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Cellular Injury and Carcinogenesis

ALKYLATION OF RIBONUCLEIC ACID OF RAT LIVER BY DIETHYLNITROSAMINE AND *n*-BUTYLMETHYLNITROSAMINE *IN VIVO*

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Dimethylnitrosamine causes severe centrilobular haemorrhagic necrosis of the liver when administered to several laboratory animal species in doses near to the LD₅₀ (Barnes & Magee, 1954) and induces tumours of the liver, kidney and lung on repeated administration (Magee & Barnes, 1956, 1962; Schmähl & Preussmann, 1959; Zak, Holzner, Singer & Popper, 1960; Argus & Hoch-Ligeti, 1961). Diethylnitrosamine is less acutely toxic (Heath, 1962) but causes a similar acute liver necrosis in doses approaching the LD₅₀. This compound induces liver tumours in the rat (Schmähl, Preussmann & Hamperl, 1960; Argus & Hoch-Ligeti, 1961; Thomas, 1961; Grundmann & Sieburg, 1962) and tumours of the lung, nasal cavity and liver in the Syrian golden hamster (Dontenwill & Mohr, 1961; Herrold & Dunham, 1963). Very recently diethylnitrosamine has been shown to

induce malignant liver tumours in the guinea pig (Druckrey & Steinhoff, 1962; Argus & Hoch-Ligeti, 1963). This observation is of great interest since the guinea pig has been regarded hitherto as remarkably resistant to hepato-carcinogenesis.

n-Butylmethylnitrosamine is nearer in toxicity to dimethyl- than to diethyl-nitrosamine (Heath, 1961, 1962). It produces the typical acute hepatic injury in appropriate doses and is carcinogenic after repeated administration to the rat. In contrast *tert*.-butylmethylnitrosamine is considerably less toxic and does not cause necrosis of the liver (Heath, 1961, 1962). It does not appear to have been tested for carcinogenicity.

Druckrey and his colleagues have studied the relationship between chemical structure and carcinogenic activity in a series of nitrosamines and have found a large number of active compounds, several of which induce tumours of the oesophagus (Druckrey, Preussmann, Schmähl & Müller, 1961).

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Di-*n*-butylnitrosamine induced tumours of the urinary bladder as well as the liver and oesophagus (Druckrey, Preussmann, Schmähl & Müller, 1962). In a study of unsymmetrical dialkyl nitrosamines the same group suggested the possibility of correlation between this type of chemical structure and capacity to induce oesophageal tumours. It is of interest that *tert*.-butylethyl nitrosamine was found not to be carcinogenic (Druckrey, Preussmann, Blum, Ivankovic & Afkham, 1963).

Evidence is now accumulating which indicates that the nitrosamines require metabolic activation before they become biologically active (Heath, 1962). It has been suggested that the compounds are converted into alkylating agents within the target cells and that these metabolites are responsible for the tissue injury and carcinogenesis (Hultin, Arrhenius, Löw & Magee, 1960; Druckrey *et al.* 1961; Emmelot & Mizrahi, 1961). Evidence that dimethylnitrosamine acts as a methylating agent *in vivo* comes from the demonstration of methylation of proteins of rat-liver slices incubated with the compound *in vitro* (Magee & Hultin, 1962) and of RNA and DNA in liver and kidney of rats treated with dimethylnitrosamine (Magee & Farber, 1962; Craddock & Magee, 1963). Methylation of liver RNA has recently been found in mice, hamsters and guinea pigs treated with dimethylnitrosamine (Lee, Lijinsky & Magee, 1964).

The present paper describes experiments on the metabolism of diethylnitrosamine, *n*-butylmethylnitrosamine and *tert*.-butylmethylnitrosamine, all labelled with ^{14}C , in which the incorporation of the isotope into RNA and protein of rat liver and other organs was studied *in vivo*.

A preliminary communication of some of this work was presented at a conference on Hepato-toxicity of Therapeutic Agents held by the New York Academy of Sciences in October 1962.

MATERIALS AND METHODS

Chemical reagents

Nitrosamines. Diethylnitrosamine was obtained from British Drug Houses Ltd., Poole, Dorset, and purified by distillation (b.p. 172°). *n*-Butyl- and *tert*.-butyl-methylnitrosamine were prepared by Dr A. R. Mattocks as described by Heath & Mattocks (1961). *n*-Butyl- ^{14}C methylnitrosamine, *n*- ^{14}C butylmethylnitrosamine, *tert*.-butyl- ^{14}C methylnitrosamine, *tert*.- ^{14}C butylmethylnitrosamine and ^{14}C diethylnitrosamine were prepared and purified by Dr D. F. Heath and Dr A. R. Mattocks by their previously described method (Heath & Mattocks, 1961).

Special chemicals. Samples of 7-methylguanine and 7-ethylguanine were gifts from Dr P. Brookes (Chester Beatty Research Institute, London) and another sample of 7-methylguanine was a gift from Dr G. H. Hitchings (Wellcome Research Laboratories, Tuckahoe, N.J., U.S.A.).

Conduct of experiments

Wistar albino rats of the Porton strain were maintained on M.R.C. diet 41 B (Bruce & Parkes, 1956) or on Rockland rat diet. Animals of either sex, weighing about 200 g., were used. The labelled nitrosamines, suitably diluted with the non-radioactive compound, were dissolved in 0.9% (w/v) NaCl and injected intraperitoneally. The dose of nitrosamine and of radioactivity is given in the text. The experiments were conducted essentially as previously described (Magee & Farber, 1962; Lee *et al.* 1964). RNA and protein preparations were made by slight modifications of the methods of Kirby (1956) and of Schneider (1945) respectively. Acid hydrolysis of RNA was in N-HCl at 100° for 1 hr. to yield purine bases and pyrimidine nucleotides (Vischer & Chargaff, 1948). Alkaline hydrolysis was in 0.3 N-KOH at 37° for 18 hr. to yield purine and pyrimidine nucleotides (Davidson & Smellie, 1952). Ion-exchange chromatography of acid hydrolysates was carried out as before, with Dowex 50 cation-exchange resin (H^+ form) and gradient elution by HCl , and of alkaline hydrolysates with Dowex 1 anion-exchange resin (formate form) by a slight modification (Magee & Hultin, 1962) of the method of Brumm, Potter & Siekevitz (1956). Paper chromatography, spectrophotometry and radioactivity measurements were also as before. For end-window assay, a Nuclear-Chicago gas-flow counter with Micromil window was used and for scintillation counting a Packard Tricarb liquid-scintillation spectrometer. Materials were prepared for assay of radioactivity as previously described (Magee & Farber, 1962; Lee *et al.* 1964).

RESULTS

Experiments with *n*-butyl- and *tert*.-butyl-methylnitrosamine. Two rats were injected with *n*-butyl- ^{14}C methylnitrosamine and *n*- ^{14}C butylmethylnitrosamine respectively, each receiving 75 mg. and $30\text{ }\mu\text{C/kg}$. body wt., and were killed 7 hr. later. Another two animals received *tert*.-butyl- ^{14}C methylnitrosamine (75 mg. and $150\text{ }\mu\text{C/kg}$. body wt.) and *tert*.- ^{14}C butylmethylnitrosamine (75 mg. and $75\text{ }\mu\text{C/kg}$. body wt.) respectively and were killed after 24 hr. The times chosen were such that little unchanged nitrosamine would be present in the animals according to data on the rates of metabolism of the two compounds (Heath, 1962). The dose of *n*-butylmethylnitrosamine was sufficient to induce a severe haemorrhagic centrilobular necrosis of the liver. The tertiary isomer does not cause hepatic necrosis but may induce some periportal fatty change. RNA and protein preparations from liver and total plasma protein were isolated and assayed for radioactivity, with the results shown in Table 1. There is clearly much higher labelling of nucleic acids and proteins when either isomer is labelled in the methyl group than when labelled in the butyl group (normal or tertiary). The ratio of specific radioactivities of RNA to protein with the *n*-butyl- ^{14}C methyl compound (4.5) is very close to that (4.9) obtained previously with ^{14}C dimethylnitrosamine (Magee

Table 1. Incorporation of radioactivity into ribonucleic acid and proteins of liver in rats treated with ¹⁴C-labelled isomeric butylmethylnitrosamines

Female rats (about 200 g. body wt.) received the labelled compound by intraperitoneal injection and were killed after the intervals shown.

Labelled nitrosamine	Dose of labelled nitrosamine		Time after injection (hr.)	Sp. radioactivity of RNA (counts/min./cm. ²)	Sp. radioactivity of protein (counts/min./cm. ²)	Sp. radio-activity of RNA/sp. radioactivity of proteins
	(mg./kg. body wt.)	(μc/rat)				
<i>n</i> -Butyl[¹⁴ C]methylnitrosamine	75	6	7	1120	249	4.5
<i>n</i> -[1- ¹⁴ C]Butylmethylnitrosamine	75	6	7	11	46	0.24
<i>tert.</i> -Butyl[¹⁴ C]methylnitrosamine	75	30	24	898	1681	0.53
<i>tert.</i> -[2,2- ¹⁴ C ₂]Butylmethylnitrosamine	75	15	24	2	22	0.09

Table 2. Distribution of radioactivity in ribonucleic acid and proteins of organs of rats treated with *n*-butyl[¹⁴C]methylnitrosamine

Four female rats (about 200 g. body wt.) received *n*-butyl[¹⁴C]methylnitrosamine (75 mg./kg. body wt., 3 μc/rat) and were killed 6 hr. later. Specific radioactivities of proteins and RNA from pooled organs were measured at 'infinite thickness'; plasma proteins were assayed individually.

Organ	Sp. radioactivity of protein (counts/min./cm. ²)	Sp. radioactivity of RNA (counts/min./cm. ²)
Liver	151	752
Kidney	110	186
Pancreas	264	3
Spleen	49	100
Plasma (mean and range for four samples)	108 (91-120)	—

& Farber, 1962). With the *tert.*-butyl[¹⁴C]methylnitrosamine, however, the protein specific radioactivity was higher than the RNA (ratio 0.53). The distribution of incorporated radioactivity from *n*-butyl[¹⁴C]methylnitrosamine into RNA and protein of liver, kidney, spleen and pancreas was then studied (Table 2). The pattern of isotopic incorporation, specific radioactivities of RNA samples being expressed as percentages of that in the liver, was: liver (100), kidney (25), pancreas (0.4) and spleen (13), which was quite similar to that observed previously with [¹⁴C]dimethylnitrosamine [i.e. liver (100), kidney (26), pancreas (1.3) and spleen (32)]. The only marked difference was in the spleen, where the relative labelling was considerably lower with the butylmethyl than with the dimethyl compound. These results suggested that some of the methyl groups of *n*-butylmethylnitrosamine might follow metabolic pathways similar to those shown previously with dimethylnitrosamine (Magee & Farber, 1962) and might therefore methylate RNA and other cell components. This possibility was tested by acid hydrolysis of the labelled RNA under conditions yielding free purine bases and pyrimidine nucleotides (Visher &

Fig. 1. Ion-exchange chromatography of acid hydrolysates of RNA from rats treated with labelled isomeric butylmethylnitrosamines. (A) RNA (19.7 mg.) from liver of rat treated with *n*-butyl[¹⁴C]methylnitrosamine (75 mg./kg. body wt., 6 μc/rat) 7 hr. earlier. (B) RNA (18.5 mg.) from liver of rat treated with *tert.*-butyl[¹⁴C]methylnitrosamine (75 mg./kg. body wt., 30 μc/rat) 24 hr. earlier. Dowex 50 (H⁺ form), 1 cm. × 10 cm. column, with gradient elution from 1 to 4 N-HCl. ●, *E*₁ cm. at 260 mμ; ○, radioactivity (counts/min.). G, Guanine; A, adenine.

Chargaff, 1948) followed by ion-exchange chromatography.

The elution pattern (Fig. 1A) was almost identical with that previously obtained with [¹⁴C]-dimethylnitrosamine and showed one major peak containing nearly all the radioactivity, which emerged between guanine and adenine. This is the known position of 7-methylguanine, which is the methylated base expected almost exclusively when RNA is methylated with relatively low concentration of the alkylating agent (Brookes & Lawley, 1961) and which was found previously in hydrolysates of liver RNA from rats treated with

dimethylnitrosamine (Magee & Farber, 1962). More material was obtained from six rats, each given *n*-butyl[^{14}C]methylnitrosamine (75 mg./kg. body wt., $3\mu\text{C}/\text{rat}$). The liver RNA was hydrolysed and subjected to ion-exchange chromatography as before. Almost all the radioactivity again appeared as a single peak between guanine and adenine, and these fractions were combined and the hydrochloric acid was removed by repeated evaporation to dryness under vacuum at 50° . The material was then taken up in a small volume of water and applied to paper as a streak, which was subjected to descending chromatography with propan-2-ol-hydrochloric acid (Wyatt, 1951). All the radioactivity was found in a band whose R_f (0.31) was close to that of authentic 7-methylguanine (0.29) applied to the same paper. Under ultraviolet light, the radioactive band and the reference material appeared as a light-blue fluorescent band well separated from guanine and merging into the darker-blue band of adenine. A strip containing much of the radioactivity but very little adenine was cut from the paper and eluted. This material was again concentrated and applied to paper for descending chromatography with propan-2-ol-ammonia solvent (Markham & Smith, 1952), an authentic sample of 7-methylguanine again being applied. The radioactivity again remained in one band which corresponded to an ultraviolet-absorbing band (R_f 0.29) well separated from a faint adenine band. The R_f of the authentic 7-methylguanine was 0.29. Both materials were then eluted from the paper and their ultraviolet spectra were plotted against the appropriate blanks at pH 2, 6 and 9 respectively. The curves at pH 2 and pH 9 are shown in Fig. 2. Although the curves of the unknown radioactive material and the authentic 7-methylguanine do not superimpose exactly at all wavelengths, the agreement is quite good, and, taken with the chromatographic evidence, they probably justify the conclusion that the unknown material is 7-methylguanine.

Acid hydrolysis and ion-exchange chromatography was then carried out in the same way on the labelled liver RNA from the rat treated with *tert*-butyl[^{14}C]methylnitrosamine (Table 1). The elution pattern is shown in Fig. 1(B). Here there was almost no detectable radioactivity in the region of 7-methylguanine, but it nearly all appeared in two large peaks whose profiles corresponded exactly with those of guanine and adenine respectively, with a small peak emerging very early in the region of the pyrimidine nucleotides. This pattern is clearly quite different from that obtained with the methyl-labelled *n*-butyl isomer and indicates that methylation of RNA is negligible or absent after treatment with the *tert*-butyl compound. The incorporation of radioactivity into RNA of liver

after administration of both isomers labelled in the butyl groups was so low that chromatography was obviously not worth while and indicated that neither alkylation by the butyl groups nor incorporation of metabolic products into normal base components had occurred to any significant extent.

Experiments with diethylnitrosamine. The time-course of labelling of protein and RNA in rat liver and kidney was studied, the animals being killed at intervals of 8 hr. after injection of [^{14}C]diethylnitrosamine (Table 3). It is apparent that maxi-

Fig. 2. Ultraviolet-absorption spectra of 7-methylguanine. Smooth curves are for an authentic sample and points for the major peak of unknown radioactive material isolated from the liver of a rat treated with *n*-butyl[^{14}C]methylnitrosamine (see Fig. 1A). ●, pH 2; ○, pH 9.

Table 3. Incorporation of radioactivity into ribonucleic acid and proteins of liver and kidney of rats at increasing time-intervals after treatment with [^{14}C]diethylnitrosamine

Female rats (about 200 g. body wt.) received [^{14}C]diethylnitrosamine (200 mg./kg. body wt., $8\mu\text{C}/\text{rat}$) by intraperitoneal injection and were killed at 8 hr. intervals.

Time after injection (hr.)	Sp. radioactivities of RNA and proteins (disintegrations/min./mg.)			
	Liver		Kidney	
	RNA	Protein	RNA	Protein
8	67	74	30	90
16	143	92	102	158
24	356	234	129	177
32	81	97	56	87
40	60	86	36	70
48	47	54	30	47

mum labelling in both organs occurred at about 24 hr. and this time after injection was therefore chosen in later experiments. After allowing for the lower specific radioactivity of the injected nitrosamine, and the greater efficiency of scintillation counting, the labelling of the RNA samples and proteins is considerably lower with [^{14}C]diethylnitrosamine than with *n*-butyl[^{14}C]methylnitrosamine and still lower than that observed previously with [^{14}C]dimethylnitrosamine (Magee & Farber, 1962). Since methylation of liver RNA *in vivo* had been observed with *n*-butylmethylnitrosamine and with dimethylnitrosamine, it was clearly possible that ethylation might occur in animals treated with diethylnitrosamine. This possibility was tested by acid hydrolysis followed by ion-exchange chromatography of the labelled liver RNA. A preliminary experiment under conditions similar to those with the butylmethylnitrosamines (Fig. 1) gave a chromatographic profile with most of the radioactivity emerging from the column very early, in the region of the pyrimidine nucleotides, and another, considerably smaller, radioactive peak which merged into but did not superimpose on adenine. The elution gradient of hydrochloric acid was therefore reduced from 1–4*N* to 1–2.5*N* and this was tested on an acid hydrolysate of ethylated guanylic acid prepared according to Reiner & Zamenhof (1957), to which a small quantity of marker adenine had been added. With the lower elution gradient there was much wider separation of adenine from guanine and a small ultraviolet-absorbing peak emerged just before and quite distinctly separated from adenine. This material, which had the ultraviolet spectrum of a guanine derivative, was assumed to be 7-ethylguanine, and these elution conditions were adopted in experiments on hydrolysates of labelled RNA from livers of rats treated with [^{14}C]diethylnitrosamine. Since the earlier experiments had shown that labelling was relatively low, ethylation of RNA could not have been extensive and material from a larger number of rats treated with non-radioactive diethylnitrosamine was therefore combined with that from the livers of a smaller number of animals treated with [^{14}C]diethylnitrosamine. In this experiment RNA from 25 rats given non-radioactive diethylnitrosamine was hydrolysed with *N*-hydrochloric acid as usual and applied to a large ion-exchange column; elution was with *N*-hydrochloric acid. All fractions between guanine and adenine were combined and repeatedly evaporated to dryness under vacuum. This material was then taken up in a small volume of *N*-hydrochloric acid and added to an acid hydrolysate of liver RNA from four rats treated with [^{14}C]diethylnitrosamine. The combined hydrolysates were applied to the small ion-exchange column and eluted with the less

steep gradient (1–2.5*N*). The elution pattern of ultraviolet absorption and radioactivity is shown in Fig. 3. Again the major part of the radioactivity appeared in the region of the pyrimidine nucleotides, but a smaller peak now emerged quite separately from adenine. All the fractions corresponding to this smaller second radioactive peak were evaporated to dryness repeatedly under vacuum. This material was dissolved in a small quantity of water and applied as a streak (about 2 cm.) to paper. On descending chromatography with propan-2-ol-ammonia solvent (Markham & Smith, 1952), the unknown material gave an ultraviolet-absorbing spot whose R_F (0.70) was identical with that of authentic 7-ethylguanine (0.70) applied to the same paper. Both spots were eluted from the paper and their ultraviolet-absorption spectra were plotted against the appropriate paper blanks at different pH values. The spectra at pH 2 and pH 6 were compared and there was good agreement between the curves of the authentic 7-ethylguanine and the unknown material in the range 220–300 $m\mu$. It appears therefore that treatment of rats with diethylnitrosamine under these conditions is followed by the appearance of 7-ethylguanine in the liver. The nature of the larger early-appearing peak of radioactivity was not clarified by these experiments, however, and its appearance with the pyrimidine nucleotide so soon after the start of elution might well have been fortuitous. This possibility was

Fig. 3. Ion-exchange chromatography of acid hydrolysate of RNA from liver of rats treated with [^{14}C]diethylnitrosamine. Hydrolysate of liver RNA from 25 rats treated with unlabelled diethylnitrosamine (200 mg./kg. body wt.) 24 hr. earlier was chromatographed on a large column (18 cm. $\times$ 3.5 cm.), and the material eluted between guanine and adenine was concentrated and rechromatographed on a smaller column (9 cm. $\times$ 1.5 cm.) with acid hydrolysate of RNA (50 mg.) from four rats similarly treated with [^{14}C]diethylnitrosamine (200 mg./kg. body wt., 50 μC /rat). Dowex 50 (H^+ form), with gradient elution from 1 to 2.5*N*-HCl. ●, $E_{1\text{cm}}$ at 260 $m\mu$; ○, radioactivity (counts/min.). G, Guanine; A, adenine.

Fig. 4. Ion-exchange chromatography of alkaline hydrolysate of RNA (44 mg.) from livers of rats treated with [^{14}C]-diethylnitrosamine (200 mg./kg. body wt., 50 μC /rat) 24 hr. earlier. Dowex 1 (formate form), 1.5 cm. $\times$ 21 cm. column, with gradient elution from 1 to 4N-formic acid. ●, $E_{1\text{ cm.}}$ at 260 m μ ; ○, radioactivity (counts/min.).

tested by alkaline hydrolysis of another sample of the labelled RNA, under conditions liberating free purine nucleotides as well as pyrimidine nucleotides, followed by ion-exchange chromatography with an anion-exchanger (Dowex 1; formate form). Under these conditions the pyrimidine nucleotides are widely separated. The resulting elution pattern (Fig. 4) showed a radioactive peak merging into guanylic acid, which appeared to correspond to a similar peak observed previously with dimethylnitrosamine (Magee & Farber, 1962), and thought probably to be the formamido pyrimidine derivative known to be liberated on alkaline hydrolysis of alkylated nucleic acids (Lawley & Wallick, 1957; Haines, Reese & Todd, 1962). Both cytidylic acid and uridylic acid contained radioactivity, probably accounting for some of the early peak observed in the acid chromatography, and there was an additional peak, emerging before the nucleotides, which was not identified.

DISCUSSION

The different patterns of labelling of the liver RNA and proteins after treatment with the methyl- and butyl-labelled isomeric butylmethylnitrosamines are interesting because of their

implications for the mechanism of metabolism of these compounds. With *n*-butyl[^{14}C]methylnitrosamine the pattern of labelling (Tables 1 and 2) in RNA and protein is very similar to that obtained previously with [^{14}C]dimethylnitrosamine (Magee & Farber, 1962) in liver, kidney and pancreas, but incorporation is considerably lower into spleen. The chromatographic profile (Fig. 1A) of the acid hydrolysate of the labelled RNA is also almost identical with that obtained with [^{14}C]dimethylnitrosamine and nearly all of the radioactivity is present as 7-methylguanine. Reasons for considering this as indicative of methylation of the nucleic acid by a methylating agent formed metabolically from the nitrosamine have been discussed (Magee & Farber, 1962; Lee *et al.* 1964). The nature of the postulated alkylating agent has also been extensively discussed (Hultin *et al.* 1960; Magee & Farber, 1962; Heath, 1961, 1962; Druckrey *et al.* 1961; Emmelot, Mizrahi & Kriek, 1962; Mizrahi & Emmelot, 1962), possibilities being the corresponding monoalkylnitrosamine, a diazo alkane or a carbonium ion. All these suggestions involve an initial enzymic oxidative *N*-dealkylation of the nitrosamine. It appears therefore that *n*-butylmethylnitrosamine gives rise to a methylating agent but there can be very little butylation of either the RNA or the proteins since the incorporation of radioactivity into these components is extremely low after treatment of the animal with *n*[1- ^{14}C]butylmethylnitrosamine (Table 1). Since the rate of oxidation of both alkyl groups of *n*-butylmethylnitrosamine to carbon dioxide is nearly the same (Heath, 1962), there is clearly no marked difference in rate of *N*-demethylation and *N*-debutylation but the overwhelming predominance of methylation over butylation of the nucleic acid can probably be explained by more successful competition of the methylating agent for available guanine moieties. Since the *n*-butyl group is probably oxidized via a C_2 rather than a C_1 intermediate the lower incorporation of radioactivity into spleen RNA may be explained because the latter (e.g. formate) is a more active precursor of normal purine and pyrimidine bases than the former (e.g. acetate). The presence of radioactivity in the RNA of the liver of the rat treated with *tert*.-butyl[^{14}C]methylnitrosamine (Table 1) is almost entirely because of incorporation into major normal base components (Fig. 1B), probably via the ' C_1 intermediate' pathway after oxidative *N*-demethylation with no evidence of significant alkylation. There was virtually no labelling of RNA or protein in the liver of the rat given *tert*.-[2,2- $^{14}\text{C}_2$]-butylmethylnitrosamine (Table 1), which is consistent with the observation of Heath (1962) that labelled *tert*.-butyl groups of *tert*.-butylmethylnitrosamine, *tert*.-butylamine and 2-methylpropan-

2-ol are not oxidized to labelled carbon dioxide, indicating considerable metabolic stability of the group.

The presence of 7-ethylguanine in acid hydrolysates of liver RNA from rats treated with diethylnitrosamine is of interest since earlier workers maintained that ethylation, in contrast with methylation, did not involve the guanine moieties of nucleic acids (Reiner & Zamenhof, 1957; Alexander & Stacey, 1958). More recently, however, 7-ethylguanine has been identified in nucleic acids treated with various ethylating agents *in vitro* (Bautz & Freese, 1960; Brookes & Lawley, 1961), and this supports the conclusion that diethylnitrosamine, as does dimethyl- or *n*-butylmethyl-nitrosamine, alkylates nucleic acids and other cell components *in vivo* after metabolic activation. The smaller extent of alkylation by diethylnitrosamine than by the methylating nitrosamines probably reflects the lower activity of ethylating than methylating agents *in vitro* (Brookes & Lawley, 1961). The lower total incorporation into nucleic acids can probably be explained by the lower incorporation of 'C₂' than 'C₁ intermediates'. The attainment of maximal incorporation into RNA about 24 hr. after an approximately LD₅₀ dose of diethylnitrosamine is different from that with dimethylnitrosamine at about 6 hr. after injection (Craddock & Magee, 1963) and may reflect the lower efficiency of ethylation. The evidence for ethylation of RNA and therefore probably of other cell components by diethylnitrosamine and methylation by *n*-butylmethyl-nitrosamine give further support for the alkylation hypothesis of nitrosamine action *in vivo*, since both these compounds induce acute hepatic necrosis and are carcinogenic in the rat (Schmähl *et al.* 1960; Heath & Magee, 1962). In contrast, *tert.*-butylmethyl-nitrosamine does not induce hepatic necrosis (Heath, 1962) and does not significantly alkylate cell components *in vivo* (Table 1, Fig. 1). This compound does not appear to have been tested for carcinogenic activity but *tert.*-butylethyl-nitrosamine has been tested in the rat (Druckrey *et al.* 1963) and found inactive.

The possible role of nucleic acids in the induction of cancer and similarities between basic mechanisms of carcinogenesis and mutagenesis have been widely discussed (e.g. Haddow, 1958; Bendich, 1961; Arley & Eker, 1962; Burch, 1963). In particular, the possible significance of alkylation of RNA in carcinogenesis by diethylnitrosamine has been discussed by Oehlert & Hartje (1963). Although all the work on nucleic acids reported here has been done with RNA it must be emphasized that this has been largely for reasons of technical convenience. DNA is also methylated by dimethylnitrosamine *in vivo* (Magee & Farber,

1962; Craddock & Magee, 1963), and it seems probable that this is also the case with diethyl- and *n*-butylmethyl-nitrosamine. It is also very likely that proteins and other cellular components are alkylated *in vivo* by these nitrosamines. It seems reasonable to suppose that the acute liver damage which follows treatment with these compounds is caused by alkylation of many intracellular components, the total effect being varying degrees of cellular damage culminating in cell death. On the other hand, the induction of cancer is more likely to be related to alkylation of one or both of the nucleic acids since these are now regarded as the hereditary material of the cell. Several methylating and ethylating agents have been shown to be mutagenic in various species (Westergaard, 1957), and it is of some interest that dimethyl- and diethyl-nitrosamine are mutagenic in *Drosophila* but *tert.*-butylethyl-nitrosamine is not (Pasternak, 1962, 1963). Moreover, dimethyl- and diethyl-nitrosamine are without mutagenic activity in bacteria (Geissler, 1962) and in *Neurospora* (Marquardt, Schwaier & Zimmermann, 1963).

SUMMARY

1. Rats were injected intraperitoneally with the hepatotoxic carcinogens diethylnitrosamine and *n*-butylmethyl-nitrosamine and with *tert.*-butylmethyl-nitrosamine, which has low hepatotoxicity. The nitrosamines were labelled in various positions with radioactive carbon. The dose, with the more toxic compounds, was adequate to cause acute necrosis of the liver.

2. Incorporation of radioactivity from the isomeric butylmethyl-nitrosamines labelled in the methyl groups was found in RNA and proteins of liver, kidney, spleen and pancreas. The ratio of specific radioactivity of RNA to that of protein was considerably higher with the *n*-butyl compound (4.5) than with the *tert.*-butyl isomer (0.53). Incorporation of radioactivity from both isomers labelled in the butyl groups was relatively very low.

3. The labelled RNA from the livers of rats treated with the methyl-labelled isomers was hydrolysed with acid and analysed by chromatography and ultraviolet spectroscopy. Most of the radioactivity in the liver RNA from animals treated with *n*-butylmethyl-nitrosamine was found to be in 7-methylguanine. After treatment of the rats with *tert.*-butylmethyl-nitrosamine the radioactivity of the liver RNA was nearly all in major normal components and no 7-methylguanine was detected.

4. The time-course of incorporation of radioactivity from labelled diethylnitrosamine was studied and found to be maximal in RNA and proteins of rat liver and kidney 24 hr. after injection.

5. Acid hydrolysis of the labelled RNA from rats injected with [¹⁴C]diethylnitrosamine, followed by chromatography and ultraviolet spectroscopy, showed the presence of 7-ethylguanine in the hydrolysate.

6. These results are considered to give further support for the hypothesis that the dialkylnitrosamines owe some of their biological actions to the metabolic production of alkylating agents in the cells of susceptible organs. Possible similarities between the mechanisms of chemical carcinogenesis and mutagenesis by nitrosamines are discussed.

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