SEPARATION AND DETECTION OF VOLATILE AND NON-VOLATILE N-NITROSAMINES

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A considerable range of N-nitrosamines derived from dialkyl, alkaryl and heterocyclic amines, etc., together with some N-nitrosamides studied are very potent carcinogens in a range of species[2, 3] so that their detection at low levels in environmental sources is of great importance.

Of the two most sensitive techniques for the detection and estimation of N-nitrosamines, polarography is more specific than gas liquid chromatography since the half wave potentials of authentic compounds of this type cover only a limited range of voltage. The specificity of their detection is enhanced in differential polarography in which advantage is taken of the degradation of all N-nitrosamines studied on irradiation with ultraviolet light in solution in 0.2N HCl to products devoid of a polarographic response at equivalent concentration. Thus a differential polarogram of a N-nitrosamine solution with reference to a further aliquot subjected to ultraviolet photolysis comprises a peak which is not influenced by other components stable to such irradiation. Unlike most N-nitrosamines, other polarographically active nitrogenous compounds are reduced at the dropping mercury electrode at both acid and alkaline pH values. Differentiation between N-nitrosamines and unsaturated aldehydes and ketones between which confusion is possible in differential polarography can be effected by the photolysis of the former in alkaline solution to nitrite, in the manner of Daiber and Preusmann.

The separation of N-nitrosodimetylamine from fish subjected to nitrite treatment is readily achieved by distillation in steam. The identity of the amine moieties contributing to N-nitrosamines available in biological systems will not, however, generally be known. In detecting and estimating small amounts of unknown N-nitrosamines in biological systems it is imperative to separate these compounds as a class as selectively as possible since the sensitivity of polarographic detection in homogenates of tissues is very poor. By no means all N-nitrosamines found to be carcinogenic can be separated by destillation in steam but of such compounds examined all were adsorbed from aqueous solution in large measure on activated carbon. After aqueous washing of the separated carbon, most adsorbed N-nitrosamines other than those containing phenyl residues can be eluted, at least in part, in boiling methanol, with separation from any inorganic nitrite originally present. Desorbed N-nitrosamines in aqueous methanpl can therefore be detected and estimated by differential polarography and by photolysis to nitrite under alkaline conditions.

EXPERIMENTAL

Polarography.

Polarographic observations were made with a Southern Analytical differential Cathode Ray Polarograph type A1660. In this instrument, the voltage sweep is applied simultaneously to two identical cells, the output currents of the being in opposition to each other. The wave heights appertaining to a range of concentrations of various N-nitrosamines were observed on the graticule superimposed on the cathode ray tube used for their display and were employed

for calibration curves, in conjunction with the pre-determined amplification factor. The deoxygenation of solutions was accomplished with a stream of oxygen free nitrogen.

Ultraviolet photolysis.

Ultraviolet irradiation of solutions of N-nitrosamines, etc., was undertaken with a Hanovia 1 litre photochemical reactor modified with a quartz vessel constructed to take six samples of 10 ml or less which was supplied by Quartz Fused Products, Shepperton, Middlesex.

Reagents.

N-nitrosodiumethylamine, diethylamine, di-n-propylamine and di-n-butylamine were obtained from Eastman Kodak Ltd., Kirkby, Liverpool; streptozotocin[4] was kindly provided by the Upjohn Company, Kalamazoo, Michigan, U.S.A., and C-nitro compounds by the British Petroleum Company, Sunbury-on-Thames. We are indebted to Dr. R. Preussmann of the Forscherhruppe Praeventivmedizin, Freiburg, Germany for samples of the N-nitrosamines of methyl-2-hydroxyethylamine, dicyclohexylamine, methyl-4-picolylamine, methylethylamine, methylamylamine, di-n-amylamine, dibenzylamine, diethanolamine and N-nitrodimethylamine, to Professor E. Boyland of the Institute for Cancer Research, London, for N-nitrosopyrrolodine, piperidine and N-methylaniline, to Dr. J.M. Barnes of the M.R.C. Laboratories, Carshalton, Surrey, for N-nitrosomethylurea and to Dr. E.K. Weisburger of the National Cancer Institute, Bethesda, Maryland, U.S.A. for 1:4-dinitroso-piperazine. N.nitrosoproline was prepared according to the method of Sander[5] and recrystallized from Benzene.

Granular activated carbon 8-12 BSS mesh was kindly donated by Sutcliffe, Speakman & Co. Ltd., Leigh, Lancashire.

Nitrite determination. These were based on the method of Nicholas and Nason[6].

RESULTS

Properties of N-nitrosamines.

Table 1 records the peak potentials, with reference to the mercury pool anode, in derivative polarography of a range of authentic N-nitrosamines in solution in aqueous 0.2N HCl. In solution in absolute methanol containing 0.1 volume aqueous 2N HCl, the peak potentials were 50-80 mv more negative. Well formed derivative peaks were observed on polarography of dialkyl, alkaryl and heterocyclic N-nitrosamines at a concentration of 0.1 p.p.m. the response disappearing in almost all instances on raising the pH above 2-3. Only in the case of 1: 4-dinitroso-piperazine was a wave evident at pH 8.4 (potential - 1.34v) comparable in size with that in 0.2N HCl (potential - 0.76v). The sensitivities of response of simple dialkyl-N-nitrosamines tested were of the same order with millimolar diffusion currents in direct polarography in aqueous 0.2N HCl for N-nitroso-dimethylamine, diethylamine, di-n-propylamine and di-n-butylamine of 26, 26, 39 and 37 A respectively. The polarographic waves of all N-nitrosamines examined disappeared after irradiation with ultraviolet light in 0.2N HCl within 1.1/2 - 3 hours. Thus a difference polarogram of a solution of a N-nitrosamine with reference to an aliquot subjected to irradiation comprises a peak characteristic of the original before photolysis whilst polarographically active contaminants not subject to photolysis do not contribute to the resultant wave.

The distilation at atmospheric pressure of an aqueous solution of a simple N-nitroso-

mine to half its original volume leads generally to its concentration in the distillate, particularly if NaOH to 2-3 N or 20% NaCl has been added initially. From Table I it is apparent that the introduction of an hydroxyl group into a simple dialkyl-N-nitrosamine as in N-nitrosomethyl-2-hydoxyethylamine almost completely eliminated the volytility in steam of the former, although the compound remained unchanged in the distillation residue even in the presence of 3N alkali initially.

The N-nitrosamines studied were removed from aqueous 10 p.p.m. solution virtually quantitatively on shaking for 2 hours with activated carbon (2 g/100 ml) with the exception of N-nitrosodimethylamine and N-nitroso-methyl-2-hydoxyethylamine (Table 1) for which the efficiency of adsorption was somewhat less. The capacity of carbon to adsorb N-nitrosodiethylamine as an instance, persisted at least over the range of pH from that of 0.2N HCl to 0.2 M phosphate buffer pH 6.0. Adsorption on other reagents (silica gel, alumina, etc.) was irregular andn rarely complete.

After centrifugation and acqueous washing of the carbon adsorbent, elution with boiling methanol (20 ml per g dry carbon) proceeded with all N-nitrosamines without a phenyl residue except N-nitroso-di-n-pentylamine but the proportion recovered varied considerably with the amine moiety of the nitrosamine, as is demonstrated in Table 1. A second similar desorption with further methanol was sufficient for the quantitative removal from carbon of all N-nitroso-di-n-propylamine but a similar treatment of adsorbed N-nitroso-di-n-propylamine but a similar treatment of adsorbed N-nitroso-di-n-butylamine raised the proportion adsorbed to only 50%.

In aqueous solution or in methanol with or without water, N-nitrosamines are photolysed to yield inorganic nitrite at an alkaline pH. Using the conditions of Daiber and Preussmann[1] in aqueous Na_2CO_3, 80-100% of one nitrogen atom involved in the nitrosamino group was liberated within 3 hours. In the presence of methanol, nitrite released was itself sunject to photolytic degradation and its release from an aqueous methanolic solution of Na_2CO_3 increased initially until an optimum period of irradiation of about 15 minutes, after which the inorganic nitrite detected declined to zero. A maximum release of up to 40% of one nitrogen atom as nitrite provided confirmation of the results of Daiber and Preussmann[1] for a more restructed range of N-nitrosamines.

Properties of related compounds.

Both the N-nitrosamides studied (Table II) gaveinitially strong polarographic waves in 0.2 N HCl but that of N-nitroso-N-methylurea disappeared within a few hours by virtue of its instability. A polarographic response in this electrolyte was common to a range of other compounds representing various nitrogenous types and also to unsaturated aldehydes and ketones but acetaldehyde gave no response. Unlike the majority of N-nitrosamines, most other nitrogenous compounds gave polarographic waves also under alkaline conditions, the sensitivity of response often being greater than at acid pH and at a more negative potential after the addition of borax to pH 8.4. N-nitrodimethylamine, in fact, gave a polarographic wave only at alkaline pH. Unsaturated aldehydes and ketones resembled the simpler N-nitrosamines in that their polarographic response disappeared on raising the pH in this manner.

None of the compounds tested could be differentiated from N-nitrosamines by ultraviolet photolysis in 0.2N HCl, since all were susceptible under the conditions employed, and all were strongly adsorbed by activated carbon. However, none of the other nitrogenous com-

pounds could be detected polarographically in methanol after attempted desorption from carbon in the reluxing solvent . The unsaturated aldehydes investigated were partially released into this solvent but the unsaturated ketones resisted desorption by methanol completely.

In solution in aqueous Na_2CO_3, all nitrogenous compounds tested yielded nitrite on irradiation with ultraviolet light; amyl nitrite reacted as inorganic nitrite also without photolysis. However, this reaction provided a means of differentiation between N-nitrosamines and unsaturated aldehydes and ketones between which confusion could otherwise arise polarographically.

Recommended procedures for the separation and detection of N-nitrosamines in biological materials.

In the absance of excessive lipid, aqueous homogenates of biological materials should contain both steam volatile and non-steam volatile N-nitrosamines which can be separated from the residual matrix by high speed centrifugation.

Simple volatile N-nitrosamines such as N-nitroso-dimethylamine, -di-n-pentylamine, -dibenzylamine, piperidine, etc., are readily separated by fractional distillation of aqueous slurries containing salt and methanol. For the removal of both volatile and non-volatile N-nitrosamines, clarified aqueous homogenates are stirred for 2 hours with granular activated carbon (2 g/100 ml); the efficiency of the removal of N-nitrosamines from solution is impaired by the presence of lipid. Aqueous washing of the separated carbon does not result in appreciable loss of adsorbed nitrosamines but does allow the removal of traces of inorganic nitrite possibly remaining from the original material examined; the polarography of N-nitrosamines must proceed in an acid environment which could promote the formation of artifacts if secondary amines are concurrently extracted.

After two or three washings of the carbon adsorbent with distilled water and decantation of residual liquid, N-nitrosamines are desorbed in refluxing methanol for two hours. Examination by derivative polarography of the methanolic eluate containing HCl to 0.2N reveals any waves attributable to N-nitrosamines at potentials within the relevant range, the concentration being estimated by the response on addition of an authentic N-nitrosamine preferably with a peak potential close to that observed. The disappearance of the wave without the appearance of a further wave at a more negative potential on addition of borax to pH 8.4 provides additional evidence of the presence of a N-nitrosamine. Similarly, the wave of a N-nitrosamine at acid pH should disappear on irradiation with ultraviolet light for two hours.

The determinations of inorganic nitrite upon aliquots of methanol eluates containing 0.1% Na_2CO_3 before and after ultraviolet irradiation for 15 minutes will indicate an increase due to exposure to the light if a N-nitrosamine (s) is present. The reaction is a complex one and the yield of nitrite is dependent upon the presence of other compounds of biological origin. For the estimation of N-nitrosamines present, it is therfore necessary to determine the yield of nitrite from an authentic N-nitrosamine in the same environment on making incremental additions to further aliquots of the methanol eluates. According to the results presented by Daiber & Preussmann[1], the yield of nitrite on alkaline irradiation of various N-nitrosamines is a linear function of their molar concentrations up to at least 0.2 mM.

DISCUSSION

The problem of the estimation of small levels of N-nitrosamines in biological sources is virtually unique in that it requires the detection of many such compounds all of potential physiological importance but with very different physical properties and with only a nitrosamino grouping (s) in common. It will be apparent from the foregoing that the methods of detection advocated are not unequivocal in characterization and provide an incomplete extraction at best. Thus the results obtained are most likely to represent underestimates of levels at which N-nitrosamines are present, though the absolute concentrations in environmental sources are seldom of great concern.

Other nitrogenous compounds simulating N-nitrosamines are unlikely to be formed without relatively drastic conditions of reaction and their confusion for N-nitrosamines would introduce an error on the side of safety. Some of the N-nitrosamines which would be lost using the separation techniques involving adsorption on carbon such as N-nitroso-diphenylamine are not, in fact, considered to be carcinogenic[2]. However, in spite of its obvious limitations, the method does provide a means of analysis of many N-nitrosamines which would not be detected using the customary distillation technique but which are nevertheless of potential physiological significance.

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TABLE I

PROPERTIES OF N-NITROSAMINES

N-Nitrosamine	Peak potential[+] in derivative polarography in 0.2N HCl (V)	Percent nitrosamine recovered on distilation to half volume from 20% NaCl	Percent nitrosamine adsorbed by carbon from aqueous solution	Percent[++] nitrosamine eluted from carbon with methanol
N-nitroso:				
Dimethylamine	-0.94	70	75	75
Methylamine	-0.92	70	100	66
Methylamine	-0.83	75	94	28
Diethylamine	-0.88	80	96	94
Di-n-propylamine	-0.84	85	100	72
Di-n-butylamine	-0.79	100	100	26
Di-n-pentylamine	-0.78	78	93	0
Dibenzylamine	-0.75	93	94	0
Dicyclohexylamine	-0.86	80	94	16
4. Picolyl-methylamine	-0.81	5	100	13
Methyl-2-hydroxyethylamine	-0.93	4	70	70
Diethanolamine	-0.90	0	92	60
Piperidine	-0.79	96	100	84
Pyrrolidine	-0.84	82	95	78
N-Methylaniline	-0.69	95	100	0
Proline	-0.79	0	90	72

[+] With respect to the mercury pool

[++] Based upon the amount of nitrosamine treated with carbon originally

TABLE II
PROPERTIES OF COMPOUNDS RELATED TO N-NITROSAMINES

Substance	Peak potential in derivative polarography in aqueous (V)		Elution from carbon with methanol	Photolysis in alkali to nitrite
N-nitrosamides:	0.2N HCl	pH8.4 buffer		
N-nitroso-N-methylurea	-0.68	-1.06	Unstable	+
Streptozotocin	(-0.40		-	+
	(-0.62	-1.06		
N-Nitramines:				
N-nitrodimethylamine	-	-1.34	+	+
-nitroarginine	-0.54	-1.15	-	+
C-nitro compounds:				
Nitromethane	-0.82	-1.00	-	+
1-nitropropane	-0.73	-0.95	-	+
Alkyl nitrite:				
Amyl nitrite	-0.99	-1.10	-	Reacts as nitrite
Alkyl nitrate:				
Ethyl nitrate	(-0.77			+
	(-1.02	no wave	+	
Unsaturated aldehydes:				
Crotonaldehyde	-1.03	no wave	+	-
Cinnamaldehyde	-0.66	-1.12	-	-
Furfuraldehyde	-0.99	-1.45	+	-
Unsaturated ketones:				
-Ionone	-1.01	no wave	x -	-
-Ionone	-0.86	no wave	-	-

+ With respect to the mercury pool anode