kept at 4° for 12 hr. The crystalline flavianate was filtered off, recrystallized five times from water and decomposed with $Ba(OH)_2$. The base was finally obtained in the form of its hydrated sulphate as previously described (Fearon & Bell, 1955). Yield 2·8 g., m.p. (after shrivelling at 162°) 170–171° (decomp.) not depressed when mixed with authentic canavanine sulphate (Found: C, 20·9; H, 5·5; N, 19·4; α-NH_2 N, 5·3. Calc. for $C_5H_{12}O_3N_4,H_2SO_4,H_2O$: C, 20·5; H, 5·5; N, 19·2; α-NH_2 N, 4·8%). $[\alpha]_D^{18} +18 \pm 2°$ in water (c, 10); canavanine sulphate monohydrate from *Colutea arborescens* (Fearon & Bell, 1955) had $[\alpha]_D^{18.5} +18.6$.

Finely ground seed (100 g.) of *Medicago sativa* was extracted as described for *Anthyllis vulneraria*. The two extracts were combined, concentrated to 200 ml. by distillation under reduced pressure and filtered before the addition of 5 vol. of ethanol. The amino acid was purified by recrystallizing its flavianate and finally isolated as the sulphate. Yield 1·5 g.; m.p. (after shrivelling at 162°) 172° (decomp.) not depressed when mixed with authentic canavanine sulphate (Found: C, 20·6; H, 5·4; N, 18·9; α-NH_2 N, 4·5%). $[\alpha]_D^{18} +18 \pm 2°$ in water (c, 2·2).

SUMMARY

1. Methods are described for the identification of canavanine in biological extracts containing compounds which mask or inhibit the guanidoxy colour reaction given by canavanine with trisodium pentacyanoammonioferrate.

2. It has been shown that canavanine occurs in the seeds of sixteen leguminous plants; quantitative values for eight are given. The acid has been isolated from two of them.

3. In addition to canavanine other compounds which, like the guanidoxines, react with trisodium pentacyanoammonioferrate at pH 7 have been detected in the seeds of leguminous plants.

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The Detection of Metabolic Products from Dimethylnitrosamine in Rats and Mice

By D. F. HEATH and ANNE DUTTON

Toxicology Research Unit, M.R.C. Laboratories, Woodmansterne Road, Carshalton, Surrey

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The toxic properties of dimethylnitrosamine have been described by Barnes & Magee (1954), Magee & Barnes (1956) and O'Leary, Wills, Harrison & Oikemus (1957). Doses of 20–40 mg./kg. produced acute liver necrosis and death in several species. Prolonged feeding of rats with lower doses led to malignant liver tumours.

Dimethylnitrosamine is metabolized rapidly *in vivo*. Magee (1956) showed that its half-life in rats was about 4 hr., and that its disappearance in rabbits followed an exponential law. Dutton & Heath (1956*b*) concluded that the compound was rapidly demethylated in rats and mice, since much of the ^{14}C administered in a labelled sample was expired as carbon dioxide. Since the metabolic products of demethylation are chemically much more reactive than the rather inert parent compound, it is possible that the toxic action may be exerted by metabolites. We have therefore made a search for metabolites in rats and, to a lesser extent, in mice, in the hope that this might throw some light on the primary biochemical lesion. The experiments were of a preliminary nature, aimed only at discovering systems likely to repay detailed study later, and consequently very few animals were used.

The problem has been approached in three ways. In the first, [^{14}C]dimethylnitrosamine was given, and the distribution of ^{14}C between the various tissues of rats and mice was found, to ascertain whether the ^{14}C not expired as carbon dioxide was particularly concentrated in any part of the body. In the other two approaches attention was concentrated on the liver, as this was the only organ damaged, and on the urine, which might contain a high proportion of metabolites, and in which, as it is less biochemically active, they were more likely to be preserved. Thus in the second approach we carried out distribution studies with dimethylnitros-

amine labelled with isotopic nitrogen in both of the possible ways, ^{15}NC ($Me_2{}^{15}N \cdot NO$) and ^{15}NO ($Me_2N \cdot {}^{15}NO$), fractionating the urine and liver in various ways.

Usually this could give no information on what the metabolites were. In the last approach therefore we applied chemical tests for specific metabolites to urine and liver homogenates, after protein precipitation. To set limits to this aspect of the work, we assumed that dimethylnitrosamine was demethylated, but that this might have been preceded or succeeded by reduction of the nitroso group to a hydroxylamino or an amino group. As methylnitrosamine is unstable in water, this led us to test for methylamine, hydrazine, nitrite and hydroxylamine. No special test was devised for methylhydrazine, which gives the same colour reaction as hydrazine (McKennis, Weatherby & Witkin, 1955), or applied for ammonia, which was unlikely to be found as such except in the urine, where it would be heavily diluted by natural ammonia and urea. Most of these groups can conjugate with acids in biochemical systems (Speck, 1947, 1949; Elliot, 1948; Webster & Varner, 1955), leading to methylamides, hydroxamic acids, oximes and hydrazides, for which a search was also made.

At most stages of this work the analytical methods already published, although basically sound, proved inadequate and had to be modified considerably.

METHODS

Compounds. [^{14}C]Dimethylnitrosamine, $(^{14}CH_3)_2N \cdot NO$, 74 μc/m-mole, was prepared as described by Dutton & Heath (1956*a*) and [^{15}NO]dimethylnitrosamine, $Me_2N \cdot {}^{15}NO$ (64 atom % ^{15}N), as described by Heath (1957). [^{15}NC]Dimethylnitrosamine, $Me_2{}^{15}N \cdot NO$, was prepared according to the scheme: $NH_4NO_3 \rightarrow NH_3 \rightarrow$toluene-*p*-sulphonamide$\rightarrow$*N*-dimethyltoluene-*p*-sulphonamide$\rightarrow$dimethylnitrosamine.

$^{15}NH_4NO_3$ (61 atom % ^{15}N, 5·5 m-moles) was treated with NaOH (2 ml. of 12·5 N) in a vacuum apparatus. The NH_3 released was dried with CaO, collected in liquid N_2 and dissolved in $CHCl_3$ (20 ml.) at $-70°$, and the solution warmed to room temperature. Toluene-*p*-sulphonyl chloride (5·8 m-moles in 10 ml. of $CHCl_3$) and Et_3N (14 m-moles in 5 ml. of $CHCl_3$) were run in, and the reaction was allowed to proceed for 21 hr. at room temperature. The sulphonamide was then extracted with 3 equal vol. of 3 N-NaOH, and converted into the *N*-dimethylsulphonamide by adding Me_2SO_4 (9·1 g.) and holding at 90° for 1 hr. The product was extracted and converted into dimethylnitrosamine (Dutton & Heath, 1956*a*). The yield, estimated polarographically (Heath & Jarvis, 1955), was 27 % on NH_4NO_3.

Animals. Animals were albino rats (Porton strain) and albino mice.

Administration. Dimethylnitrosamine was injected subcutaneously in 1–2 % aq. soln. at 50 mg./kg. (rat) and 25 mg./kg. (mouse).

Estimation of ^{14}C in samples. Samples of tissue were weighed wet, and oxidized to CO_2 by wet combustion. The CO_2 was converted into $BaCO_3$ and counted at infinite thickness, by using a thin end-window Geiger–Müller tube.

Nine published methods of oxidation were tried, but all gave low and variable recoveries with dimethylnitrosamine, probably because of its high volatility. The following composite method gave recoveries of 98–100 %, and was used on all samples. The combustion fluid was prepared as described by Evans & Huston (1952), omitting potassium iodate (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). The combustion was carried out in the apparatus of Lindenbaum, Schubert & Armstrong (1948), at atmospheric pressure, the CO_2 being swept over by a slow stream of CO_2-free air. The CO_2 was absorbed by saturated $Ba(OH)_2$, which was back-titrated at the end of the reaction with 0·1 N-HCl to a phenolphthalein end point, thus avoiding filtering $Ba(OH)_2$ solutions (Calvin *et al.* 1949). A drop of saturated $Ba(OH)_2$ was then added to ensure CO_2 retention, and the precipitate and supernatant were left overnight to flocculate. The precipitate was then collected on a sintered-glass filter (25 mm. diam., porosity 4) made for us by Baird and Tatlock Ltd. by cutting down a Gooch crucible to a height of 2 mm. above the sinter. To obtain an even deposit the supernatant was decanted through the filter under suction, followed by the $BaCO_3$, which was washed with ethanol–ether (3:1, v/v). The precipitate was washed with CO_2-free water, dried with acetone ether, stored in a desiccator to constant weight and weighed and counted. Infinite thickness of $BaCO_3$ required 142 mg./sinter. Sample sizes were chosen to give 140–250 mg. of $BaCO_3$.

All samples were counted to at least 1000 counts above background, and the background was determined each day by recording at least 1000 counts.

Estimation of ^{15}N. The nitrogen contents of various fractions were converted into NH_3 by micro-Kjeldahl techniques. Because some nitrogen compounds might be present in oxidized states, samples containing 2–3 mg. of nitrogen were first reduced with HI (Friedrich, 1933) and then oxidized for 24 hr. with the reagent of Chibnall, Rees & Williams (1943). Dimethylnitrosamine was extracted from biological samples before combustion. The method oxidized very little dimethylnitrosamine, but quantitative yields of NH_3 were obtained from it and from dimethylamine by reduction with HI followed by heating for 6 hr. with the oxidizing mixture used for the experiments with ^{14}C. After oxidation, all bases were separated by distillation from alkaline solution in a micro-Kjeldahl apparatus and estimated by titration. Ammonia was also estimated in some instances by Nessler's reagent to check that oxidation was complete. The samples were assayed for ^{15}N by Mr G. Dickinson, National Institute for Medical Research, Mill Hill, London.

Collection of urine. Urine from rats in a metabolism cage was separated from faeces and collected in a tube cooled by solid CO_2–acetone. Protein was precipitated with an equal volume of 0·25 % perfluoro-octanoic acid (Klevens & Ellenbogen, 1955).

Fractionation of urine for determinations of ^{15}N. Dimethylnitrosamine was removed after protein precipitation by six extractions with equal volumes of $CHCl_3$. The $CHCl_3$ extracts were rejected. Volatile bases and urea NH_3 were separated by aeration as described by Peters & Van Slyke (1932) for NH_3, with urease for urea NH_3. The ^{15}N in the protein fraction was also determined.

Fractionation of liver for determinations of ^{15}N. Rats were bled to death 4 hr. after injection, and the livers weighed immediately. The whole liver was homogenized at 0° in six to eight times its weight of 0·25M-sucrose in a Potter–Elvejhem type homogenizer with a Perspex pestle for 2 min. at 1500 rev./min., and made up with 0·25M-sucrose to contain 10% by wt. of liver.

Half was fractionated into nuclei, microsomes, mitochondria and supernatant (Schneider & Hogeboom, 1950). The protein in the supernatant was precipitated with 0·2 vol. of 30% trichloroacetic acid, and the resultant supernatant extracted immediately six times with equal volumes of $CHCl_3$ to remove dimethylnitrosamine.

The other half was fractionated into protein powder, nucleic acids, lipids and acid-soluble fractions. A portion (10 ml.) was treated with 10 ml. of 10% trichloroacetic acid, and the supernatant extracted six times with equal volumes of $CHCl_3$. The aqueous residue was taken as the acid-soluble fraction. In the longer procedure given now the dimethylnitrosamine was left in contact with trichloroacetic acid for some time, during which a little was decomposed by acid hydrolysis. It was therefore not suitable as a method of obtaining the acid-soluble fraction. In this procedure protein was precipitated from a 10 ml. portion as before. The precipitate was washed three times with 10% trichloroacetic acid, dissolved in 10–15 ml. of N-NaOH, left for 15 min., acidified slightly with 6N-HCl and reprecipitated with 10–15 ml. of 5% trichloroacetic acid. This procedure ensured the removal of all dimethylnitrosamine from the precipitate. This was then held at 90° for 15 min. in 10 ml. of 5% trichloroacetic acid to extract nucleic acids, and the residue washed twice with 5% trichloroacetic acid. Lipids were removed with acetone, alcohol–chloroform (1:2, v/v), alcohol–ether (3:1, v/v) and ether (twice). The fat solvents were evaporated to dryness to obtain the lipids. The residue of protein powder was dried at 100°. All fractions were assayed for ^{15}N in the usual way.

Chemical estimations

Compounds were determined colorimetrically in deproteinized urine and liver homogenates (10%, w/v, liver, deproteinized with trichloroacetic acid) from rats. Optical densities were measured with a Unicam spectrophotometer SP. 500. Sensitivities are given in terms of wet weight of liver and volume of urine before deproteinizing.

Hydrazine. Hydrazine in liver was determined by the method of Watt & Chrisp (1952) on 0·5 ml. of homogenate. In urine, urea and pigments interfered, but could be allowed for by using two wavelengths, 420 and 470 mμ, with 0·1 ml. of urine/analysis. Then the concentration of hydrazine = 67 (E_{470}–E_{420}/22) μg./ml. of urine, where values of E are the optical densities in 1 cm. cuvettes. The factor 22, which gives the difference between the densities in the absence of hydrazine, varies by about ±3 from urine to urine, so that E_{470}–E_{420} was only significant when it exceeded 0·02. This simple formula ignores the absorption at 420 mμ due to hydrazine. As the optical density of the urine alone at 420 mμ is in the range 1–1·5, it can be shown that for values of E_{470} less than 0·4 the error introduced by ignoring the hydrazine absorption at 420 mμ is less than the uncertainty introduced by the variations in the factor. The concentrations of hydrazine likely to be encountered correspond to optical densities much less than 0·4 at 470 mμ. The equation can be modified to allow calculation of results from higher optical densities, with the absorption spectra given by Watt & Chrisp (1952), but we did not need to do this.

Hydrazides. The method of Rapi (1953) was used on 4 ml. samples except that the colour was separated from the pigments by extraction with butanol. When using *iso*nicotinic hydrazide in recovery tests, this reduced the optical density by 25% at all concentrations. It was assumed that other hydrazides would give similar colour reactions.

Methylamine and methylamides. Methylamine is volatile from weakly alkaline solutions containing high concentrations of salts. Methylamides are fairly stable at room temperature under such conditions, but give methylamine on prolonged hydrolysis in boiling dilute acid. Methylamine can be estimated by the method of Ormsby & Johnson (1950). Thus in principle both methylamine and methylamides can be estimated, assuming that only compounds containing methylamide groups give methylamine on acid hydrolysis. In practice, fairly consistent recoveries of methylamine added to urine before deproteinization were obtained by strict adherence to the method given below, but the recoveries of methylamine from liver homogenates and of methylacetamide from both urine and liver homogenates were dependent on concentration and were very low (less than 10% at 5 μg. of nitrogen/g. of liver or/ml. of urine, and 20–30% at 25 μg. of nitrogen/g. of liver or/ml. of urine), so that the tests on these systems were only qualitative.

For methylamine a 10 ml. portion (deproteinized urine or liver homogenate) was placed in a 100 ml. two-necked flask in an ice bath, and neutralized with 5N-NaOH, of which 0·3 ml. more was then added, and followed by 4 g. of anhydrous K_2CO_3. A vacuum leak and side arm were put on quickly, and the side arm was connected with rubber tubing to a removable tap in one arm of a U-tube immersed in liquid N_2 containing 1 ml. of 2N-HCl and evacuated to 1 mm. Hg. The flask was evacuated slowly over several minutes. The tap was then opened fully, the flask warmed to 25° and at least 4 ml. allowed to condense in the U-tube. The tap was closed, the vacuum in the U-tube was released, the tap placed on the other arm and the first stoppered, and the U-tube re-evacuated for a few seconds. The tube was then allowed to warm to room temperature under vacuum, and shaken, and the contents were transferred with washing to a 25 ml. standard flask. If the tube was not re-evacuated the increase of pressure on warming forced the stopper out.

For methylamides, deproteinized-liver homogenates were first extracted six times with equal volumes of $CHCl_3$ to remove dimethylnitrosamine, acid hydrolysis of which yields nitrous acid, which destroys methylamine. This step was omitted in recovery tests, as methylacetamide is extractable from water by $CHCl_3$, unlike the methylamides of amino acids. Then 2 ml. of 36% (w/w) HCl were added to 10 ml. of liver homogenate or 5 ml. of urine + 5 ml. of water, and refluxed for 20–24 hr. The condenser was washed down with water, and the solution cooled; then 5 ml. of 12·5N-NaOH was added and the methylamine separated by distilling half the total volume into an iced receiver. The volume was made up to 25 ml. This gave methylamine + methylamides, from which methylamides were calculated by difference.

The methylamine in the 25 ml. samples was determined by the method of Ormsby & Johnson (1950), twice the volumes being used. For their method, the concentrations of NH_3 and NaOH are critical. The NH_3 was therefore determined in the samples from methylamine estimations by back-titration of a portion with NaOH, and in those from methylamide estimations by direct titration of a portion with acid. Ammonia, NaOH and water were added to the portions taken for methylamine analysis to give the final concentrations recommended. The method appeared very sensitive to the precise methods of incubation, so that a standard of pure methylamine solution was always determined with each batch, greatly increasing the precision. The optical densities were measured in 4 cm. cuvettes.

Recoveries of methylamine from urine are given in Table 1. They averaged 70%, so values obtained were multiplied by 1·43 to correct for this. The urine of untreated animals normally contained 5–10 μg. of methylamine nitrogen/ml., and the use of the method was limited by the variations in these control values, rather than by inaccuracies in the method.

Nitrite. This compound was estimated by the method of Shinn (1941). For liver a 5 ml. sample was used. For urine, pigments interfered with the determination, so the dye produced was extracted quantitatively into butan-2-ol at pH 7–8. The butan-2-ol layer was transferred to a 50 ml. flask, acidified with an equal volume of N-HCl, and made up with ethanol. The colour was then read at 545 mμ in a 4 cm. cuvette. Optical density, 0·87/10 μg. of NO_2^-.

Hydroxylamine, hydroxamic acids and oximes. All these can be estimated as nitrous acid by modifications of the methods of Raschig (1924) and Csaky (1948). On pure aqueous solutions the method below gave quantitative results for hydroxylamine, benzohydroxamic acid and propionohydroxamic acid, and 70–90% of the theoretical for dimethylglyoxime, benzoin oxime and acetone oxime.

For hydroxylamine and the hydroxamic acids a 5 ml. portion of solution, containing 0·5–10 μg. of hydroxylamine, was acidified, adjusted to pH 3–4 with NaOH, and in turn were added 0·5 ml. of 40% (w/v) sodium acetate, 1 ml. of 0·1 N-I_2 in acetic acid and 5 ml. of 0·2% sulphanilamide. After 3–10 min., 1 ml. of 5% ammonium sulphamate was added; after a further 2 min., 2 ml. of 0·1 N-sodium arsenite; and, after a further 2 min., 1 ml. of 5 N-HCl and 1 ml. of 0·1% *N*-(1-naphthyl)ethylenediamine dihydrochloride. From the addition of I_2 to the addition of arsenite the reaction had to be carried out in the dark. The total volume was made up to 50 ml., and the solution left 15 min. and the colour read at 545 mμ. in 4 cm. cuvettes.

For oximes, 1 ml. of 5 N-HCl was added to a 5 ml. sample, and the mixture held at 100° for 1 hr., before continuing as above. The concentration of HCl which could be used was limited, as high concentrations of NaCl interfered with colour development.

Liver homogenates gave lower and variable recoveries, so that the methods became semi-quantitative only. For hydroxylamine, 5 ml. samples were extracted four times with equal volumes of $CHCl_3$ to remove dimethylnitrosamine. The supernatant was then analysed for hydroxylamine. Recoveries of 60–95 % were obtained (nine samples from homogenates of two livers). In experiments with treated animals, blanks were provided by liver homogenates of untreated animals. Blanks varied a little from liver to liver, not due to the presence of hydroxylamine, but due to general pigment and cloudiness. As a result, at the lowest concentrations judgment by eye proved more reliable than measurement by the spectrophotometer. The least discernible by eye was 0·5 μg./g. of liver; the least concentration certainly significant on the spectrophotometer was 2 μg./g. of liver. For oximes the homogenates were made faintly alkaline and concentrated to half the volume by boiling to remove dimethylnitrosamine (this also destroyed free hydroxylamine). The volume was made up to 5 ml. and the oximes were hydrolysed with HCl as already described. Recoveries varied from 19 to 65% (19 results on five homogenates). Concentrations equivalent to 0·9 μg. of hydroxylamine/g. of liver gave positive results, as judged by eye, in all eight samples tested at this concentration, although two of the readings were less than the variations in the blanks determined on the spectrophotometer.

Urine samples could not be treated similarly as pigments interfered, and no method was found for oximes. The following method was used for hydroxylamine and benz- and propion-hydroxamic acids. Deproteinized urine (5 ml.) was adjusted to pH 1–2, held at 100° for 5 min., and cooled and filtered slowly through an ion-exchange column (1 cm. × 3 cm.) of Dowex 50 resin (Dow Chemical Co., Midland, Mich., U.S.A.) or Amberlite IR-120 (British Drug Houses Ltd.) both 50–100 mesh in the H^+ form. The column was washed with water and eluted with 5 ml. of 2 N-HCl followed by water. The acid fractions (volumes about 20 ml.) were analysed for hydroxylamine as usual. Above 5 μg. of hydroxylamine recoveries were 50–60%; below 1 μg. of hydroxylamine recoveries were very low. Failure to obtain a colour probably only indicated that the urine sample contained less than 2 μg. of hydroxylamine.

Sensitivities of the chemical methods. Assuming that metabolites of dimethylnitrosamine were distributed evenly throughout the animal and its urine, all the tests except those for methylamine and methylamides were sensitive enough to detect metabolites equivalent to 7% of an injected dose of 50 mg./kg. The sensitivity for methylamine in urine was about 50%. Assuming even distribution, methylamides in urine and methylamine and methylamides in liver were not detectable, the lowest quantities detectable being equivalent to 10, 5 and 5% of the total dose of dimethylnitrosamine injected.

Table 1. *Recoveries of methylamine from rats' urine*

Methylamine hydrochloride was added to rats' urine before deproteinization, and the urine was analysed for methylamine as described under Methods.

Methylamine added (μg. of N/ml.)	0	5·0	23
Methylamine found (μg. of N/ml.)	5·9, 6·1, 6·1	9·3, 9·3, 9·3	24, 23, 23, 22
Recovery of added methylamine (μg. of N/ml.)	—	2·8, 2·8, 2·8	18, 17, 17, 16

Table 2. *Distribution of* ^{14}C *in a mouse and a rat treated with* [^{14}C]*dimethylnitrosamine*

A 40 g. mouse was given 1 mg. of dimethylnitrosamine containing 1 μc of ^{14}C. Expired air, faeces and urine were collected. At 6 hr. the mouse was killed, and ^{14}C in the organs, etc., was determined as $BaCO_3$. Similarly, a 100 g. rat was given 5 mg. of dimethylnitrosamine containing 5 μc of ^{14}C, and killed at 24 hr. Specific activities of the $BaCO_3$ samples are given, with standard errors, as counts/min., and the total activity in each fraction as a percentage of the total ^{14}C injected. Weights of the organs of the mouse were obtained by weighing; those of the rat were from the table given by Caster, Poncelet, Simon & Armstrong (1956). No radioactivity is given for the expired air from the rat as several air samples were taken at different times.

	Mouse		Rat	
	Radioactivity (counts/min.)	% of activity injected	Radioactivity (counts/min.)	% of activity injected
Expired air	374±4	64·0	—	44·0*
Urine	226±5	6·0	120±5	6·0†
Faeces	1·7±0·32	—	41·8±1·5	0·8
Blood	6·3±0·43	1·0	22·2±0·90	0·8
Liver	24·2±0·74	8·0	28·0±1·1	2·6
Kidney	17·7±0·80	1·4	30·0±1·2	0·3
Lung	7·7±0·49	0·2	23·2±0·95	0·2
Spleen	7·2±0·48	—	53·1±1·9	0·3
Testis	1·0±0·31	0·0	13·0±0·64	0·3
Heart	6·1±0·43	0·2	12·8±0·66	0·1
Brain	4·8±0·40	0·3	—	—
Thymus	9·9±0·54	0·1	—	—
Muscle	4·0±0·37	—	10·9±0·58	11·2
Stomach	5·0±0·46	3·0	23·7±0·97	0·7
Intestine	16·2±0·73	3·6	48·0±1·7	5·3
Cartilage	—	—	7·0±0·47	2·5
Carcass and fat	1·9±0·33	10·8	15·7±0·72	3·2 (fat only)
Skin and hair	3·2±0·28	3·0	13·0±0·64	5·8
Total recovered		101·6		84·1

* Later results suggest that normally at least 60% is expired in 24 hr.
† Results from another experiment with a similar rat.

Table 3. *Distribution of* ^{15}N *in livers of rats* 4 *hr. after injection with* [^{15}NC]- *and* [^{15}NO]-*dimethylnitrosamine*

A 200 g. rat was injected with 10 mg. of either [^{15}NC]- or [^{15}NO]-dimethylnitrosamine, and bled to death 4 hr. later and the liver excised, homogenized and fractionated in two ways. One way gave nuclei, mitochondria, microsomes, residual protein and supernatant; the other gave acid-soluble fraction, nucleic acids, lipids and protein powder. Results are expressed as atom % excess of ^{15}N. The minimum excess of ^{15}N detectable was 0·003%.

Fraction	^{15}N (atom % excess) [^{15}NC] expt.	[^{15}NO] expt.
Nuclei	0·006	0·006
Mitochondria	0·004	0·006
Microsomes	0·009	0·009
Residual protein	0·003	0·006
Supernatant	0·025	0·013
Acid-soluble fraction	0·023	0·009
Nucleic acids	0·003	0·003
Lipids	0·006	0·006
Protein powder	0·003	0·006

RESULTS

Distribution of ^{14}C *in injected animals.* A mouse and a rat were given [^{14}C]dimethylnitrosamine and kept in a metabolism chamber until the dimethylnitrosamine had been completely metabolized. The ^{14}C in the expired air, excreta and organs was then determined, with the results shown in Table 2.

Much of the ^{14}C was expired as $^{14}CO_2$. Carbon compounds in the urine were relatively rich in ^{14}C, and the total ^{14}C excreted in the urine of the rat (6%) was considerably greater than the quantity of dimethylnitrosamine (1–2%) found by Magee (1956) to be excreted from rats treated similarly. Otherwise the specific activities found in the various fractions were fairly constant, with no marked preponderance in the liver.

Distribution of ^{15}N *in rats injected with* [^{15}N]-*dimethylnitrosamine.* Different rats were injected with dimethylnitrosamine labelled in the ^{15}NC and ^{15}NO positions respectively and killed by bleeding 4 hr. later. The liver was fractionated as already described, and the ^{15}N content of the various fractions determined. Results are shown in Table 3.

Four rats for each compound were similarly injected, and the urine was collected over the first 8 hr., and then from 8 to 24 hr. Results are shown in Table 4.

The total ^{15}N content of the liver was low, and fairly evenly distributed, the supernatant fractions containing somewhat more than the rest. At 4 hr. half of an injected dose is decomposed

(Magee, 1956) and the total ^{15}N content of the liver was therefore consistent with the assumption that the ^{15}N was evenly distributed throughout the nitrogen-containing constituents of the rat. Thus the liver, which contains about 5 % of the nitrogen in a rat, contained about 2 % of the ^{15}N given. If the ^{15}N was evenly distributed, it should have contained 2·5 % of the ^{15}N. This agrees with the calculated value within the experimental error of the determinations of ^{15}N, as the concentrations of ^{15}N were very low. The urine collected in the first 8 hr. contained two to three times as much ^{15}N as the liver, mainly as urea. The concentration of ^{15}N was highest in the free base fractions, but these contained only about 7 % as much nitrogen as the urea. The concentration dropped in the next 16 hr., but the rate of excretion remained high.

The position of labelling made no marked difference to the distribution of ^{15}N. Such differences as there were are discussed later. Urinary protein contained the same low percentage of ^{15}N (0·004 %) as plasma protein. Higher percentages found in urinary protein in some earlier experiments were probably due to inadequate washing.

Chemical tests. Tests for hydrazine, nitrites, hydroxylamine, hydroxamic acids, oximes and methylamine + methylamides were carried out on homogenates of the livers of treated rats 4 hr. after injection. Traces of hydroxylamine were found ($<2\,\mu$g./g. of liver) in two out of four rats, and traces of methylamine + methylamides in each of two rats. These tests are only qualitative, but probably indicated the presence of methylamine, either free or conjugated, equivalent to 5–10 % of the dimethylnitrosamine given.

Similar tests were carried out on urine collected over the first 8 hr., and then from 8 to 24 hr. In one of two experiments a trace of hydroxylamine was found (about 2 μg./ml.). Both control and treated rats excreted traces of nitrite.

To find whether more methylamine was excreted by treated rats, tests for free methylamine and total methylamine (methylamine + methylamides) were carried out on the urine from groups of rats on several successive days. These rats were then injected and the tests repeated. The injected rats urinated at about four times the usual rate, which lowered the accuracy of the tests. Ten experiments were carried out on a total of 20 rats. The control rats excreted free methylamine at about 60 μg. of nitrogen/day, but the actual rates varied by a factor of two from day to day. The qualitative test for methylamides showed that at least 100 μg. of methylamide nitrogen/day was excreted. If the methylamine excreted after injection was compared with the average daily excretion from the same rats in each experiment, then the following results were obtained. In no experiment was the total methylamine excreted in the first day after injection less than the average. In seven experiments it was over 50 % higher, and in three experiments 300 % higher. In six experiments, including the three in which the total methylamine increased less than 50 %, methylamine and methylamides were estimated separately. In all these experiments the excretion rates of free methylamine in the first 8 hr. were higher than the averages by an amount equivalent to an optical density of 0·02 or more, i.e. by significant amounts. From 8 to 24 hr. the rate was always higher than in the controls, but by insignificant amounts. The methylamide-excretion rates did not increase on the average. As in six experiments the excretion increased significantly, and in all ten experiments an increase was recorded, there seems no doubt that the excretion of methylamine was increased (on the average by about 60 μg. of nitrogen/day).

Isolation of [^{14}C]*methylamine.* Methylamine and methylamide methylamine were separated from the

Table 4. *Distribution of* ^{15}N *in the urine of rats treated with* [^{15}NC]- *and* [^{15}NO]-*dimethylnitrosamine*

Four 200 g. rats were used for each experiment, each being injected with 10 mg. of dimethylnitrosamine. The urine was collected in a container cooled with solid CO_2–acetone. Urine samples collected from 0 to 8 hr. and from 8 to 24 hr. were treated separately. Each sample was treated with an equal volume of 0·25 % perfluoro-octanoic acid to precipitate protein, and the ^{15}N in protein and supernatant was determined. The supernatant was immediately extracted six times with chloroform, and the residual ^{15}N in the aqueous layer was determined. The ^{15}N of the free bases in the supernatant, and of the free bases and the ammonia liberated by urease, was then estimated. Results are expressed as atom % excess of ^{15}N.

	[^{15}NC] expts.		[^{15}NO] expts.	
Fraction	0–8 hr.	8–24 hr.	0–8 hr.	8–24 hr.
Total supernatant	—	—	0·054	0·047
Supernatant after chloroform extraction	0·051	0·020	0·025	0·025
Free bases	0·108	0·026	0·085	0·042
Free bases + ammonia after treatment with urease	—	0·017	0·019	0·017
Protein	0·004	0·017	0·004	—

urine obtained over 1 day from a 100 g. male rat given 5 mg. of dimethylnitrosamine containing 5 μc of ^{14}C. Methylamine hydrochloride (200 mg.) was added to the distillates (i.e. the methylamine in the urine was diluted about 2000-fold), and the solution was evaporated to dryness. The residue was dissolved in ethanol (10 ml.), which was concentrated to 5 ml., and cooled with an ice–salt mixture. The methylamine content of the mixture of methylamine hydrochloride and ammonium chloride which crystallized out was determined colorimetrically. Portions were oxidized as usual and counted as $BaCO_3$. The specific activity of the $BaCO_3$ from diluted free methylamine was 24 ± 1 counts/min. and from the total methylamine was 37 ± 1 counts/min. It was calculated that the free methylamine contained 17 % of the total ^{14}C in the urine, and the methylamides 8 % of the total ^{14}C. The only radioactive compound which might have interfered was dimethylamine. The total ^{14}C in the urine was equivalent to 180 μg. of dimethylamine, assuming that it was derived from dimethylnitrosamine. As dimethylamine hydrochloride is considerably more soluble in ethanol than methylamine hydrochloride and was present at less than one-thousandth of the concentration, it is unlikely that it would have crystallized out. Thus some of the extra methylamine excreted after injection was derived from dimethylnitrosamine.

DISCUSSION

The results throw little light on the biochemical lesion which leads to injury to the livers of rats and mice, but do provide an outline of the major reactions undergone by dimethylnitrosamine *in vivo*.

Experiments with [^{14}C]dimethylnitrosamine showed that much of the ^{14}C was respired as carbon dioxide, and the remainder was distributed fairly evenly throughout the tissues. These results are consistent with the assumption that the compound was demethylated to one carbon-atom intermediates, which were either further oxidized to carbon dioxide or used in the normal metabolic processes of the body. Such a process implies that the compound was demethylated to monomethylnitrosamine, which, as it is unstable, would give methylamine, and this, by further demethylation, ammonia. Traces of methylamine were found in the liver, and some labelled methylamine in the urine, a few hours after treatment. That much of the amino nitrogen was converted into ammonia was shown by experiments with [^{15}NC]dimethylnitrosamine. The urea ammonia was relatively heavily labelled (Table 4) and the nitrogenous fractions of the liver were evenly and lightly labelled (Table 3), as would be expected. It is interesting, however, that the distribution of ^{15}N after injection of [^{15}NO]-dimethylnitrosamine was very similar. Thus some of the nitroso groups were reduced to ammonia, as shown by the labelling of the urine fractions (Table 4), but it cannot be assumed that this was so in the liver fractions. The nitroso group is likely to be liberated as nitrite, and then can be either oxidized to nitrate or reduced to hydroxylamine or ammonia, any of which might react with protein. The labelling was, however, remarkably unspecific, and was consistent with the assumption that both the NC and NO nitrogen atoms became distributed evenly throughout the nitrogenous constituents of the body, although Magee (1956) has shown that the liver is the only organ capable of metabolizing the compound. This favours the assumption that the nitroso group was reduced to ammonia, on the grounds that nitrite and hydroxylamine, being more generally reactive, would be more likely to react near where they are formed, whereas ammonia would readily enter the normal metabolism.

The only conclusion about the biochemical lesion which can be drawn from these results is that it cannot involve the attachment of more than minute quantities of any metabolite of dimethylnitrosamine to liver constituents. The isotopic analyses would have revealed the attachment of metabolites equivalent to less than 1 % of the injected dose. In this, dimethylnitrosamine differs from the amino-azo dyes, residues of which are incorporated into liver protein (e.g. Miller & Miller, 1947, 1953; Hultin, 1956). Whether dimethylnitrosamine itself or one of its metabolites exerts the toxic action is therefore still undecided.

SUMMARY

1. Mice were treated with [^{14}C]dimethylnitrosamine, and rats with unlabelled dimethylnitrosamine, and with [^{14}C]-, [^{15}NO]- and [^{15}NC]-dimethylnitrosamine.

2. In rats and mice much ^{14}C was respired as carbon dioxide. The remainder was rather evenly distributed between the tissues.

3. In rats treated with [^{15}N]dimethylnitrosamine, liver fractions (nuclei, mitochondria, microsomes, protein from supernatant and residual supernatant; and liver protein, nucleic acids, lipids and acid-soluble fractions) were evenly and lightly labelled. In the urine free bases and urea nitrogen were heavily, and protein lightly, labelled. Plasma protein was lightly labelled.

4. Urine and acid-soluble fractions were tested microchemically for hydrazine, hydrazide, methylamine, methylamide, nitrite, hydroxylamine, hydroxamic acids and oximes by modifications of previously published methods. Results were

negative except for traces of methylamine in liver, and more in urine, some of which was derived from dimethylnitrosamine.

5. The results indicate that the dimethylamino groups are oxidized to one carbon-atom intermediates and ammonia, and that the nitroso group is partly reduced to ammonia.

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Nitrogenous Compounds and Nitrogen Metabolism in the Liliaceae

4. ISOLATION OF AZETIDINE-2-CARBOXYLIC ACID AND EVIDENCE FOR THE OCCURRENCE OF $\alpha\gamma$-DIAMINOBUTYRIC ACID IN *POLYGONATUM**

By L. FOWDEN and MARY BRYANT
Department of Botany, University College, Gower Street, London, W.C. 1

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Azetidine-2-carboxylic acid, the lower imino acid analogue of proline, has been isolated from two members of the Liliaceae family. Fowden (1955, 1956) isolated it from *Convallaria majalis* (lily of the valley) and characterized it chemically by comparison with synthetic material. An identical substance was isolated from *Polygonatum officinalis* (a Solomon's seal) by Virtanen and co-workers (Virtanen, 1955*a*). A survey of liliaceous and related plants by Fowden & Steward (1957) demonstrated that the imino acid was present in about one-quarter of the ninety species examined. Its distribution was practically confined to the members of the Liliaceae, although a few species of the Agavaceae contained small amounts of the acid. The observation that it is present in certain members of the latter family, a group of plants split off rather recently from the Liliaceae, is interesting since there is no report of its occurrence in other families. The imino acid often accumulates in very large amounts and may represent the major proportion of the non-protein-nitrogen of particular plants. This is the case for the two species from which it was isolated. Azetidine-2-carboxylic acid is not known to occur in plant proteins.

The present paper describes the isolation of azetidine-2-carboxylic acid and $\alpha\gamma$-diaminobutyric acid from a liliaceous plant. Diaminobutyric acid,

* Part 3: Zacharius, Cathey & Steward (1957).