0}$	$\frac{1}{3.4}$	$\frac{1}{17}$	$\frac{1}{37}$	$\frac{1}{5}$	$\frac{1}{1.7}$	$\frac{1}{2}$	$\frac{1}{4.8}$	$\frac{1}{3.3}$

Hexamethylenenitrosamine — 820 mg of hexamethyleneimine hydrochloride after nitrosation, adding alkali and distilling to dryness gave (1) 12 g of (alkaline) distillate containing 11.9 mg/g, (2) 65 g of distillate containing 5.7 mg/g and (3) 12 g of distillate containing no nitrosamine. Distillates 1 and 2 were slightly acidified with dilute acid and extracted with, respectively, 3 times 15 ml and 3 times 50 ml ether. The ether extracts were backwashed twice with one fifth the volume of water and the ether removed at room temperature in a stream of nitrogen. The residual oil was dissolved in approximately 50 ml water and the concentration of nitrosamine determined spectrometrically; the specific activity was determined on a weighed aliquot of the solution and is given in table 2. Again some of the nitrosamine was lost by evaporation and in the back extraction. The total activity of tritiated water and of nitrosamine were calculated and the ratio of activity of carbon-bound to nitrogen-bound tritium in the original amine was determined.

Methylcyclohexylnitrosamine — 800 mg of methylcyclohexylamine hydrochloride after nitrosation, adding alkali and distilling to dryness gave (1) 9 g of (alkaline) distillate containing 196 mg (some of the nitrosamine separated as an oil), (2) 56 g of distillate containing 4.65 mg/g, and (3) 19 g of distillate containing less than 0.3 mg/g of nitrosamine. Distillates 1 and 2 were slightly acidified with dilute acid and extracted with, respectively, 3 times 15 ml and 3 times 50 ml of ether. The ether extracts were backwashed twice with one fifth the original volume of water and the ether evaporated at room temperature in a stream of nitrogen. The residual oil was dissolved in approximately 50 ml water and the nitrosamine concentration and specific activity assayed (table 2). There was very little loss of nitrosamine by evaporation, but the yield was low (less than 50%). This might have been due to the presence of solvent in the original sample of tritiated amine hydrochloride, leading to an overestimate of the amount of starting material; the same explanation probably applies to the previous preparation. Trial preparations of the two nitrosamines using cold amines gave almost quantitative yields. The ratio of the activity of carbon-bound to nitrogen-bound hydrogen in the original amine was determined as before.

Nitrosomethylaniline — 815 mg of methylaniline hydrochloride after nitrosation, adding alkali and distilling to dryness gave (1) 58 g of distillate from which the nitrosamine separated as an oil, and (2) 17 g of distillate containing no nitrosamine. Distillate 1 was slightly acidified and extracted with 3 times 50 ml of ether. The ether extract was backwashed twice with 20 ml water and the ether evaporated at room temperature in a stream of nitrogen. The residual oil, containing a small amount of water, was dissolved in approximately 40 ml ethanol, the concentration of nitrosamine estimated spectrometrically and the specific activity determined (table 2). To ascertain whether any reduction of the benzene ring had taken place during exposure to tritium gas, an aliquot of the nitrosomethylaniline solution was chromatographed on a thin

layer plate [5] which, after development, was cut into several bands, including the strongly UV absorbing band containing the nitrosamine. Each band was eluted with a mixture of ether and methanol, made up to a standard volume and assayed for radioactivity. Negligible activity was present other than in the nitrosomethylaniline zone (which contained 90% of the total activity). The band corresponding to nitrosomethylcyclohexylamine contained only a minute amount of radioactivity. From the total activity in water and nitrosamine, the ratio of carbon-bound to nitrogen-bound activity in the original amine was calculated.

Nitrosomorpholine—460 mg of morpholine hydrochloride after nitrosation, adding alkali (20 ml of 65% NaOH) and distilling to dryness gave (1) 5 g of distillate containing 13 mg/g, (2) 50 g of distillate containing 7.6 mg/g, and (3) 9 g of distillate containing no nitrosamine. Distillate 2 was extracted with 4 times 25 ml chloroform, backwashed twice with 10 ml water and the chloroform extract distilled to a small volume. The residual liquid was extracted 4 times with 6-7 ml water and the concentration of nitrosomorpholine and its specific activity determined in the combined aqueous solution (table 2). There were, apparently, large losses in this procedure, which led to the revised method involving evaporation of solvent at room temperature in subsequent preparations.

Nitrosopiperidine — 470 mg of piperidine hydrochloride after nitrosation, adding alkali and distilling to dryness gave (1) 46 g of distillate containing 9.5 mg/g, (2) 9 g of distillate containing 0.9 mg/g, and (3) 13 g of distillate containing no nitrosamine. Distillate 1 was slightly acidified and extracted with 5 times 25 ml of ether, the ether solution backwashed twice with 15 ml water and the ether evaporated in a stream of nitrogen. The residual oil was dissolved in approximately 30 ml water and the concentration of nitrosopiperidine and its specific activity determined.

Nitrosopyrrolidine — 590 mg of pyrrolidine hydrochloride after nitrosation, adding alkali (20 ml of 65% NaOH) and distilling to dryness gave (1) 6 g of distillate containing 18 mg/g, (2) 41 g of distillate containing 9.7 mg/g, and (3) 7 mg of distillate containing no nitrosamine. Distillate 2 was slightly acidified and extracted with 4 times 25 ml chloroform; the chloroform solution was backextracted twice with 10 ml water and the chloroform distilled to a small volume. The residue was extracted with 4 times 10 ml (approximately) of water, the aqueous solution assayed for nitrosopyrrolidine and for radioactivity and the specific activity determined (table 2).

Dinitrosopiperazine — 930 g of piperazine hydrochloride after nitrosation, adding alkali and distillation gave two distillates of 11 g and 16 g, which contained negligible quantities of nitrosamine (dinitrosopiperazine is only slightly

volatile under these conditions). The solution remaining was extracted 4 times with 40 ml of chloroform, the chloroform solution backwashed twice with 20 ml water and the chloroform evaporated in a stream of nitrogen. The residual nitrosamine was a crystalline solid. The specific activity was determined with a small quantity of the crystals (table 2).

Since much of our biochemical work involved dimethylnitrosamine, it was necessary to carry out several preparations of this compound labeled with tritium. A second preparation of DMN from the same sample of dimethylamine hydrochloride-H^3, but after a three-year-time interval yielded a nitrosamine of lower specific activity (table 2); the water produced in this preparation had a considerably higher activity than before. This showed that, in this time, there had been a noticeable exchange of tritium between the methyl groups and ionizable hydrogen in the solution. Whether or not a similar slow exchange takes place in the dilute solutions of the prepared nitrosamines has not been ascertained.

A second preparation of tritium labeled dimethylamine hydrochloride was made using a more rapid method than Wilzbach exchange. This was a short term exposure to tritium gas during passage of an electric arc [6]. While this was expected to yield material of higher specific activity, as shown in table 2, in fact the product had a much lower specific activity than was the case with the Wilzbach exchange.

Several conclusions can be drawn from the experiments described here. Firstly, several of the amines, although highly purified, gave rise to impurities of high specific activity during tritiation. These appeared not to be secondary amines, since they survived nitrosation, but were rendered unextractable with ether or chloroform by acidification of the aqueous solution. They also tended to distill with the first few ml of water in the preparation of the nitrosamines. Secondly, the proportion of tritium gas incorporated under identical conditions in the various amines varied very widely; it seemed to be highest in methylaniline and lowest in morpholine, but there appeared to be no sharp distinction between the aliphatic compound (dimethylamine) and the ring amines. Thirdly, the ratio between the tritium exchanged with carbon-bound hydrogen and that exchanged with nitrogen-bound hydrogen varied considerably (from 1 : 2 to 1 : 40). The reason for these differences is not known.

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