cellulose and on insoluble swollen cellulose, but the bacterial extracts, which are even more effective on carboxymethylcellulose than the fungal enzyme preparation, are inert towards swollen cellulose. Neither preparation is particularly effective against undegraded cellulose of cotton fibres, although cell-free filtrates from *M. verrucaria* produced about 10% solubilization of this substrate, as measured by the alkaline-digestion procedure.

Carboxymethylcellulase appears to be relatively stable to heat, as is evident from the increased reactivity at about 50°, thus confirming work by Reese, Siu & Levinson (1950). In addition, Fig. 2 illustrates that cellulase not only has an optimum temperature similar to that of carboxymethylcellulase, but also a relatively greater hydrolytic effect at 50° than has that enzyme. This can be attributed to the corresponding difference in their susceptibilities to heat treatment at 50° (cf. 'treatment A' of Fig. 6, Halliwell, 1961).

Both enzymes are relatively stable to the pH of the medium during short periods of exposure at pH 5-7 but outside this region activities drop sharply in a manner resembling the pH-activity curve described above.

Existing cellulase preparations fail to solubilize undegraded cellulose found in, for example, cotton fibres, and it was thought that this might be due to proteolytic attack on cellulolytic systems. However, digestion experiments indicated that both cellulase and carboxymethylcellulase lost no more activity in the presence of pepsin or trypsin than could be ascribed to the acidity of the medium.

This report extends the findings of Halliwell (1961) on this subject by defining the conditions required for the quantitative determination of cellulase and carboxymethylcellulase, as well as enumerating more fully the situations which govern the reaction of cellulase on its insoluble substrate.

SUMMARY

1. The action of cell-free filtrates from *Myrothecium verrucaria* and from rumen bacteria was examined on soluble carboxymethylcellulose, insoluble swollen cellulose powder and on cotton fibres.

2. Conditions are defined for reproducible and quantitative determinations of carboxymethylcellulase. The Michaelis constant of rumen bacterial carboxymethylcellulase is 0.8 g. of carboxymethylcellulose/l.

3. Maximum activity of carboxymethylcellulase and cellulase is evident at 50°.

4. Carboxymethylcellulase and cellulase exhibit maximum stability between pH 5 and 7.

5. Neither enzymic system is inactivated by digestion with pepsin or trypsin.

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The Decomposition and Toxicity of Dialkylnitrosamines in Rats

By D. F. HEATH

Toxicology Research Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey

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Dimethylnitrosamine inhibits protein synthesis (Magee, 1958; Brouwers & Emmelot, 1960; Emmelot & Mizrahi, 1961), causes centrilobular necrosis of the liver (Barnes & Magee, 1954; Magee, 1958) and is a potent carcinogen, producing cancers

in the liver, kidney and lung (Magee & Barnes, 1956, 1959, 1962; Schmähl & Preussmann, 1959; Zak, Holzner, Singer & Popper, 1960; Argus & Hoch-Ligeti, 1961; Dontenwill & Mohr, 1961). Several other dialkylnitrosamines have been

shown to cause one or more of these effects (Schmähl, Preussmann & Hamperl, 1960; Druckrey, Preussmann, Schmähl & Müller, 1961*a*, 1962; Druckrey, Schmähl & Schildback, 1961*b*; Argus & Hoch-Ligeti, 1961; P. N. Magee, personal communication; Heath, 1961).

Dimethylnitrosamine is decomposed both *in vivo* (Magee, 1956; Heath & Dutton, 1958) and *in vitro* (Magee & Vandekar, 1958; Brouwers & Emmelot, 1960; Hultin, Arrhenius, Löw & Magee, 1960). As biochemical effects are only observed after a time-lag (Magee, 1958; Hultin *et al.* 1960), and as decomposition *in vivo* is substantially confined to the liver, which is the only organ severely affected by acute doses (Magee, 1956), it has generally been assumed that the toxic compound is a metabolite. Formaldehyde (Brouwers & Emmelot 1960), nitrite (Heath & Dutton, 1958) and diazomethane (Rose, 1958; R. Schoental, personal communication) have been suggested, and have been discussed by Hultin *et al.* (1960). There is no evidence, however, that the biochemical effects observed could kill the animal, and, consequently, no hypothesis as to the nature of the toxic substance was ruled out. In this paper I attempt to elucidate the nature of the toxic substance.

Some of the possible compounds have properties which exclude any direct approach, since they are probably too unstable to find, and, if they were found, could not be given to an animal in such a way as to confine their effects to the liver, so that it would be difficult to assess their importance. Diazomethane is of this type. Others are essentially normal metabolites, e.g. formaldehyde, and could only be toxic by being produced in a particular molecular environment.

I therefore investigated quantitatively the metabolism, toxicities and mutual effects of dimethylnitrosamine and three related compounds in intact rats. From these results, and the effects of inhibitors of nitrosamine metabolism, the nature of the toxic compounds was elucidated.

The work falls into four parts: (1) The rates of metabolism were estimated from the rates of fall of nitrosamine concentrations in the blood.

Allowance had to be made for losses of unchanged nitrosamines by excretion in urine and expired air. (2) The rates of expiration of ^{14}C -labelled carbon dioxide were shown to be related to rates of decomposition, so that the latter could be found from the former, with an increase in accuracy and speed. Rates of decomposition of one, labelled, nitrosamine could also be found in the presence of another, unlabelled. (3) The rates of decomposition were determined for all four compounds at various doses in the presence and absence of inhibitors. (4) The relevant toxicity tests were carried out. Statistical estimates of error were made at every stage. Biological variations proved to be small.

MATERIALS AND METHODS

Nitrosamines. Unlabelled dimethyl- and diethyl-nitrosamines were redistilled before use. *n*-Butylmethyl- and *tert*-butylmethyl-nitrosamines were prepared by Dr A. R. Mattocks as described by Heath & Mattocks (1961). Bis-2-hydroxyethyl- and ethyl-2-hydroxyethyl-nitrosamines were gifts from Dr L. Preussmann, Chirurgische Universitäts-Klinik, Freiburg-im-Breisgau, Germany. The first was also prepared by Dr Mattocks. The preparations and tests for purity of the ^{14}C -labelled nitrosamines have been described by Heath & Mattocks (1961). The labelling and specific activities are listed in Table 1. The [1- ^{14}C]- and [2,2- $^{14}\text{C}_2$]-*tert*-butyl compounds contained about 4% of a labelled impurity, probably *tert*-butyl alcohol. The remainder were at least 99% pure, both chemically and in labelling.

Other compounds. Dimethyl- and diethyl-formamides were redistilled before use. *n*-Butylmethyl- and *tert*-butylmethyl-formamides were prepared by Dr Mattocks and fractionated, b.p. 203–204° and 198° (uncorr.) respectively.

I thank the following individuals and firms for gifts of monoamine-oxidase inhibitors. JB516 (2-phenylisopropylhydrazine), from the Lakeside Laboratory, Milwaukee, Wis., U.S.A.; Marsalid (*N*-isonicotinoyl-*N'*-isopropylhydrazine), from Dr A. N. Davison, Guy's Hospital Medical School, London; SKF 4648 [10-(3-dimethylaminopropyl)-2-trifluoromethylphenothiazine hydrochloride] from Smith, Kline and French Laboratories Ltd., Coldharbour Lane, London, S.E. 5. SKF 525A (2-dimethylaminoethyl 2,2-diphenylbutyrate) was also used.

[$\text{Me-}^{14}\text{C}$]Dimethylformamide was prepared from labelled dimethylammonium chloride (Dutton & Heath, 1956)

Table 1. *Labelling and specific activities of dialkylnitrosamines*

Compounds are of the type: $\text{RR}'\text{N}\cdot\text{NO}$, where R and R' are alkyl groups.

Compound		Labelling	Specific activity (mc/m-mole)
R	R'		
Methyl	Methyl	Methyl	1.0
Ethyl	Ethyl	[1- C]Ethyl	1.8
Butyl	Methyl	[1- C]Butyl	0.5
		Methyl	1.0
<i>tert</i> -Butyl	Methyl	[1- C]- <i>tert</i> -Butyl	0.12
		[2,2- C_2]- <i>tert</i> -Butyl	0.09
		Methyl	0.21

(1 m-mole, 0.2 ml.) by treating it with 12.5N-NaOH (0.3 ml.) in a vacuum manifold. The dimethylamine released was partially dried by passing it under a pressure of several mm. over anhydrous calcium sulphate, and was collected in a tube cooled with liquid N_2 . Methyl formate (2 m-moles) was condensed into it, and the mixture was left at room temperature for 90 min. The unchanged dimethylamine and most of the excess of methyl formate were then removed by cautious evacuation. The product was distilled to free it from dimethylammonium formate, and stored at -30° . The yield was 55% on the basis of dimethylamine. The specimen was shown to be radiochemically pure by a method similar to that used for the nitrosamines, and to contain less than 3% of methyl formate (unlabelled) by Hestrin's (1949) method.

Dr Mattocks prepared $[2,2-^{14}C]$ tert.-butyl alcohol and $[2,2-^{14}C]$ tert.-butylamine by slight modifications of the methods given by Heath & Mattocks (1961).

Animals. Only female rats were used (albino, Porton strain, maintained for several years as a closed colony in this Laboratory). With a few exceptions, weights were in the range 165–185 g., and always so in experiments on rates of decomposition.

Methods of dosing. Compounds were injected intraperitoneally in 0.9% NaCl solution. Unlabelled compounds were given on a mg./kg. basis, and dose rates within each group were equal and precise to within $\pm 2\%$. For labelled compounds each rat in a group received the same dose ($\pm 1\%$) regardless of the exact weight of the rats. Only the average dose rates are stated.

Determination of dialkyl nitrosamines in vivo. The method, basically that of Heath & Jarvis (1955), was modified to increase the sensitivity threefold. Treated rats were asphyxiated by coal gas, and blood (3 ml.) was taken from the vena cava and transferred to aq. 2.5% (w/v) sulphosalicylic acid (6 ml.). The mixture was stirred, centrifuged and filtered into a tube containing 12.5N-NaOH (0.1 ml.). (In weakly alkaline solution the compounds can be stored in the dark without measurable loss for up to 24 hr. at 4° ; but in acid solution losses by hydrolysis are appreciable.) Of the filtrate, 5 ml. was mixed with solid NaOH (1–2 g.) and half the solution was distilled over. The nitrosamines were determined polarographically in the distillate. Bright light, which decomposes dilute nitrosamine solution, was avoided throughout.

When nitrosamines were added to blood, only dimethylnitrosamine could be recovered quantitatively. Recoveries of the rest were 80–90%. Therefore, to establish a relation between the quantity in the animal and the concentration in the solution prepared from the blood, the following 'recovery test' was applied. Several rats were given the same dose intraperitoneally. Some were killed 30–40 min. after injection, the rest 60–120 min. after injection. The concentrations of nitrosamine in the solutions prepared from the blood were found, and plotted against the time. The process was repeated with different doses. For each dose the best straight line was calculated, and was extrapolated to zero (injection) time, as shown in Fig. 1A. When the concentration falls linearly with time, the intercepts at $t = 0$ should be proportional to the dose, as in Fig. 1B. At the lowest concentrations of the dimethyl and tert.-butyl compounds the fall was not linear (Fig. 3), so the extrapolated lines were probably a little low. At the highest concentration of the tert.-butyl compound (300 mg./kg.)

the fall was not linear until an hour after injection because distribution was not complete. The slope was therefore calculated from concentrations at 70–250 min. Distribution of dimethylnitrosamine was certainly complete by 30–40 min. as identical results were got when the compound was given intravenously.

The proportionality between the concentration in the solution prepared from the blood and the dose can be expressed in the form: $\mu\text{g./ml.} = \alpha \text{ mg./kg.}$, where α is the proportionality constant. The values of $\alpha \pm \text{s.e.}$ were: dimethyl- 0.800 ± 0.006 , diethyl- 0.715 ± 0.007 , butylmethyl- 0.486 ± 0.010 , tert.-butylmethyl-nitrosamine 0.556 ± 0.015 . If a nitrosamine were distributed evenly throughout the body water, α should be 0.80, the value for dimethylnitrosamine. Magee (1956) showed that dimethylnitrosamine was distributed throughout the body water of rabbits. Other compounds gave lower constants because recoveries from blood were not complete, and because they are more fat-soluble.

The reliability of individual estimates was calculated for each compound. Whenever for a given dose, compound and time after injection, at least four values had been found within the first 2 hr. after injection (4.2 hr. for the tert.-butyl compound), the coefficient of variation was calculated. The coefficients did not vary significantly with dose for any compound and appeared to be distributed normally. For each nitrosamine the overall coefficient of all the results was calculated to be: dimethyl-, 5.3%; diethyl-, 3.2%; butylmethyl-, 7.6%; tert.-butylmethyl-nitrosamine, 4.2%. These coefficients allow, of course, for all sources of variation except differences in rates of decomposition—the last had not had time to show themselves.

Excretion in urine. For each experiment five rats were dosed and kept for 24 hr. in a funnel-shaped container with

Fig. 1. Relationship between concentrations of diethylnitrosamine in rats and in solutions prepared from their blood. A, The concentrations in $\mu\text{g./ml.}$ found in solutions prepared from blood are plotted against time after injection of four doses. The best straight lines, calculated statistically, are shown. B, The intercepts at $t = 0$ are plotted against dose in mg./kg. The s.e. on the intercepts are shown, and the best straight line is given.

water, but not food. Urine was collected in a tube cooled with acetone-solid CO_2 . Solid NaOH (3 g.) was added to urine (25 ml.). Half the solution was distilled, and the distillate was made just acid to methyl red. NaCl (1.5 g.) was added as a salting-out agent and about two-thirds of the mixture was distilled. This second distillation separated the nitrosamines from ammonia and other bases that interfered with the polarography. The volume was recorded, and the nitrosamine in the distillate was estimated polarographically. Quantitative recoveries were got with diethylnitrosamine added to urine.

Expiration of ^{14}C carbon dioxide. The respiration chambers consisted of glass cylinders (10 cm. int. diam. $\times$ 45 cm. long) made of industrial Pyrex pipe-line, with two standard end-pieces fixed with bolts. Each contained a wire tray to support the rats, and a supply of drinking water. The exit end was attached to two traps in series. Each was a 1 l. bottle with a downwards-directed hemispherical sintered-glass bubbler, of porosity 2, containing NaOH solution (500 ml.). These led to a third trap, with a plain tube bubbler, containing dilute sulphuric acid, connected via a tap to a filter pump. The acid trap stopped alkaline spray from blocking the tap. In operation, five rats were placed in a chamber immediately after injection. A run was deemed to have started after the injection of the third rat (the injection of five rats took 3–5 min.). The flow of air was started, and adjusted to 3–4 l./min., measured at the input end. The cylinder was then covered with a dark cloth to obviate the lens effect of the curved glass. Without it bright light distressed the rats. The first of the two caustic traps was changed at set times, and the $^{14}\text{CO}_2$ absorbed as carbonate was determined as BaCO_3 on portions. The BaCO_3 was filtered off through a sintered-glass planchet (Heath & Dutton, 1958), weighed and counted with an end-window Geiger-Müller tube at infinite thickness. Every second to fourth time of changing the first trap the second was changed also, and the contents were treated similarly. The concentrations of NaOH in the first trap depended on the time over which CO_2 was to be collected: if for 3 hr. or less, 1N; for 3–6 hr., 2N; for over 6 hr., 3N. In the second trap the concentration was always 1N.

Results were always calculated as follows. Let f be the fraction of the contents of the first trap taken for analysis, and let this yield x g. of BaCO_3 giving a count of y counts/min. corrected for background and for variations in counting efficiency from planchet to planchet. Then the total ^{14}C in the trap is given by xy/f , in the arbitrary units (counts/min.) $\times$ (g. of BaCO_3). Call this T_1 . Suppose that the second trap was changed at the same time as the first trap was changed for the n th time, and that the second trap contained z counts $\times$ g./min. This trap did not contain all the ^{14}C which escaped the first trap, but a rough correction factor was found by switching the first and second traps in trial runs. Let the correction factor be c . Then the total $^{14}\text{CO}_2$ expired in the period covered by n changes of the first trap and one of the second is given by

$$T = \sum_1^n T_1 + cz.$$

The quantity expired during the collection in the first trap is then given, in terms of the $^{14}\text{CO}_2$ in that trap, T_1 , by the expression

$$TT_1 / \sum_1^n T_1.$$

This expression is the mathematical equivalent of sharing out the $^{14}\text{CO}_2$ which escapes the first trap amongst the samples collected in it. The total ^{14}C injected was calculated from the count rate given by BaCO_3 got by combustion of the labelled material with known quantities of carrier as described by Heath & Dutton (1958). Results were expressed as those percentages of the total ^{14}C injected which were expired as $^{14}\text{CO}_2$ in the intervals stated. The results of a typical experiment are shown in Fig. 2.

With some exceptions (discussed below) the method gives results with the following characteristics. The rate of expiration of $^{14}\text{CO}_2$ was initially slow, as was expected (this effect cannot be shown on a small Figure). During this period the compound becomes distributed in the rat, and the $^{14}\text{CO}_2$ liberated becomes mixed with the rat's hydrogen carbonate pool. The induction period was followed by a nearly constant rate of expiration, which was maintained until most of the compound had decomposed. The rate then dropped rapidly. The percentage of the total ^{14}C injected ('per cent ^{14}C ') expired/hr. in the period immediately after the first hour was taken as the expiration rate corresponding to the dose given. The total per cent ^{14}C expired by the time the compound had been wholly decomposed for a few hours was also calculated for each run as a check that the experiment had been carried out correctly.

The error was analysed in detail. Duplicate runs were always carried out. The coefficient of variation of the mean was about 6%. The sum of the squares of the coefficients of variation contributed by errors in injection, weighing, counting etc. was about 15. The residual error, about $(6^2 - 15)^{1/2}$, i.e. 4–5%, must be attributed to biological variation, which was therefore the greatest source of error. In a particular series of experiments with *tert*-butyl- ^{14}C -methyl nitrosamine great care was taken to minimize all non-biological variations, and the coefficient in these experiments was 5.0%.

Distillation ratios. Although the pure nitrosamines are less volatile than water, distillation of an aqueous solution yields a distillate more concentrated than the distilland. In the absence of reflux the ratio at any moment of the concentration in the distillate to the concentration in the distilland is called the distillation ratio. At low concentrations well below saturation the ratio is independent of concentration, and does not usually change rapidly with temperature.

Fig. 2. Expiration of $^{14}\text{CO}_2$ from rats given 50 mg. of ^{14}C dimethylnitrosamine/kg. The $^{14}\text{CO}_2$ expired is shown as a percentage of the total ^{14}C injected. The results were from two groups of five rats, and the means are shown. The time-scale is changed at 10 hr.

Distillation ratios were found as follows. A dilute solution (about 50 $\mu\text{g./ml.}$) of the nitrosamine (250 ml.) was distilled at about 10° under reduced pressure until about 3 ml. of distillate had been collected in a trap cooled in acetone-solid CO_2 . The concentrations of both solutions were then determined polarographically. As the tiny fraction distilled did not change the concentration of the distillate appreciably, the distillation ratio was given by the ratio of the concentrations found. The mean values for duplicate determinations were: dimethyl- 1.2, diethyl- 2.5, butylmethyl- 2.5, *tert.*-butylmethyl-nitrosamine 2.2, with errors of about 5%.

Expiration of nitrosamines. From the distillation ratios it can be shown that significant amounts of nitrosamines may be excreted in the expired air. For a dose of 200 mg. of diethylnitrosamine/kg. expiration could account for 10% of the rate. The calculation was based on the assumption that the nitrosamine freely permeates the lung wall, and that the expired air is saturated with it. 'Minute volumes' were values taken from Crossfill & Widdicombe (1961).

Expired nitrosamines could not be collected reproducibly in traps immersed in acetone-solid CO_2 or liquid O_2 . Satisfactory results were got when the nitrosamine expired from groups of five rats held in the usual respiration chambers was collected by passing the expired air through sintered-glass bubblers into 400 ml. of water in traps of the type described above. The traps were covered with a dark cloth to avoid decomposition of the nitrosamines by light; and the bottoms of the respiration chambers were lined with layers of filter paper to absorb any urine. The air flow was $3.0 \pm 0.1 \text{ l./min.}$ A run was started 0.5 hr. after injection, and continued for only 100–120 min., as in longer runs evaporation of nitrosamines excreted in urine, and re-evaporation by the rapid air stream of nitrosamines already absorbed in the trap gave spurious results. The aqueous solutions were concentrated by successive distillations from 3*N*-NaOH and the nitrosamines were estimated polarographically. Recovery tests were carried out by spreading 1 mg. of nitrosamine in 0.2 ml. of solution over about 30 cm.^2 of filter paper propped up in the respiration chamber. Air was then passed through the chamber at the standard rate. Evaporation from the filter papers was not complete after 30 min. but was after 1 hr. These rates of release of nitrosamines were similar to those from rats receiving the particular doses given. The results of the recovery tests are given in Table 2. The low recoveries cannot be attributed to losses by re-evaporation from the traps, or to losses attendant on distillations from 3*N*-NaOH, both of which were shown to be negligible. They result from the low efficiency with which the traps absorb the nitrosamines in the first place.

Table 2. *Recovery of nitrosamines evaporated from filter papers*

The factor is the value by which the quantity found in experiments with rats must be multiplied to give the quantity expired.

Nitrosamine	Percentage recovered	Factor
Dimethyl	68.7, 61.8	1.53
Diethyl	65.1, 66.4	1.52
Butylmethyl	59.2, 61.8	1.67
<i>tert.</i> -Butylmethyl	59.0, 71.8	1.53

Toxicity tests

(1) The LD_{50} values of several compounds were determined on four groups of three, four or six rats per test. The results were calculated as described by Weil (1952).

(2) The effect on the LD_{50} of dimethylnitrosamine of giving the compound in divided doses instead of as a single dose was studied. Care was taken to use a group of rats of the same age and weight, and the comparison experiments were started within 2 hr. of each other. The LD_{50} values were calculated as above.

(3) The effect of dimethylformamide on the LD_{50} of dimethylnitrosamine and on the time to death were studied. For reasons given below it was expected that dimethylformamide would increase the LD_{50} only slightly, and would have a greater effect on the time to death of rats given dimethylnitrosamine in doses near or below the LD_{50} than of rats given higher doses. Some rats were given 700 mg. of dimethylformamide/kg. (about one-fifth of LD_{50}) as a 25% solution in 0.9% NaCl. After 0.5 hr. they were given dimethylnitrosamine intravenously by tail vein in graded doses, ten rats for each dose level. These were compared with groups of ten rats given dimethylnitrosamine intravenously at the same doses, as near as possible at the same time. The order of dying was then recorded, comparisons being confined within the pairs of groups which had received the same dose of dimethylnitrosamine. If those rats receiving dimethylformamide are denoted by B, and those not receiving it by A, a typical order of death might be: AAABAABABB, ten surviving. The probability of this distribution is recorded. The method of calculation is given by Heath & Irwin (1962).

(4) The liver necrosis caused by *n*-butylmethyl nitrosamine was studied. The LD_{50} of the *n*-butyl compound is 120–150 mg./kg. At these doses some rats die fairly fast, probably of a lung lesion. Others survive for 2–3 days, and show both the lung lesion and liver damage. Because some rats would die of the lung lesion before the liver lesion had had time to appear, the liver lesion was studied at sublethal doses. Rats were given graded doses of the *n*-butyl compound, five rats for each dose. After 2 days all the rats were killed, and their livers excised. The further treatment was carried out by Dr P. N. Magee.

Sections of the livers were prepared, the labels covered, and the slides doubly shuffled by two people. The sections were then graded, according to the degree of liver necrosis observed, in the rankings 0, 1, 2 and 3, as shown in Table 16: 0 represented no discernible necrosis, 3 severe necrosis, and 1 and 2 represented intermediate grades. The dose giving an average ranking of 1.5 was then calculated as follows: the total scores for each group of five rats were expressed as a fraction of 15, and the regression with dose and the variances were calculated by probit analysis. This use of probit analysis is discussed by Finney (1947), who shows that the median dose should be correct, but that the variances may be in error. A simpler but less reliable method was used to assess the effects of inhibitors on the necrotic action of dimethyl- and diethyl-nitrosamines (Table 14).

(5) The liver lesions produced by *tert.*-butylmethyl nitrosamine were compared with those produced by the *n*-butyl compound as described above. The *tert.*-butyl compound is rapidly lethal at doses above about 700 mg./kg. The signs resemble those produced by *tert.*-butylmethylformamide.

Rats were therefore given successive sublethal doses spread over 4 days. The experimental details are given in the Results section (16).

RESULTS

(1) *Excretion in urine*

Table 3 shows the quantities of the four nitrosamines excreted under various conditions. The losses can be significant.

Table 3. *Excretion of nitrosamines in urine*

Urine was collected for a 24 hr. period after injection. The first-order constants used in later calculations are given under *k'* (see section 4).

Nitrosamine	Dose (mg./kg.)	Amount excreted (% of dose)	<i>k'</i> (hr. ⁻¹)
Dimethyl	50	3.5, 4.5	0.0085
	50*	12.8, 15.8	—
Diethyl	200	11.0, 11.1	0.0116
	150	6.8	—
	100	2.8, 1.4	—
	50	0.54	—
Butylmethyl	100	4.1	0.0142
	40	1.5	—
<i>tert.</i> -Butylmethyl	400	2.0	0.002
	300	2.1	—

* Dimethylformamide (700 mg./kg.) was also given.

(2) *Expiration of nitrosamines*

Rates of excretion in the expiration stream are given in Table 4 together with rates calculated as described in the Materials and Methods section. A little more was expired than expected, but it is doubtful if this was significant. It is assumed below that the rate of expiration is proportional to the concentration remaining in the rat (one of the assumptions on which the calculations were based) and that the experimental values in the Table represent the rates of expiration at the stated doses.

(3) *Rates of fall in nitrosamine concentrations in vivo*

These were found by polarographic estimation of the nitrosamines in the blood of rats killed at different times after treatment. The units adopted throughout are mg./kg./hr. Results fall into four groups: (a) After most treatments the concentrations *in vivo* fell linearly with time within experimental error (Table 5). (b) Diethylnitrosamine at 200 mg./kg. was a marginal case. The rate of fall appeared to be faster near the beginning of the run than later. This was confirmed by calculating the gradients over the ranges 97–73 % and 73–11 % of the initial dose. These rates (Table 5) are

Table 4. *Expiration rates of nitrosamine*

The method is described in the Materials and Methods section. The calculated doses are the average doses present in the rats over the period of collection. The first-order constants used in later calculations are given under *k''* (see section 4).

Nitrosamine	Dose (mg./kg.)		100 × Expiration rate (% of dose/min.)		<i>k''</i> (hr. ⁻¹)
	Injected	Calc.	Found	Calc.	
Dimethyl	49	41	0.66, 0.55	0.27–0.50	0.0043
Diethyl	200	185	1.13, 1.34	0.64–1.17	0.0080
Butylmethyl	100	85	0.61, 0.65	0.45–0.82	0.0045
<i>tert.</i> -Butylmethyl	302	300	0.78, 0.70	0.68–1.25	0.0044

Table 5. *Linear rates of fall in concentration of nitrosamines in rats*

Groups of four or more rats were killed at set times after the injection of the nitrosamines. When dimethylformamide was also given, it was injected about 30 min. before dimethylnitrosamine. The average rates of fall were calculated statistically (see, e.g. Snedecor, 1946). Rates of decomposition were calculated as in section (4).

Nitrosamine	Dose (mg./kg.)		Fall in concn. (mg./kg./hr. ± S.E.)	Rate of decomp. (mg./kg./hr.)	Fall in concn. (%)		No. of times of sampling
	Nitrosamine	Dimethylformamide			From	To	
Dimethyl	49	—	5.68 ± 0.34	5.33	95	20	5
	16.2	98	1.86 ± 0.18	1.75	89	37	4
	16.3	500	0.76 ± 0.07	0.59	97	66	4
Diethyl	49	500	1.99 ± 0.22	1.47	96	71	4
	19.6	—	5.20 ± 0.45	5.20	82	42	3
	80	—	7.61 ± 0.39	6.96	93	46	6
Butylmethyl	24.1	—	10.9 ± 0.8	10.9	77	40	3
	102	—	10.2 ± 0.8	9.44	96	32	4
Diethyl	200	—	12.2 ± 1.0	9.0	97	73	4
	200	—	10.4 ± 0.7	8.7	73	11	3

probably different. (c) The *tert.*-butyl compound and dimethylnitrosamine at the lowest doses given (25 and 16.2 mg./kg. respectively) gave curves rather than straight lines (Fig. 3). Both sets of results could be fitted adequately by simple exponential functions from which the average rates over any chosen period could be calculated. (d) The *tert.*-butyl compound at 100 and 300 mg./kg. gave markedly sigmoid curves when the concentration *in vivo* was plotted against time. The results are given in Table 6. At the higher dose little weight can be put on the s.e., as the spread of results was so great that the distribution could not be normal.

Fig. 3. Decomposition of low doses of nitrosamines in rats. Concentrations $\pm$ s.e. in rats are plotted against time after injection. The values at $t = 0$ were calculated from the doses and the constants in Table 2. A, Dimethylnitrosamine (16.2 mg./kg.). The full curve is $C = C_0 e^{-0.007t}$, where C and C_0 are the concentrations at times t and 0 respectively. The broken curve is calculated from the Michaelis-Menten equation (section 11). B, *tert.*-Butylmethyl nitrosamine (25 mg./kg.). The curve is

$$C = C_0 e^{-0.00277t}$$

(4) Determination of rates of decomposition from rates of fall in concentration

The rate of decomposition is given by the rate of fall in concentration less the rates of excretion in urine and of expiration. Later calculations and discussion are simplified if this relationship can be made mathematically explicit. A satisfactory equation is

$$dC/dt = -k - (k' + k'')C \quad (1)$$

which on integration gives:

$$C_1 - C_2 = (C_0 + k/k_1) [\exp(-k_1 t_1) - \exp(-k_1 t_2)] \quad (2)$$

where C_0 , C_1 and C_2 are the concentrations *in vivo* in mg./kg. at times 0, t_1 and t_2 ; k , k' and k'' are the rate constants for decomposition, excretion and expiration respectively; and $k_1 = k' + k''$. The equations are based on the following considerations: (a) The rate of decomposition is nearly independent of concentration, except for the *tert.*-butyl compound, under conditions where excretion and expiration make a significant contribution to the overall rate. A more refined treatment is given in section (11), but the zero-order approximation is good enough for the present purpose. (b) Expiration is proportional to concentration, as stated in section (2). Values of k'' are listed in Table 4. (c) Excretion in urine is proportional to concentration. If the expiration term is ignored (it is always less than 15% of the overall rate) eqn. (1) gives:

$$a = e^{(1-F)a} - 1 \quad (3)$$

where F is the total fraction of the dose excreted, and $a = C_0 k/k'$. This equation fits the results in Table 3 for dimethyl- and *n*-butylmethyl-nitrosamines excellently. As excretion only makes a big contribution to some dimethylnitrosamine results, those in which decomposition is slowed (k reduced) by inhibitors of oxidation, the overall eqn. (2) is highly satisfactory where its application is most

Table 6. Rates of fall of *tert.*-butylmethyl nitrosamine concentrations in rats

For the last two columns, see section (5).

Dose (mg./kg.)	Time-interval after injection (min.)	Amount left at end (% of dose)	Fall in concn. (mg./kg./hr.)	Decomposition		Rate of expiration of $^{14}\text{CO}_2$ (% of ^{14}C injected/hr.)	Ratio (A:B)
				(mg./kg./ hr.)	(%/hr.) (A)		
300	80-420	91.7	4.1 $\pm$ 1.0	1.9	0.63	1.24	0.5
	420 $\pm$ 770	66.3	13.5 $\pm$ 3.1	11.5	3.8	2.6	1.46
	770 $\pm$ 1100	33.6	17.9 $\pm$ 4.1	16.9	5.6	4.2	1.33
	1100-1440	10.9	7.2 $\pm$ 2.5	7.0	2.3	3.0	0.77
100	70-250	86.4	2.5 $\pm$ 0.8	2.0	2.0	2.01	1.00
	250-430	66.1	6.7 $\pm$ 1.6	6.3	6.3	3.5	1.74
	430-770	31.5	6.1 $\pm$ 0.8	5.9	5.9		

needed. The excretion equation also fits the *tert*-butyl results fairly well. It does not fit the diethylnitrosamine results; the value of k' calculated from the excretion after 200 mg./kg. in Table 3 seriously overestimates rates of excretion after lower doses. At these doses, however, the calculated contribution of excretion to the overall rate is less than its s.e. As eqn. (2) makes the correct allowance for expiration, it is convenient to use it with the excretion constant calculated from the highest dose, where the excretion correction is also important. The values of k' are shown in Table 3.

Tables 5 and 6 show the rates of decomposition calculated by eqn. (2). The differences between rates of decomposition and rates of fall in concentration are fairly small, so the s.e. values on the former are likely to be the same as on the latter.

(5) *Comparison of rates of expiration of ^{14}C carbon dioxide with rates of decomposition*

To use the rates of expiration of labelled carbon dioxide to determine the rates of decomposition a relationship between the two must be established. To do this experiments were carried out with ^{14}C -labelled nitrosamines closely comparable with those described in section (4). The results for all but the *tert*-butyl compound are shown in Table 7. The rates of expiration are compared with the rates of decomposition in the same units.

The calculation of the s.e. values of the ^{14}C -

carbon dioxide runs was as follows. For dimethylnitrosamine at 16.4 mg./kg. eight runs were carried out spread over several months as a test that the rats behaved uniformly. The s.e. was calculated in the usual way from these results. The remaining results are given in Table 7.

For dimethylnitrosamine at 16.4 mg./kg. the average rate from 30 to 150 min. was calculated from the exponential equation of Fig. 3A, and was compared with the expiration of labelled carbon dioxide from 60 to 180 min. The ratio, 1.65, is in satisfactory agreement with the other ratios for dimethylnitrosamine.

For dimethyl- and diethyl-nitrosamines under a wide variety of conditions, rates of decomposition are proportional to rates of expiration of labelled carbon dioxide, so the former can be calculated from the latter by using the conversion ratios in the last column.

The *n*-butyl compound at the lowest dose gave a very high ratio, i.e. the rate of expiration was slower than expected. This can be explained by the time-lag between the decomposition of the compound and the appearance of labelled carbon dioxide in the expired air. The time-lag will have the greatest effect near the start of the run. As in this instance 43% of the *n*-butyl compound was decomposed/hr., ratios can only be calculated from rates near the start. In this paper ratios are mainly used to calculate inhibited rates, which were fairly slow, so that we can ignore high ratios which can be explained in this way.

Table 7. *Rates of decomposition from polarographic estimations and rates of expiration of ^{14}C carbon dioxide compared*

All errors are s.e. All $^{14}\text{CO}_2$ runs were carried out in duplicate. The differences between duplicates were consistent with the assumption that for all runs the coefficient of variation of the mean was 6.1%. This variation was assumed in calculating the s.e. of the ratios. Doses refer to the ^{14}C runs, which do not always match precisely those given for 'polarographic' runs in Table 5. Corrections were made to allow for the small differences.

Nitrosamine	Dose (mg./kg.)		Rate of decomposition (%/hr.) (A)	Rate of expiration of $^{14}\text{CO}_2$ (% of ^{14}C injected/hr.) (B)	Ratio (A:B)	
	Nitrosamine	Dimethyl- formamide			Calc.	Taken
Dimethyl	16.4	—	24.3	14.7 ± 0.45	1.65	
	48	—	10.9 ± 0.7	5.99	1.82 ± 0.16	
	16.7	100	10.5 ± 1.1	6.38	1.65 ± 0.20	
	16.1	500	3.66 ± 0.43	2.07	1.77 ± 0.23	
	48.5	500	3.03 ± 0.45	1.77	1.71 ± 0.28	1.74
Diethyl	20	—	26.0 ± 2.3	13.3	1.95 ± 0.21	
	82.8	—	8.41 ± 0.47	5.27	1.60 ± 0.13	
	204*	—	4.42 ± 0.49	2.26	1.96 ± 0.25	
	204†	—	4.27 ± 0.34	2.30	1.86 ± 0.19	1.84
Butylmethyl	25.5	—	43.6 ± 3.2	14.8	2.95 ± 0.28	
	102.5	—	9.21 ± 0.78	4.4	2.09 ± 0.22	2.1

* Rates from 0.7 to 4.3 hr. after injection.

† Rates from 4.3 to 16.5 hr. after injection.

For the *tert.*-butyl compound comparable results are given in Table 6. The ratios are not constant. A graph can, however, be constructed from the [^{14}C]carbon dioxide results which is consistent with the range of concentrations determined polarographically. This suggests that the main errors were in the latter method. A relationship can be established from the [^{14}C]carbon dioxide runs only. (a) For this and section (6), nine sets of duplicate runs were carried out with *tert.*-butyl[^{14}C]methyl nitrosamine. The differences between duplicates were consistent with the assumptions that the errors were constant and that the coefficient of deviation from the mean of any pair was 5.0%. Thus runs were reproducible and the errors low. A relationship can therefore be assumed. The low S.E. values of these runs imply that the big variations in concentrations determined polarographically arose from interference with the method by products in the poisoned rats. Accordingly in polarographic runs variance was greatest at the highest doses, and increased as the runs proceeded. (b) Ratios of percentage decomposed to percentage ^{14}C expired were calculated for 24 hr. runs, by the end of which nearly all the compound had been decomposed or excreted. The ratios were 1.59, 1.53 and 1.48 for doses of 25, 100 and 300 mg./kg. respectively. Ratios calculated in this way for the compounds and treatments listed in Table 7 are consistently about 15% low, because some of the ^{14}C was initially incorporated into fairly stable compounds. This was not, therefore, taken into account when ratios were calculated in the usual way, but some of it was released as labelled carbon dioxide in 24 hr. after injection. The ratios for the *tert.*-butyl compound should, therefore, be increased by 15%, giving an average ratio of 1.80. This is similar to the ratio for the other compounds. (c) At the low dose of 25 mg./kg. interference with the polarographic method should be minimal, and in fact the

results were highly reproducible. A satisfactory ratio should be obtainable in the usual way from these results. The ratio found is 2.1.

One possibility remains. If the *tert.*-butyl compound were converted into a derivative which, though fairly stable, gave labelled carbon dioxide slowly, the results might still be reproducible, and give satisfactory ratios at 24 hr., but rates of decomposition could not be calculated from the rates of expiration of labelled carbon dioxide. In section (6) it is shown that this is unlikely. The ratio 1.8 is therefore used in section (10).

(6) *Inhibition of the decomposition of tert.-butyl[^{14}C]methyl nitrosamine by diethylformamide*

The results are shown in Table 8. It is shown below that inhibition is often strictly competitive in analogous cases. If this is so here, any two percentage inhibitions can be calculated from the third. The calculated and found inhibitions are in excellent agreement. This is inconsistent with the hypothesis that the *tert.*-butyl compound goes to a fairly stable intermediate, unless the metabolism of the intermediate is inhibited to a very similar degree, which seems unlikely.

(7) *Decomposition of dimethylnitrosamine and dimethylformamide*

Rates of decomposition of dimethylnitrosamine were calculated from [^{14}C]carbon dioxide-expiration runs, and are listed in Table 9. Some rates are not the same as those given in section (5) for the same treatments. In section (5) the average rates over fairly long periods were needed for comparison with the rates determined polarographically. In this section the rates at known doses are needed, and these are best calculated from the rates of expiration between 1 and 3 hr. after injection. The differences arise because most of the inhibited rates

Table 8. *Rates of expiration of [^{14}C]carbon dioxide from rats given tert.-butyl[^{14}C]methyl nitrosamine and diethylformamide*

Rats received 300 mg. of the labelled compound/kg. at zero time and 600 mg. of diethylformamide/kg. at the times stated. The average rates of expiration of $^{14}\text{CO}_2$ were determined over the stated time intervals after the injection of the labelled compound, and are compared with the rates in rats which did not receive diethylformamide. Two groups of five rats were used for each comparison. Assuming the enzymes were saturated with substrate, percentage inhibition is given by: $100 kI/(C + kI)$, where C and I are the average nitrosamine and formamide concentrations. From the first value, $k = 0.58$. The remaining values were calculated from this constant.

Time of injection of inhibitor (hr.)	Interval over which rate was determined (hr.)	Rate (%/hr.)		Percentage inhibition	
		With inhibitor	Without inhibitor	Found	Calc.
-0.3	1-7	0.56	1.24	54 ± 6	54
7	7-13	1.03	2.54	59 ± 6	59
13	13-18	1.50	4.81	64 ± 6	69

Table 9. *Decomposition of dimethylnitrosamine in vivo from [¹⁴C]carbon dioxide-expiration runs*

Rates were calculated as described in the text, by using the ratio of 1.74 given in Table 7. It is assumed in this and subsequent sections that decomposition and ¹⁴CO₂ expiration rates were strictly proportional, even though the results in section (5) established proportionality only within about 10 %, because the errors in polarographic runs are greater than in ¹⁴CO₂ expiration runs. The factors in Table 7 are also assumed to be correct. This may introduce systematic errors of a few per cent, which are, however, self-cancelling in later calculations. Duplicate runs were used for each determination, and the means are given. The coefficient of variance is about 6 %.

Expt.	Dose of dimethyl-nitrosamine (mg./kg.)	Inhibitor	Dose (mg./kg.)	Rate of decomposition (mg./kg./hr.)	K _i /K _m
1	16.4		—	4.20	—
2	48.0		—	5.43	—
3	16.7	Dimethylformamide	95	1.90	2.79
4	16.1		500	0.51	2.32
5	48.5		500	1.31	2.34
6	51.6		700	1.07	2.31
7	16.4	Diethylnitrosamine	50	1.87	1.4
8	16.8		154	0.87	1.5
9	17.3	Butylmethylnitrosamine	40	1.51	0.91
10	16.6		100	0.91	0.99
11	16.6	tert.-Butylmethylnitrosamine	52	2.16	2.3
12	16.4		207	0.98	2.7
13	16.8	Bis-2-hydroxyethylnitrosamine	1200	3.43	270
14	16.5	Ethyl-2-hydroxyethylnitrosamine	750	1.78	—
				2.29	—
15	22.0	Diethylformamide	600	0.33	1.54

Fig. 4. Abnormal ¹⁴CO₂ expiration curves. ●, [¹⁴C]-Dimethylnitrosamine (16.1 mg./kg.) 30 min. after 500 mg. of dimethylformamide/kg. The rate of ¹⁴CO₂ expiration increases in each successive period. ▲, [¹⁴C]Diethylnitrosamine (83.2 mg./kg.) 30 min. after 200 mg. of dimethylnitrosamine/kg. The rate of ¹⁴CO₂ expiration decreases in each successive period.

varied with time because the inhibitors were both reversible and decomposed at different rates from the nitrosamines (see Fig. 4 for examples). The most extreme case is shown as Expt. 14 in Table 9, where the rate increased from 1.78 mg./kg./hr.

between 1 and 2 hr. after injection to 2.29 mg./kg./hr. between 2 and 3 hr. after injection.

Remmer (1959) showed that young rats decomposed amidopyrine much more rapidly *in vivo* after repeated doses of phenobarbitone sodium. The decomposition of dimethylnitrosamine was not affected in a similar experiment. Young female rats (40 g.) were given 80 mg. of phenobarbitone sodium/kg. on three successive days. They decomposed a dose of 35 mg. of dimethylnitrosamine/kg. at 6.1 mg./kg./hr., which does not differ significantly from the rate found in controls, namely 6.3 mg./kg./hr.

Dimethylformamide was the most stable inhibitor of oxidation, as measured by expiration of labelled carbon dioxide, but it was mostly decomposed in a day. The curve for excretion of labelled carbon dioxide after 52 mg. of [¹⁴C]-dimethylformamide/kg. is shown in Fig. 5. The rate of expiration remained very slow indeed for about 7 hr., and in 2 days only about 25 % of the total ¹⁴C was expired. More was excreted into urine (42 % in 1 day) of which less than half was unchanged dimethylformamide, as shown by a low yield of [¹⁴C]dimethylamine recovered after hydrolysis of the urine with boiling 4N-hydrochloric acid. Thus dimethylformamide was metabolized, but the expired labelled carbon dioxide was released by the slow oxidation of a metabolite. The rates of metabolism could not, therefore, be determined from the rates of expiration of labelled carbon dioxide.

(8) *Decomposition of diethylnitrosamine*

Results analogous to those obtained with dimethylnitrosamine are shown in Table 10. With dimethylnitrosamine as an inhibitor the rate of expiration of labelled carbon dioxide fell with time, as shown in Fig. 4. Rates were therefore calculated

Fig. 5. Expiration of $^{14}\text{CO}_2$ after $[\text{Me-}^{14}\text{C}]$ dimethylformamide. The dose was 52 mg./kg. Two runs are shown.

from the maximum rates of expiration found in the first 3 hr. after injection, and must be somewhat low.

(9) *Decomposition of n-butylmethylnitrosamine*

Results obtained with the ^{14}C methyl compound are given in Table 11. In addition a few results were got with the $[1\text{-}^{14}\text{C}]$ butyl compound. The rates were the same within the errors.

(10) *Decomposition of tert.-butylmethylnitrosamine*

Some results got with the ^{14}C methyl compound are given in Table 12. Others are in Table 8, and may be converted into the same units by multiplication by 5.4. All the rates refer to the first few hours after injection.

Groups of rats were also given 25.5 mg. of the $[2,2\text{-}^{14}\text{C}_2]$ - and $[1\text{-}^{14}\text{C}]$ -tert.-butyl compounds/kg. respectively. Only 1.6 and 1.1% of the ^{14}C injected were recovered as labelled carbon dioxide in 24 hr. The tert.-butyl group proved difficult to oxidize *in vivo*. From rats given 50 mg. of labelled tert.-butyl alcohol and tert.-butylammonium chloride/kg. about 1.2% and less than 0.5% of the

Table 10. *Decomposition of diethylnitrosamine in vivo*

The details are as in Table 9.

Expt.	Dose of diethylnitrosamine (mg./kg.)	Inhibitor	Dose (mg./kg.)	Rate of decomposition (mg./kg./hr.)	K_i/K_m
1	20.0	—	—	4.92	—
2	82.8	—	—	8.11	—
3	204	—	—	8.91	—
4	82	Diethylformamide	200	4.80	2.22
5	82	—	500	3.11	2.30
6	197	—	500	5.44	2.08
7	20.0	Dimethylformamide	150	1.69	2.2
8	20.0	—	600	1.11	4.7
9	82	—	600	4.01	6.6
10	202	—	1000	7.40	24.3
11	20.5	Dimethylnitrosamine	50	1.50	0.5
12	20.0	—	200	1.32	1.9
13	82	—	50	4.72	0.7
14	83	—	200	4.37	2.3

Table 11. *Decomposition of n-butyl ^{14}C methylnitrosamine*

The details are as in Table 9.

Expt.	Dose of butylmethylnitrosamine (mg./kg.)	Inhibitor	Dose (mg./kg.)	Rate of decomposition (mg./kg./hr.)	K_i/K_m
1	25	—	—	7.7	—
2	72.0	—	—	9.0	—
3	102.5	—	—	9.4	—
4	25.0	Dimethylformamide	600	2.80	—
5	25.1	Dimethylnitrosamine	50	4.24	2.1
6	24.7	—	200	3.01	4.6
7	24.6	tert.-Butylmethylnitrosamine	200	4.28	—

Table 12. *Decomposition of tert.-butyl[¹⁴C]methylnitrosamine*

The details are as in Table 9.

Expt.	Dose of tert.-butylmethyl-nitrosamine (mg./kg.)	Inhibitor	Dose (mg./kg.)	Rate of decomposition (mg./kg./hr.)
1	25.1	—	—	2.25
2	102.5	—	—	3.67
3	300	—	—	6.60
4	306	Dimethylformamide	1050	5.02
5	24.9	Dimethylnitrosamine	50	1.34
6	24.5		200	1.09
7	107.6		200	2.63
8	301	Diethylformamide	600	3.05
9	106	Diethylnitrosamine	200	1.62

¹⁴C injected was recovered as labelled carbon dioxide in 24 hr.

(11) Mathematical treatment of the results

Can the inhibitors be regarded as competitive, and can any of the non-toxic inhibitors be used as substitutes for the toxic ones, e.g. dimethylformamide for dimethylnitrosamine? If only one enzyme is involved, and both substrate and inhibitor obey the Michaelis-Menten equation on it, then in the absence of inhibitor:

$$v = V_{\max}S/(S + K_m) \quad (4)$$

and in the presence of inhibitor:

$$v_i = V_{\max}S/(S + K_m + K_m I/K_i) \quad (5)$$

where v values are the rates, S and I are the concentrations of substrate and inhibitor, K_m and K_i are their adsorption constants, the $V_{\max}$ is the maximum rate.

Of most interest is the constancy of K_i/K_m . To calculate this ratio from eqn. (5) rough values of K_m are needed. From rates no. 1 and 2 of Table 9, $V_{\max}$ and K_m for dimethylnitrosamine are 6.19 mg./kg./hr., and 4.8 mg./kg. respectively [by substituting the average concentrations during which the rates were determined in eqn. (4)]. Unless the rates are in error by more than their s.e. values, $V_{\max}$ should be correct to 0.5, and K_m to 1.5. These values were substituted into the integrated form of eqn. (4) to calculate the decomposition curves. The calculated curve for a dose of 48 mg./kg. was substantially linear until at least 80 % of the dose had disappeared, in agreement with experiment. The results at 16.2 mg./kg. were also reproduced fairly well (see Fig. 3A), provided that little weight was given to the point at zero time. This is probably justifiable, as decomposition cannot obey the equation in the first few minutes after injection. The K_m and $V_{\max}$ values for the diethyl and *n*-butylmethyl compounds were: diethyl, $V_{\max} = 9.45$ mg./kg./hr., $K_m = 11.6$ mg./kg.; *n*-butylmethyl, $V_{\max} = 9.79$ mg./kg./hr., $K_m = 3.7$ mg./kg. From these constants the values of K_i/K_m given in Tables 9–11

Fig. 6. Dependence of dimethylformamide concentration in rats on time. $\log(C_0/C)$ is plotted against time, where C and C_0 are the concentrations at times t and 0. The method of calculation is given in the text. The gradient corresponds to the first-order constant, 0.13/hr. Δ , From a run with 16.1 mg. of dimethylnitrosamine/kg. plus 500 mg. of dimethylformamide/kg. $\bullet$, From a run as above, but 48.5 mg. of dimethylnitrosamine/kg. $\square$, Rats were given 700 mg. of dimethylformamide/kg., and 51.6 mg. of dimethylnitrosamine/kg. 7 hr. later. The average rate from 1 to 3 hr. after the second injection was determined.

were calculated by substituting in eqn. (5) the average concentrations present during the rate determinations. Trial calculations showed that values could not be more than 30 % in error unless at least one rate constant used in the calculation was in error by more than its variance. From the constancy of K_i/K_m , the decomposition of dimethylnitrosamine appears to have been inhibited competitively by the other three nitrosamines.

Inhibition by dimethylformamide is of particular interest. K_i/K_m cannot be calculated until its concentrations *in vivo* are known. Now, when dimethylformamide was given 7 hr. before a dose of dimethylnitrosamine it was a far less effective inhibitor than when it was given shortly before. Assuming competitive inhibition the results of an experiment of this sort (see Fig. 6) showed that the average rate of decomposition in one case was about 50 mg./kg./hr. The decomposition kinetics could not be generally of zero order, or in Expt. 3

of Table 9, all the formamide would have been decomposed before the rate of decomposition of the nitrosamine was determined, and no inhibition would have been observed. This suggested that the rate might be represented by an exponential function: $I = I_0 e^{-kt}$. Accordingly, it was found that a value of K_i/K_m could be chosen such that the concentrations of inhibitor calculated from eqn. (5) varied exponentially with time (Fig. 6), with $k = 0.13/\text{hr}$. The rate equation for the loss of dimethylnitrosamine from rats given dimethylformamide also can now be written:

$$v = V_{\max} S / (S + K_m + K I_0 e^{-kt}) + (k' + k'') S \quad (6)$$

where $K = K_m/K_i$, and the last term allows for the loss of undecomposed nitrosamine by expiration and excretion, when $k' + k'' = 0.0128/\text{hr}$. (see Tables 3 and 4). The average rates were known over intervals of 2–3 hr. for each of Expts. 3–5 (see Fig. 4, for example), so a value of K_i/K_m could be calculated for each interval. The average value for each run is given (Table 9). The values are very similar, i.e. inhibition was competitive. Moreover, eqn. (6) is likely to give more accurate values of the concentrations of dimethylnitrosamine left in rats during runs with dimethylformamide present than can be got from direct calculation from the labelled carbon dioxide expired, as the equation includes all the results from the early parts of all the runs, and is not affected by the expiration of labelled carbon dioxide in the later parts of long runs from the metabolism of relatively stable products. This advantage remains even if the theory can be faulted.

Another inhibitor, diethylformamide, requires similar treatment. From the results of Expts. 15 of Table 9 and 4–6 of Table 10, it was concluded that this compound inhibited the decomposition of both dimethyl- and diethyl-nitrosamines competitively, and that it was decomposed with a rate constant of 0.14/hr. In these cases the resulting equations reproduced excellently rates calculated from the expiration of labelled carbon dioxide more than 3 hr. after injection, but were less successful at reproducing rates in the first 3 hr. The average values of K_i/K_m are given as before. Rates again can be represented by equations like (6), with $k = 0.14/\text{hr}$. For diethylnitrosamine,

$$k' + k'' = 0.0196/\text{hr.},$$

but, as pointed out in section (4), the excretion rate of diethylnitrosamine is not adequately represented by a term of the form $(k' + k'')S$, so calculations based on the equation will be somewhat in error.

The other values of K_i/K_m in Tables 10 and 11 lead to the following conclusions: (a) Dimethylformamide and dimethylnitrosamine were not simple competitive inhibitors of the decomposition

of diethyl- and *n*-butylmethyl-nitrosamines, as shown by the lack of constancy of K_i/K_m . One must postulate that at least two enzymes decomposed these compounds, only one of which was inhibited. The values of $V_{\max}$ and K_m given above for these compounds are, then, average values. (b) Dimethylformamide inhibited a very similar range of enzymes to dimethylnitrosamine. K_i/K_m is 2.3, so a dose of dimethylformamide should be a little more effective an inhibitor than one-third the dose of the nitrosamine. Comparisons in Table 10 of Expts. 7 and 11, 8 and 12, and 9 and 14, and in Table 11 of Expts. 4 and 6, bear this out. Dimethylformamide, which is of very low toxicity, can therefore be used instead of dimethylnitrosamine as an inhibitor.

(12) Inhibition of nitrosamine decomposition by inhibitors of microsomal oxidation

The enzymes decomposing nitrosamines are microsomal (Magee & Vandekar, 1958; Brouwers & Emmelot, 1960), and bear some other likenesses to those described by Brodie and co-workers (see, for example, Axelrod, Reichenthal & Brodie, 1954). SKF 525A, Marsalid, SKF 4648A and JB 516 were therefore tried against dimethylnitrosamine, and JB 516 also against the other three nitrosamines. Four of these treatments caused less than 25% inhibition. Marsalid (50 mg./kg.) against dimethylnitrosamine and JB 516 (5 mg./kg.) against dimethyl- and *n*-butylmethyl-nitrosamines caused about 40% inhibition. The last two were investigated further, and the rate curves are shown in Fig. 7. The inhibition was only short-lived, the rate increasing rapidly with time. Inhibition was much less and much more transient than is usually the case when such inhibitors are used against microsomal oxidation systems.

(13) Effects of inhibitors on the acute toxicities of dimethyl- and diethyl-nitrosamines

Dimethylformamide slightly increased the LD_{50} of dimethylnitrosamine, and delayed death (Table 13). The compound had no significant effect on the LD_{50} of diethylnitrosamine: the LD_{50} (fiducial limits) of the nitrosamine alone was 216 (190–247) mg./kg., and after pretreatment with 1000 mg. of dimethylformamide/kg. was 248 (203–303) mg./kg.

The effects were studied of diethylformamide and JB 516 on the dose of dimethylnitrosamine causing liver necrosis (the ED_{50}). The ED_{50} was chosen instead of the LD_{50} because effective doses of these inhibitors caused some immediate signs of poisoning in the rats, which might, therefore, affect the LD_{50} . The method is described in Table 14. Unlike that used in section (15) it may not be free from

subjective bias. The inhibitors, however, appeared to have little effect.

The effects of 600 mg. of diethylformamide/kg. on the ED₅₀ of dimethylnitrosamine were also studied, essentially by the method described in Table 14. Livers of rats given diethylnitrosamine alone showed a clear gradation of damage with dose. The doses were 190, 158, 132 and 110 mg./kg., so the ED₅₀ was 145 mg./kg. The livers of rats also given the formamide all showed nitrosamine-type damage, and several were as damaged as the least damaged of those given the nitrosamine alone; but there was no clear gradation with dose, and there were slight differences which made quantitative

comparisons difficult. Overall, the diethylformamide exerted a distinct protective effect, and it appeared likely that the ED₅₀ in its presence was in the range 200–300 mg./kg.

Table 13. *Effect of dimethylformamide on the toxicity of dimethylnitrosamine*

Some groups of rats were given dimethylformamide (700 mg./kg.) before dimethylnitrosamine; other equivalent groups were not. For each dose of dimethylnitrosamine the order in which the rats died is recorded, and the probability of this, or a more extreme order, on the null hypothesis that dimethylformamide was without effect. 'A' represents a rat which had received dimethylnitrosamine only, 'B' one which had also received dimethylformamide. 'A_n' means that *n* rats given dimethylnitrosamine only died in succession. When results are in parentheses, the order is not known. Two experiments are summarized. The LD₅₀ values, with fiducial limits in parentheses, were: without dimethylformamide, 35.9 (32.2–40.0) mg./kg.; with dimethylformamide, 52.6 (44.0–63.0) mg./kg.

Dose (mg./kg.)	Order of death	No. of survivors	Probability
57.6	A ₁₀ B ₂	5	0.0001
48	A ₂ BABABAB	6	0.0005
40	(A ₂ B ₂)BABA ₂	11	0.456
40	A ₃ B ₃ AA	12	0.136
33.3	A ₂ BA ₄	12	0.014
27.8	A ₂	18	0.236
23.2	None	20	—

Table 14. *Effects of diethylformamide and JB516 on the ED₅₀ of dimethylnitrosamine*

Five rats were used in each group. Four groups received dimethylnitrosamine only in the doses shown; five groups received the same and one higher dose of the nitrosamine 1.5 hr. after 10 mg. of JB516/kg.; and similarly 30 min. after 600 mg. of diethylformamide/kg. All injections were intraperitoneal. The rats were killed 2 days later, and liver sections were prepared. The sections were examined by Mr R. L. F. Dawes as follows. The four groups with nitrosamine only were presented with the labels covered, as groups, but in the wrong order. They were put into the correct order, i.e. the damage increased with the dose. The remaining ten groups were then all presented together out of order, and they were matched with those with dimethylnitrosamine only, i.e. six grades were used; 1–4, the grades of damage corresponding to the doses of dimethylnitrosamine only, 0 representing less damage, and 5 representing more.

Dose (mg./kg.)	Grades of damage		
	No inhibitor	Diethyl-formamide	JB516
32	—	5	4
26.7	4	4	4
22.2	3	3	4
18.5	2	0	3
15.4	1	1	2

Fig. 7. Effects of inhibitors on the persistence of nitrosamines *in vivo*. The concentrations *in vivo* after ED₅₀ doses are plotted against time. The ED₅₀ values are taken from sections 13 and 15. A: I, Dimethylnitrosamine (36 mg./kg.; LD₅₀) only; II, dimethylnitrosamine (52.6 mg./kg.; LD₅₀) 30 min. after dimethylformamide (700 mg./kg.). Both curves were calculated from eqn. (6). B: I, Dimethylnitrosamine (22.2 mg./kg.; ED₅₀) only; II, dimethylnitrosamine (22.2 mg./kg.; ED₅₀) 1.5 hr. after JB516 (10 mg./kg.). Curves I and II were calculated from quadruplicated ¹⁴CO₂ runs. The s.e. values of all but one point were less than 1 mg./kg. III, Dimethylnitrosamine (22.2 mg./kg.; ED₅₀) 30 min. after diethylformamide (600 mg./kg.), calculated by eqn. (6). C: I, Diethylnitrosamine (145 mg./kg.; ED₅₀) only; II, diethylnitrosamine (200 mg./kg.; minimum ED₅₀) 30 min. after diethylformamide (600 mg./kg.). Both curves were calculated by eqn. (6). D: I, *n*-Butylmethyl nitrosamine (72 mg./kg.; ED₅₀) only, from two ¹⁴CO₂ runs (the difference between the runs averaged 2 mg./kg.); II, *n*-butylmethyl nitrosamine (72 mg./kg.; ED₅₀) 1.5 hr. after JB516 (5 mg./kg.); the curve was calculated from quadruplicated ¹⁴CO₂ runs. Both curves are corrected for expiration and excretion. The broken lines to zero concentration are the extrapolations made in estimating ED₅₀ and LD₅₀ values as described in the Discussion section.

(14) *Cumulative toxicity of dimethylnitrosamine*

The LD₅₀ of dimethylnitrosamine was increased significantly when the compound was given in three equal doses instead of in a single dose (Table 15). The LD₅₀ for single doses was slightly higher than the LD₅₀ found in the experiment recorded in Table 13. The LD₅₀ determinations were done 2 years apart. During this time the growth rates of the rats fell, so that the rats used in the second experiments were older than those used in the first. The difference in LD₅₀ may be related to this. The rate of decomposition of dimethylnitrosamine was not, however, changed. From the results a rough allowance can be made for the time effect of varying the time of exposure to dimethylnitrosamine on its toxicity.

(15) *Liver necrotic effects of n-butylmethyl-nitrosamine*

An ED₅₀, the dose causing medium necrotization, was found for the compound both with and without pretreatment with 5 mg. of JB 516/kg. The results are shown in Table 16. The *n*-butyl compound was about one-third as effective as dimethylnitrosamine; and JB 516 was without effect.

Table 15. *Cumulative LD₅₀ values of dimethylnitrosamine*

One group of rats was given single doses of dimethylnitrosamine. The other two groups received three equal doses at the times stated. The LD₅₀ values calculated by the method of Weil (1952) are given, with fiducial limits in parentheses.

Treatment	LD ₅₀ (mg./kg.)
Single doses	42.7 (40.0-45.5)
Doses at 0, 12-16, and 24 hr.	52.4 (46.8-58.5)
Doses at 0, 24 and 48 hr.	59.8 (57.3-62.5)

Table 16. *Effect of JB 516 on the necrotic action of n-butylmethyl-nitrosamine*

JB 516 (5 mg./kg.) was given 1.5 hr. before the *n*-butyl compound. The necrotic damage was assessed in 4 grades, 0, 1, 2 and 3. The ED₅₀ values are the doses causing an average grade of 1.5. The ED₅₀ values, with fiducial limits in parentheses, are: with JB 516, 73.8 (61-89) mg./kg.; without JB 516, 73.1 (69.7-76.7) mg./kg.

Dose (mg./kg.)	Necrotic damage	
	With JB 516	Without JB 516
125	—	3, 3, 3, 3, 3
100	—	3, 2, 2, 2, (1 died)
80	3, 3, 2, 1, 1	3, 2, 2, 1, 1
64	2, 1, 1, 1, 0	1, 1, 1, 1, 0
51	1, 1, 0, 0, 0	1, 1, 1, 0, 0
41	1, 0, 0, 0, 0	—
33	0, 0, 0, 0, 0	—

(16) *Toxic effects of tert.-butylmethyl-nitrosamine*

A dose of 1400 mg. of the *tert*.-butyl compound/kg. given intraperitoneally to two rats over a period of 4 days did not cause liver necrosis of the type associated with nitrosamine poisoning. Several rats died at lower doses, which also did not give the effect, nor did oral dosing of four rats at 400 mg./kg./day for three successive days, and then 200 mg./kg./day for three successive days, i.e. a total dose of 1800 mg./kg. over 5 days. The compound was thus at least 20 times less effective than its *n*-butyl isomer.

(17) *Miscellaneous toxicity tests and observations*

The intraperitoneal LD₅₀ of dimethylformamide was 3800 (3100-4600) mg./kg., and of diethylformamide was 1740 (1390-2200) mg./kg.

n-Butylmethyl- and *tert*.-butylmethyl-formamides caused convulsions, rapid prostration, and death in a few minutes when given intraperitoneally at 600 and 700 mg./kg. respectively.

Ethyl-2-hydroxyethyl-nitrosamine was not lethal at a dose of 3 g./kg., given intraperitoneally, nor did it cause centrilobular necrosis.

Bis-2-hydroxyethyl-nitrosamine was not lethal in single intraperitoneal doses of 4.8 g./kg. (three rats), nor in two doses of 4.8 g./kg. separated by 6 hr. (three rats). The rats were killed a fortnight after treatment. All their livers showed some signs of centrilobular necrosis, severe in four cases. The compound appeared to be qualitatively a typical nitrosamine, but very weak. The possibility that it contained an active impurity cannot be completely ruled out.

DISCUSSION

I hope to show that the work described here reduces to three the possible number of simple hypotheses about the nature of the compounds responsible for acute liver poisoning by dialkyl-nitrosamines. This is feasible only because the nitrosamines behaved in a highly reproducible way in the experimental animals, female rats, and are so simple in structure that only a few compounds can be postulated.

The main results can be summarized as follows:

(1) Dimethyl-, diethyl-, *n*-butylmethyl- and *tert*.-butylmethyl-nitrosamines were all decomposed in female rats. (2) Labelled carbon dioxide was expired by rats given nitrosamines labelled in methyl, ethyl or *n*-butyl groups. (3) Relationships were established between the rates of expiration of labelled carbon dioxide and the rates of decomposition, so that the latter could be calculated from the former with a coefficient of deviation of about 6%. Allowance had to be made for the fraction of each

nitrosamine excreted unchanged in the expired air and the urine. The relationship was a little different for each compound, presumably because the proportions of metabolites incorporated and oxidized to labelled carbon dioxide varied. (4) The nitrosamines inhibited each other's decomposition, and it is likely were therefore partly decomposed at the same sites. (5) Dimethylformamide was an effective inhibitor of dimethylnitrosamine oxidation, and diethylformamide of diethylnitrosamine oxidation. (6) One other fairly effective inhibitor was JB516. (7) Inhibitors had little effect on the toxic action of the nitrosamines. (8) The *tert.*-butyl compound was without necrotic action on the liver even at high doses.

The possible metabolism of dialkylnitrosamines can be represented as shown in Scheme 1, in which dimethylnitrosamine is taken as the example. The labelled carbon dioxide expired comes from the further metabolism of formaldehyde, Y and Z. Reaction (1) is the main oxidation reaction. Presumably intermediates, represented by Int., are formed, but they must be very unstable, and pre-

Scheme 1

Table 17. ED_{50} values of nitrosamine with and without inhibitors present

The ED_{50} and LD_{50} values given in sections (13) and (15) are corrected for excretion and expiration of unchanged nitrosamines as described in the text. In one case, dimethylnitrosamine plus dimethylformamide, the LD_{50} value is also corrected for the increased time of action of the nitrosamine, i.e. the LD_{50} has been reduced by the fraction 42.7/52.4, the ratio of the LD_{50} values of dimethylnitrosamine given in single doses and in divided doses over 1 day (section 14). Calculated ED_{50} values were calculated from Fig. 7 as explained in the text, i.e. $(\text{ED}_{50} \text{ calc.})/\text{ED}_{50} = (\text{dose}) \times (\text{intercept for nitrosamine only})/(\text{intercept for nitrosamine} + \text{inhibitor})$.

Treatment	Corrected ED_{50} or LD_{50} (mg./kg.)	
	Found	Calc.
Dimethylnitrosamine (LD_{50})	34	—
Dimethylnitrosamine + 700 mg. of dimethylformamide/kg. (LD_{50})	36	9
Dimethylnitrosamine (ED_{50})	22	—
Dimethylnitrosamine + 10 mg. of JB516/kg. (ED_{50})	22	11
Dimethylnitrosamine + 600 mg. of diethylformamide/kg. (ED_{50})	19	4.4
Butylmethylnitrosamine (ED_{50})	68	—
Butylmethylnitrosamine + 5 mg. of JB516/kg. (ED_{50})	67	50

sent only in low concentrations, or their formation would interfere with the good correlation found between the rates of expiration of labelled carbon dioxide and the rates of fall in nitrosamine concentration *in vivo* (section 5). Concentrations greater than 2 mg./kg. would be difficult to reconcile with the results got with dimethylnitrosamine, for example. Reaction (2) represents possible side reactions, such as conversion into hydroxylamine derivatives by reduction of the nitroso group, or hydrolysis to dimethylamine and nitrite. The products, represented by X, might be further oxidized.

The possibilities summarized by these equations are:

(i) *The dialkylnitrosamines themselves are toxic.* This is not tenable. If this were true, any treatment which increased the persistence of a nitrosamine *in vivo* should increase its toxicity. Now the persistence of three of the nitrosamines was increased considerably with inhibitors, as shown in Fig. 7, but their toxicities were either unaffected or slightly decreased (sections 13 and 15). Direct action by the nitrosamines can only be postulated if in every case the inhibitors of oxidation protected the rats just sufficiently to compensate for the increase in persistence they brought about. This is very unlikely.

As the toxic agents are metabolites, the portion of a dose of nitrosamine that is excreted or expired unchanged cannot contribute to the toxicity. It is therefore reasonable to subtract these portions from the ED_{50} and LD_{50} values determined (referred to as ED_{50} values below), and carry the discussion further in terms of the corrected ED_{50} values so obtained. Another correction is also needed in one case. Dimethylnitrosamine was a little less toxic when it was given in divided doses over 1 day than when the whole dose was given at once (section 14). Dimethylformamide increased the persistence of dimethylnitrosamine to rather more than 1 day (Fig. 7), so that under these conditions dimethylnitrosamine should be less toxic by the same factor. Corrected ED_{50} values are given in Table 17. None is given for diethylnitrosamine, as the values obtained were markedly less accurate than the rest (section 13).

(ii) *Side reactions give toxic products.* The products are those referred to as X and Z in the scheme above, and it is assumed that most of each nitrosamine was oxidized to non-toxic products by reaction (1). The hypothesis is not tenable: inhibitors increased persistence, and should therefore have increased the yields of compounds formed by side reactions (unless these also were inhibited), and hence their toxic effects. On the assumption that the inhibitors do not also inhibit the side reaction, the ED_{50} values to be expected on the present

Nitrites can be excluded. Nitrites are supposed to be released by the hydrolysis of the monoalkylnitrosamines. The hypothesis is clearly untenable if different nitrosamines give the same yield of nitrite in the same places, as the doses of dialkyl-nitrosamines causing observable necrosis ranged from 15 mg. of dimethylnitrosamine/kg. to above 1000 mg. of *tert.*-butylmethylnitrosamine/kg. Nitrites can be the effective agents either if the yields of nitrite vary greatly, or if, in some cases, most of the nitrite is released outside the liver cell—nitrite in the blood stream does not cause liver necrosis. On the first possibility, it must be assumed that the balance of hydrolysis and other reactions varies greatly with structure. This may be so, but *tert.*-butylmethylnitrosamine cannot undergo one of the competitive reactions, the formation of a diazoalkane (Heath, 1961), and this suggests that it is not likely to give the very much lower yield of nitrite required by the nitrite hypothesis. In this context the abnormalities in the decomposition of the *tert.*-butyl compound do not seem relevant. It is unlikely that much nitrite is released outside the cell. According to diffusion theory, monomethylnitrosamine, with a life of under a second, is unlikely to diffuse in pure water more than 10μ from its point of formation before decomposing. The distance presumably will be less in the highly structured environment of a cell. As the distance diffused is proportional to the square root of the life, the lower toxicities of diethyl- and *tert.*-butylmethyl-nitrosamines can only be accounted for if diethyl- and *tert.*-butylmethylnitrosamines have half-lives of about half a minute and several minutes respectively. These changes in stability are much greater than are usually brought about by substituting one alkyl group for another.

The argument shows only that nitrite is unlikely to contribute appreciably to the acute toxic effects. It seems that hydrolysis to nitrite takes place, although this is not a reaction of monoalkylnitrosamines which has been postulated before. The other reactions all lead finally to the release of the whole nitrogenous content of the nitrosamine as gaseous nitrogen. Heath & Dutton (1958) showed that rats given dimethyl[^{15}N]-nitrosamine excreted ^{15}N -containing compounds in their urine, and that ^{15}N was incorporated into their livers. The amounts recovered showed that reactions leading to incorporation were important and possibly accounted for over half of the nitrogen. Either some other reaction takes place, such as an exchange reaction, or hydrolysis is important *in vivo*. Alkaline or non-aqueous media in which the reactions of monoalkylnitrosamines have been studied *in vitro* may have so catalysed the other, competitive, reactions that hydrolysis was insignificant.

Tenable hypotheses. Monoalkylnitrosamines themselves, or diazoalkanes or carbonium ions formed from them, can be postulated as the toxic agents. None can be ruled out.

Carbonium-ion formation has been postulated to explain the wide variety of products found when primary amines are treated with nitrous acid (see Austin, 1960; and Ridd, 1961). Carbonium ions, however, are only supposed to exist for a few molecular vibrations, and to be formed from monoalkylnitrosamines. Therefore, although in terms of reaction mechanism a clear distinction can be made between reactions of monoalkylnitrosamines themselves and of the carbonium ions released from them, the formation of carbonium ion can be regarded as a step in the reaction of monoalkylnitrosamines. The carbonium ion and monoalkylnitrosamine hypothesis lead to identical conclusions, so only the latter need be discussed.

The diazoalkane hypothesis explains the results simply. The yield of diazoalkane should not depend on the rate of decomposition of the dialkyl-nitrosamine, so corrected ED_{50} values should not be affected by inhibitors. Different diazoalkanes will react at different rates, and give different products, so different nitrosamines should have different toxicities. Diazoalkanes are highly reactive, but not so reactive that the biochemical lesion need take place in the immediate molecular environment where they are formed. Finally, the *tert.*-butyl compound cannot form a diazoalkane, and should therefore be without centrilobular necrotic action.

The monoalkylnitrosamine hypothesis explains equally well all the results but the last, but it may seem that *tert.*-butylnitrosamine should be an alkylating agent also, and hence that *tert.*-butylmethylnitrosamine should be a necrotic agent. This simple reasoning is unsound. *n*-Butylmethylnitrosamine can be oxidized in two ways, to *n*-butyl- or to methyl-nitrosamine. Were it solely debutylated, the only product would be methylnitrosamine, and it would be as toxic on a molar basis as dimethylnitrosamine. In fact it was about one-third as toxic, so the major product must have been *n*-butylnitrosamine, formed by demethylation. P. N. Magee (unpublished work) has shown, however, that *n*-butylmethylnitrosamine methylates rat liver RNA *in vivo*, but he found no evidence of butylation. Thus mono-*n*-butylnitrosamine and its products appear to be weak butylating agents. This is confirmed by Magee's (unpublished) observation that di-*n*-butylnitrosamine causes the typical lesion only at doses of about 800 mg./kg. As *tert.*-butylmethylnitrosamine cannot be debutylated by the primary oxidation reaction, it can only give *tert.*-butylnitrosamine, and it may be fairer, therefore, to compare its toxicity with that of di-*n*-

butylnitrosamine. On this comparison there is no evidence that its toxicity is particularly low. Thus monoalkylnitrosamines acting as alkylating agents cannot be eliminated. If they act in other ways they can be eliminated by the arguments that eliminated nitrite.

The discussion shows that the acute hepatotoxic action of simple dialkylnitrosamines can be explained if the compounds are converted *in vivo* into monoalkylnitrosamines, and these, or carbonium ions or diazoalkanes formed from them, react with some vital component of the liver. Other hypotheses can be maintained as indicated at various points above, but the necessary assumptions are either too far-fetched or too complex.

In one respect the hypotheses favoured are similar: all three types of compound must be presumed to act as alkylating agents. Diazoalkanes and carbonium ions act only in this way, and other reactions of monoalkylnitrosamines can be eliminated. Thus the biochemical lesion is an alkylation, and the results suggest that the study of alkylation *in vivo* can throw light on the action of nitrosamines. There is still a distinction between the hypotheses. Should the biochemical lesion involve the introduction of a methylene bridge, diazoalkanes would be favoured; and were the methylation of a very weak base necessary, one of the two other hypotheses. There is a good correlation between liver necrotic activity and carcinogenicity in the series. The di-*n*-butyl compound is appreciably carcinogenic (Druckrey *et al.* 1962). If the *tert*.-butyl compound were not, the monoalkylnitrosamine hypothesis would appear less likely.

The results show that not all simple dialkylnitrosamines are likely to be metabolized in the same way. Both bis-2-hydroxyethyl- and ethyl-2-hydroxyethyl-nitrosamines were very weak inhibitors of dimethylnitrosamine oxidation, and the second was also very transient (section 7). Probably both are too water-soluble to adsorb appreciably on the oxidative enzymes involved, which would explain their low necrotic and carcinogenic activity (Druckrey *et al.* 1961*a*). Also adsorption on the site of oxidation is not enough for oxidation to take place. Dimethylformamide was adsorbed on the same sites as dimethylnitrosamine, but it was not *N*-demethylated (section 7). This was unexpected, as the molecules are isoelectronic, and very similar in polarity.

SUMMARY

1. To find out which compounds are responsible for the acute hepatotoxic action of dialkylnitrosamines, the metabolism in female rats of the following dialkylnitrosamines was studied: dimethyl, diethyl, *n*-butylmethyl and *tert*.-butylmethyl.
2. Some of each nitrosamine was excreted un-

changed in urine and in expired air. The dependence of rate on dose was determined.

3. Rates of decomposition *in vivo* were determined from the rates of fall of blood concentrations, and from rates of expiration of ¹⁴C-labelled carbon dioxide from rats given the compounds labelled in methyl, ethyl or *n*-butyl groups. The results agreed well when allowance was made for excretion, and could be found with a coefficient of deviation of 6%. Labelled *tert*.-butyl groups of *tert*.-butylmethylnitrosamine, *tert*.-butylamine or 2-methylpropan-2-ol were not oxidized to labelled carbon dioxide.

4. From the rates of expiration of labelled carbon dioxide it was shown that the decomposition of all but the *tert*.-butyl compound obeyed the Michaelis-Menten equation; and that the oxidation of dimethylnitrosamine was inhibited competitively by the other three nitrosamines and by diethyl- and dimethylformamides. The oxidation of diethyl- and *tert*.-butylmethyl-nitrosamines was also inhibited competitively by diethylformamide.

5. Dimethylnitrosamine and dimethylformamide inhibited much the same range of sites. From their action it appeared that the other three nitrosamines were oxidized at at least two types of site.

6. The LD₅₀ or ED₅₀ values, or both, of the nitrosamines were determined. The *tert*.-butyl compound was without measurable necrotic activity.

7. The ED₅₀ values were unchanged by inhibitors that greatly increased the persistence of the nitrosamines *in vivo*.

8. The results showed that the nitrosamines were not themselves toxic, and that the toxic agent must be a product of oxidation.

9. Most possible metabolites could not be postulated as the toxic agents unless drastic assumptions were made.

10. The results were consistent with the assumption that the toxic agent in each case was a diazoalkane, or a monoalkylnitrosamine, or carbonium ions formed from it.

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The Isolation of L-Homoarginine from Seeds of *Lathyrus cicera*

By E. A. BELL

Department of Biochemistry, King's College, London, W.C. 2

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The occurrence of appreciable concentrations (1% or more as judged by the intensity of the ninhydrin spots) of free ninhydrin-reacting compounds in the seeds of 45 species of *Lathyrus* has been reported by Bell (1962a). The genus was divided into groups of species characterized by peculiar associations of these compounds in their seeds. Most of the compounds are probably non-protein amino acids and the associated groups may represent final storage products together with their respective precursors.

The distribution and relative concentrations of two of the compounds suggested that they may be related. These substances have now been isolated and identified. Both are amino acids that have not been found previously in natural sources. The first, lathyrine, was isolated from seeds of *Lathyrus tingitanus* by Bell (1961) and shown to be 2-amino-pyrimidin-4-ylalanine (Bell & Foster, 1962). The second was identified by Bell (1962a, b) as homoarginine (α -amino- ϵ -guanidinocaproic acid), and

the present paper describes its isolation from seeds of *Lathyrus cicera*. The isolated amino acid was shown to be L-homoarginine by direct comparison with the synthetic compound and by the identification of homocitrulline (α -amino- ϵ -ureidocaproic acid) and lysine as the principal products of alkaline hydrolysis.

The possible role of L-homoarginine (I) as the natural precursor of lathyrine (II), and the relationship between the groups of species in which these two amino acids occur, are discussed.