Analysis of Bases of Rat-Liver Nucleic Acids after Administration of the Carcinogen Dimethylnitrosamine

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Dimethylnitrosamine is metabolized to form an alkylating intermediate, which may have significance for its carcinogenic action. However, certain other compounds that are known to be highly mutagenic, including nitrous acid and hydroxylamine, might also be formed. Owing to the general reactivity of these compounds, it would be difficult to detect their formation in the intact animal. Instead, the nucleic acids of carcinogen-treated animals were examined for products of reaction with nitrous acid and hydroxylamine, i.e. for deamination of adenine and guanine, and formation of *N*⁶-hydroxycytosine, respectively. A double-labelling technique was used to detect very small amounts of the abnormal bases. The purine moieties of DNA in adult rat liver were labelled either with ¹⁴C or with ³H, by treating the neonatal animals with [¹⁴C]formate or with [³H]formate, and then allowing a period for normal growth. During this time, in liver, the labels were largely lost by metabolic turnover from cell components other than DNA. The pyrimidine moieties in DNA were labelled by treating the neonatal animals with [¹⁴C]orotate. The purine constituents of RNA of adult rat liver were labelled by repeated administration of [¹⁴C]- or [³H]-formate to the adult rats. The [¹⁴C]nucleic acid-labelled rat could then be treated with the carcinogen, and the [³H]nucleic acid-labelled animal could be used as a control. By this means the experimental and control tissues could be homogenized together in a single preparation, and the nucleic acids from the two tissues could be isolated, hydrolysed and analysed in a single sample. It was therefore possible to have an internal control for artifacts due to changes taking place in the nucleic acid bases during the experimental procedures. With this technique, the formation *in vivo* of 7-methylguanine in rat-liver DNA and RNA after administration of dimethylnitrosamine was confirmed, and no evidence was found for the formation of xanthine, hypoxanthine, *N*⁶-hydroxycytosine, or any other abnormal base.

The inheritable nature of the change from a normal to a neoplastic cell suggests that a change in the hereditary material of the cell might be involved (Boveri, 1914; Boyland, 1954; Strong, 1958). Primary reactions in carcinogenesis not involving the genetic material have also been suggested (Kaplan, 1959; Monod & Jacob, 1961; Pitot & Heidelberger, 1963). In agreement with these concepts, the carcinogen dimethylnitrosamine (Magee & Barnes, 1956, 1959) brings about methylation of DNA, RNA and proteins in the intact animal (Magee & Farber, 1962; Craddock & Magee, 1963), the alkylation of the nucleic acids occurring almost entirely on the 7-position of guanine.

Dimethylnitrosamine is metabolized in liver and possibly at a much smaller rate in other tissues to give an alkylating intermediate, which is possibly diazomethane. However, there is evidence that

this is not the sole metabolic pathway. If all the nitrosamine were converted into an alkylating intermediate, all the nitrogen of the molecule would be converted into gaseous nitrogen. Experiments with dimethylnitrosamine labelled with ¹⁵N (Heath & Dutton, 1958) showed that the heavy isotope appeared eventually in urea and protein. Though the intermediates of these conversions are unknown, it is conceivable that certain compounds are formed that are very highly mutagenic and that therefore may have some significance in the problem of carcinogenesis. Among these compounds are nitrous acid and hydroxylamine. Although no increase in the concentration of either of these compounds in the acid-soluble fractions of tissues or in urine after administration of dimethylnitrosamine was found (Heath & Dutton, 1958) it was possible that they may have reacted as formed

with some component of the cell and therefore would not have been detected. It was therefore necessary to look for the products of such reactions rather than for the compounds themselves.

There is evidence (Druckrey, Steinhoff, Beuthner, Schneider & Klärner, 1963) that nitrous acid is not carcinogenic in the rat. However, the biological action of a compound may be very different after administration to the intact animal from when it is formed from a precursor within the cell. In the former case it may react with compounds in the blood or the cytoplasm, and never reach the DNA of the nucleus. When applied more directly to the unprotected cellular material, nitrous acid causes inactivation of yeast (Stein & Lochmann, 1964). It is mutagenic for tobacco-mosaic virus (Gierer & Mundry, 1958) and for T2 bacteriophage (Vielmetter & Wieder, 1959), and it inactivates transforming DNA (Litman & Ephrussi-Taylor, 1959). It is therefore possible that nitrous acid, if formed within the liver cell, may act as a carcinogen.

Nitrous acid is believed to exert its biological effects by deamination of certain bases of nucleic acids (Schuster & Schramm, 1958), the relative rates of deamination of the different bases depending on the pH (Vielmetter & Schuster, 1960). However, in native DNA, there is evidence (Lochmann & Stein, 1963) that guanine is deaminated more rapidly than adenine or cytosine. If such deamination had occurred, some xanthine and hypoxanthine would be expected in the nucleic acids of animals treated with dimethylnitrosamine, but the amount of conversion would be extremely small at the assumed intracellular pH of about 7.4. Investigation of this possibility was difficult because some deamination is known to occur as an artifact during isolation and hydrolysis of nucleic acids. A method was therefore devised that allowed for an internal control. Animals to be treated with dimethylnitrosamine were prepared with their liver DNA or RNA purines labelled with ^{14}C , and control animals were similarly labelled with ^3H . The control and experimental tissues could then be homogenized together in the same tube, and the mixed nucleic acids isolated, hydrolysed and analysed by column chromatography. The amounts of xanthine and hypoxanthine in the ^{14}C - and ^3H -labelled nucleic acids could then be determined by differential counting. With [^{14}C]- and [^3H]-formate as precursors, adenine becomes labelled with both isotopes in the 2- and 8-positions, whereas guanine is similarly labelled with ^{14}C but the ^3H label occurs only in the 8-position. As shown in Scheme 1, deamination would not involve loss of either isotope.

Other conceivable metabolites of dimethylnitrosamine are hydroxylamine or derivatives. When applied *in vitro* hydroxylamine is known to

Scheme 1. Deamination of purine moieties in nucleic acids labelled by the administration of [^{14}C]- and [^3H]-formate. The isotopic carbon and hydrogen atoms are shown in bold type.

cause chromosome damage in Chinese-hamster cells (Somers & Hsu, 1962), and in mouse-embryo cells (Borenfreund, Krim & Bendich, 1964), and hydroxylamine is of course a mutagen (Freese, Bautz-Freese & Bautz, 1961). There is evidence that the carcinogenic action of certain amines may be due to the formation of *N*-hydroxy derivatives *in vivo* (Cramer, Miller & Miller, 1960).

The mechanism of the mutagenic activity of hydroxylamine is probably due to the reaction with cytosine moieties in the nucleic acids, although this may not be the only base affected *in vivo* (Tessman, Ishiwa & Kumar, 1965). Hydroxylamine reacts with 5-methylcytosine to give *N*⁶-hydroxy-5-methylcytosine (Janion & Shugar, 1965), and with cytosine to form first 4,5-dihydro-4-hydroxy-aminocytosine, which then reacts with a second molecule of hydroxylamine to form a bishydroxy-amino compound (Brown & Schell, 1965). Acid hydrolysis of a nucleic acid containing the bis-hydroxyamino compound would yield *N*⁶-hydroxy-cytosine. The intermediates in these reactions should not affect the labelling of the cytosine, as shown in Scheme 2. Therefore rats whose liver DNA was labelled in the pyrimidines with ^{14}C were treated with dimethylnitrosamine, and the DNA was isolated and hydrolysed, carrier *N*⁶-hydroxy-cytosine was added and the mixture was subjected to column chromatography to determine whether the peak of carrier *N*⁶-hydroxycytosine contained radioactivity.

At low concentrations, hydroxylamine reacts indirectly with DNA, after reacting with oxygen to form hydrogen peroxide, nitroxyl and intermediate radicals (Freese & Bautz-Freese, 1965). If

Scheme 2. Reaction of cytosine moiety in DNA, labelled by the administration of [6-¹⁴C]orotic acid, with hydroxylamine. The isotopic carbon atoms are shown in **bold** type.

reactions such as these occurred in DNA of rat liver *in vivo*, the products of the reactions, and the base deletions to which they may give rise, might not have been detected in the present experiments.

The experiments described below confirmed the formation *in vivo* of 7-methylguanine in liver DNA and RNA after the administration of dimethylnitrosamine but, within the limits of the methods used, no xanthine, hypoxanthine, N⁶-hydroxycytosine, or any other abnormal base was detected. A preliminary report of this work has been presented (Craddock & Magee, 1965).

EXPERIMENTAL

Animals. Wistar albino rats of the Porton strain were maintained on M.R.C. diet 41B (Bruce & Parkes, 1956).

Materials. Sodium [¹⁴C]formate, sodium [³H]formate, and [6-¹⁴C]orotic acid were purchased from The Radiochemical Centre, Amersham, Bucks. N⁶-Hydroxycytosine was a gift from Dr D. M. Brown, University Chemical Laboratory, Cambridge. 7-Methylguanine was a gift from Dr P. Brookes, Chester Beatty Research Institute. Dimethylnitrosamine, supplied by British Drug Houses Ltd., Poole, Dorset, was redistilled before use.

Method of labelling purines of DNA in the intact animal. A litter of newborn rats was divided into two groups, which were treated with [¹⁴C]- and [³H]-formate respectively. Each neonatal rat was given a series of 11 injections during the first 24 days of life, the first eight being subcutaneous and the last three intraperitoneal. Each rat labelled with ¹⁴C was given a total of 34.5 μC of [¹⁴C]-formate, and each animal labelled with ³H was given a total of 345 μC of [³H]formate. At the end of the injection period, the rats were maintained on a normal diet, after weaning, for a period of 9–18 weeks, to allow time for normal growth.

Method of labelling pyrimidines of DNA in the intact animal. Each neonatal rat used was given a series of nine injections, the first eight subcutaneous and the last one intraperitoneal, over a period of 19 days. The total dose of [6-¹⁴C]orotate administered was 20.0 μC. The rats were then maintained on normal diet until 13 weeks of age, when they were used.

Method of labelling purines of RNA in the adult animal. Two female rats (200g. body wt.) were treated with [¹⁴C]- and [³H]-formate respectively. Each animal was given a

total of five intraperitoneal injections over a period of 6 days. One rat received a total of 20 μC of [¹⁴C]formate and the other received 200 μC of [³H]formate. The rats were used on the day after the last injection.

Treatment of animals and isolation of nucleic acids. (a) Experiment on purines of DNA. Two rats aged 9 weeks, whose liver DNA was labelled with ¹⁴C in the purine moieties, were given an intraperitoneal injection of dimethylnitrosamine (30mg./kg.) and killed 5hr. later. At the same time two rats from the control group with ³H-labelled purines were killed. The livers from the four animals were placed in the same flask of liquid N₂ and were homogenized together, and DNA was prepared from the single homogenate by the method of Kirby (1957).

(b) Experiment on purines of RNA. Two rats, one with ¹⁴C-labelled purines and the other with ³H-labelled purines, were starved overnight. The ¹⁴C-labelled rat was given an intraperitoneal injection of dimethylnitrosamine (30mg./kg.) and both animals were killed 5hr. later. The two livers were pooled and RNA was isolated from the mixed homogenate by the method of Kirby (1962).

(c) Experiments on pyrimidines of DNA. In a preliminary experiment, to obtain a specimen of normal DNA labelled in the pyrimidines by the orotic acid treatment described above, two rats were killed and liver DNA was isolated by a modification of the method of Kirby (1957). To obtain DNA from rats treated with dimethylnitrosamine, two litter mates of the control animals, treated in the same way with orotic acid, were given an intraperitoneal injection of dimethylnitrosamine (30mg./kg.) and killed 5hr. later, and liver DNA was prepared at the same time as that of the control animals.

Analysis of nucleic acid bases. (a) Purine-labelled DNA. To minimize deamination occurring during hydrolysis, the perchloric acid method of Wyatt (1952) was used. A 40mg. sample of DNA was heated with 0.4ml. of 70% (w/w) HClO₄ at 100° for 1hr. and the hydrolysate was diluted to 2ml. and centrifuged. Carrier xanthine, hypoxanthine, and 7-methylguanine were added to the supernatant, and the mixture was run on a Dowex 50 (X12) column (10cm. × 0.9cm.), with the eluent being changed from N-HCl to a gradient approaching 5N-HCl after elution of the cytosine peak.

(b) Purine-labelled RNA. A 10mg. sample of RNA was heated with 0.1ml. of 70% HClO₄ and treated as described for the analysis of purine-labelled DNA, the change in concentration of the acid during chromatography being to 4N-HCl after collecting 220ml. of effluent.

(c) Pyrimidine-labelled DNA. The DNA samples (30–40mg.) were hydrolysed with 0.5ml. of 98% formic acid at 175° for 45 min. (Wyatt, 1951) and the hydrolysates were diluted and centrifuged. The supernatants were evaporated to dryness on a rotary evaporator, water was added and the process was repeated; the residue was finally dissolved in 0.1N-HCl. Carrier N⁶-hydroxycytosine was added and the mixture analysed on a Dowex 50 column by using a gradient from 0.1N- to 4.0N-HCl.

Treatment of effluents from column chromatography. The E₂₆₀ was determined on each fraction of the effluent by using a Unicam SP.500 spectrophotometer. Where necessary, the ultraviolet spectra were recorded by using a Unicam SP.800 automatic recording spectrophotometer.

The radioactivity of each sample was determined after evaporation to dryness in a scintillation bottle. Then

0.5 ml. of 1.0M-Hyaminate [*p*-(di-isobutylresoxyethoxy-ethyl)dimethylbenzylammonium hydroxide] was added, the bottle was warmed at 37° for 30 min. and after cooling 10 ml. of scintillator solution [0.1 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 4.0 g. of 2,5-diphenyloxazole in 1 l. of toluene] was added. Radioactivity was determined by using a Packard Tri-Carb model 3000 liquid-scintillation counter.

RESULTS

Analysis of purines of DNA. The results of a preliminary experiment in which both the ^{14}C -labelled DNA and the ^3H -labelled DNA were prepared from normal animals are shown in Fig. 1. As expected, the guanine, adenine and thymine were highly labelled. Some radioactivity appeared in the carrier hypoxanthine, presumably as an artifact due to deamination during isolation and hydrolysis. A small amount of ^{14}C was present in the cytosine peak. Both ^3H and ^{14}C showed two small peaks, *a* and *b*, which may be minor base components, or artifacts formed during the experimental procedures. Peak *b* did not coincide with the peak of added carrier 7-methylguanine, as shown by the extinction profile.

The experiment in which the rat labelled with ^{14}C had received an injection of dimethylnitrosamine is shown in Fig. 2. As in the preliminary experiment, guanine, adenine, thymine and carrier

hypoxanthine were labelled with both isotopes, cytosine showed a very low content of ^{14}C , and peaks *a* and *b* were detectable. In the ^{14}C profile, but not in the ^3H profile, there was an additional peak of radioactivity that coincided exactly with the extinction peak of carrier 7-methylguanine.

Analysis of purines of RNA. The experiment in which the purines of RNA had been labelled with ^{14}C or ^3H is shown on Fig. 3. Both isotopes are present very largely in guanine and adenine, whereas uracil, cytosine and hypoxanthine contained very little radioactivity. The ^{14}C profile, but not the ^3H profile, showed a peak that coincided exactly with the extinction peak of carrier 7-methylguanine. Two unidentified peaks, *d* and *e*, were detectable in both the ^{14}C and in the ^3H profiles.

Analysis of pyrimidines of DNA. A preliminary experiment in which carrier *N*⁶-hydroxycytosine was added to a DNA hydrolysate from a normal ^{14}C -labelled rat is shown in Fig. 4. As expected, the cytosine and thymine were relatively highly labelled, and the radioactivity profile showed no peaks in the guanine or adenine regions. The very minor peaks of radioactivity did not coincide with the extinction peak given by the carrier *N*⁶-hydroxycytosine.

Analysis of the DNA hydrolysate from the rat treated with dimethylnitrosamine is shown in Fig. 5.

Fig. 1. Column chromatography of DNA hydrolysate. The DNA sample was prepared from two rats, one with DNA labelled in the purines with ^{14}C and the other with ^3H . Both rats were otherwise normal. The positions of the peaks as indicated were determined by E_{260} readings, carrier xanthine (X), hypoxanthine (H) and 7-methylguanine (M) having been added. Other known peaks are thymine (T), cytosine (C), guanine (G) and adenine (A). *a* and *b* are unidentified radioactive peaks without detectable extinction. The total radioactivity (in disintegrations/min.) in the guanine peak was 10983 for ^3H and 26118 for ^{14}C , and in the adenine peak was 307813 for ^3H and 34068 for ^{14}C . —, ^3H profile; ----, ^{14}C profile.

Fig. 2. Column chromatography of DNA hydrolysate. The sample was prepared as described in Fig. 1, except that the ^{14}C -labelled rat had been treated with dimethylnitrosamine (30 mg./kg. body wt.) 5 hr. before being killed. The explanation of the Figure is as given in Fig. 1. Total radioactivity (in disintegrations/min.) in the guanine peak was 36505 for ^3H and 23394 for ^{14}C , and in the adenine peak was 335145 for ^3H and 33634 for ^{14}C .

Fig. 3. Column chromatography of RNA hydrolysate. The RNA sample was prepared from two rats, one with the RNA purines labelled with ^{14}C and the other with ^3H . The rat labelled with ^{14}C had been treated with dimethylnitrosamine (30 mg./kg. body wt.) 5 hr. before being killed. The explanation of the Figure is as given in Fig. 1. U is the uracil peak and *d* and *e* are unidentified radioactive peaks without detectable extinction. The total radioactivity (in disintegrations/min.) in the guanine peak was 28689 for ^3H and 6772 for ^{14}C , and in the adenine peak was 70200 for ^3H and 5946 for ^{14}C .

The minor peaks of radioactivity correspond to those found in the normal DNA hydrolysate, and no peak of radioactivity was apparent in the N^6 -hydroxycytosine peak.

Quantitative analysis of results. (a) Purines of DNA and RNA. The total counts/min. in the purines are shown in Table 1. There is substantial radioactivity in hypoxanthine in each peak, but the radioactivity in xanthine is extremely low and exceeds the background by more than 2.5 counts/min. in only one case. The one higher value is

only 9.2 counts/min. above the background. The reason for this is not clear. However, the xanthine in this case was derived from a ^3H -labelled animal that had not been treated with dimethylnitrosamine. The very low radioactivity in xanthine from the treated animals indicates that any deamination of guanine *in vivo* must have been extremely small.

As there was appreciable deamination of adenine, it is necessary to compare the extents of conversion in the treated and untreated animals. After making

Fig. 4. Column chromatography of DNA hydrolysate. The DNA was isolated from a rat previously treated with $[6\text{-}^{14}\text{C}]$ orotic acid. Carrier N^6 -hydroxycytosine (N) had been added. ----, ^{14}C profile; —, E_{260} profile. The letters designating the peaks mean the same as in Fig. 1.

Fig. 5. Column chromatography of DNA hydrolysate. Conditions were as described for Fig. 4, except that the rat had been treated with dimethylnitrosamine (30 mg./kg. wt.) 5 hr. before being killed.

Table 1. *Total radioactivity in respective peaks after column chromatography of nucleic acid hydrolysates of normal and dimethylnitrosamine-treated rats, the ^{14}C - and ^3H -labelled nucleic acid preparations being isolated in a single sample*

Experimental details are given in the text. The errors shown refer to counting errors only.

		Radioactivity (counts/min. above background)	
		^3H	^{14}C
DNA:			
^{14}C -labelled rat and ^3H -labelled rat both untreated with dimethylnitrosamine	Guanine	3185	10286
	Xanthine	-0.8 ± 0.44	1.8 ± 0.40
	Adenine	50273	13519
	Hypoxanthine	420 ± 2.9	120 ± 2.08
DNA:			
^{14}C -labelled rat treated with dimethyl-nitrosamine, ^3H -labelled rat as control	Guanine	7139	9359
	Xanthine	9.2 ± 1.1	1.7 ± 1.0
	Adenine	53897	13200
	Hypoxanthine	473 ± 3.05	117 ± 2.06
RNA:			
^{14}C -labelled rat treated with dimethyl-nitrosamine, ^3H -labelled rat as control	Guanine	4944	2702
	Xanthine	0.62 ± 0.61	2.5 ± 0.62
	Adenine	10600	2344
	Hypoxanthine	116 ± 3.78	26 ± 2.6

Table 2. *Extent of deamination of adenine in DNA and RNA from normal ^3H -labelled rats and ^{14}C -labelled rats treated with dimethylnitrosamine, the ^{14}C - and ^3H -labelled nucleic acid preparations being isolated in a single sample*

Experimental details and the calculation of extent of deamination are described in the text. The errors shown refer to the counting errors for each of the three experiments.

		Deamination (%)	
		^3H	^{14}C
DNA:			
^{14}C -labelled rat and ^3H -labelled rat both untreated with dimethylnitrosamine		0.83 ± 0.006	0.89 ± 0.016
DNA:			
^{14}C -labelled rat treated with dimethyl-nitrosamine, ^3H -labelled rat as control		0.86 ± 0.006	0.84 ± 0.015
RNA:			
^{14}C -labelled rat treated with dimethyl-nitrosamine, ^3H -labelled rat as control		0.98 ± 0.03	1.27 ± 0.13

the necessary corrections involved in double-channel counting and quench corrections to convert counts/min. into disintegrations/min., the percentage deamination of [^{14}C]adenine to [^{14}C]hypoxanthine and [^3H]adenine to [^3H]hypoxanthine was calculated in each case (Table 2). The standard errors shown are those that can be attributed to radioactivity assay. If these were the only errors involved, the percentage deamination in ^3H - and ^{14}C -labelled rats would be significantly different. However, it is more reasonable to suppose that there are other errors due to

manipulations and that, for DNA, the four results can be regarded as estimates of the average deamination under the circumstances, i.e. $0.855 \pm 0.026\%$ (s.d., $n=4$). On this basis, the probability is less than 5% that more than 0.085% deamination would have been unobserved. For RNA, owing to the lower labelling of adenine compared with DNA adenine, the counting errors appear to predominate. The deamination in the treated rat is marginally higher ($P=0.05$) than in the control.

The extent of conversion of guanine into 7-methylguanine could not be calculated as accurately

Table 3. $^3\text{H}/^{14}\text{C}$ ratios in bases isolated from nucleic acid hydrolysates of rats treated with $[\text{}^3\text{H}]$ - or $[\text{}^{14}\text{C}]$ -formate

Experimental details are given in the text. Values were calculated from total disintegrations/min. in the respective peaks, after column chromatography.

	$^3\text{H}/^{14}\text{C}$ ratio			Time after injection of $[\text{}^3\text{H}]$ - or $[\text{}^{14}\text{C}]$ -formate (weeks)
	Adenine	Hypoxanthine	Guanine	
RNA:				
^{14}C -labelled rat treated with dimethylnitrosamine, ^3H -labelled rat as control	11.8	12.0	4.2	1
DNA:				
^{14}C -labelled rat treated with dimethylnitrosamine, ^3H -labelled rat as control	10.0	10.2	1.6	9
DNA:				
^{14}C -labelled rat and ^3H -labelled rat both untreated with dimethylnitrosamine	9.1	8.4	0.4	18

as the extent of deamination, as the 7-methylguanine peak was not so well separated from other components. However, the extent of methylation of guanine in RNA was approx. 1%, which agrees with the value found previously with $[\text{}^{14}\text{C}]$ dimethylnitrosamine (Craddock & Magee, 1963).

An observation of possible significance is the $^3\text{H}/^{14}\text{C}$ ratio in adenine and guanine from the different samples. The isotopic precursors were administered in the $[\text{}^3\text{H}]\text{formate}/[\text{}^{14}\text{C}]\text{formate}$ ratio 10:1. A $^3\text{H}/^{14}\text{C}$ ratio 10:1 would therefore be expected in the adenine peaks, but this ratio would not be expected to be exact, as it would depend not only on their specific radioactivities, but also on the amounts of the two nucleic acids obtained from each liver. As the molecule of guanine contains two carbon atoms derived from formate, but only one hydrogen from this source, the ratio would be expected to be 5:1.

Table 3 shows that an approximate ratio 10:1 was found in the adenine, as expected. A similar ratio was found in the hypoxanthine, confirming that it had in fact been formed from adenine. The ratio in the guanine, however, was lower than 5. That of the DNA sample prepared from 9-week-old rats was 1.6, and that of 18-week-old animals had fallen to 0.4. There appears to be an exchange of the hydrogen on C-8 of guanine moieties in DNA. An enzymic exchange of ^3H from C-6 of glucose has been postulated (Dunn & Strahs, 1965), but the nature of the loss of ^3H from guanine is not yet known.

(b) Pyrimidines of DNA. As shown in Figs. 4 and 5, most of the radioactivity was in the pyrimidines, with virtually no labelling of the purines. The total radioactivity of cytosine in the control DNA (32.4 mg.) was 240485 counts/min. and in the DNA from the treated rat (39.6 mg.) the total radioactivity was 257953 counts/min. Although

there are minor peaks of radioactivity in the region of the carrier N^6 -hydroxycytosine, their profiles do not correspond to the extinction profile of the carrier. Inspection of the chromatograms indicates that a total of 250 counts/min. in the N^6 -hydroxycytosine would have been easily detected. This would correspond to hydroxylation of 0.1% of the cytosine.

DISCUSSION

The experiments described were carried out on nucleic acids of liver, after the animal had received a single injection of dimethylnitrosamine. This situation is known to be carcinogenic for kidney, but not for liver, the latter organ developing tumours only when dimethylnitrosamine is fed in the diet for an adequate period (Magee & Barnes, 1956, 1959). However, the reactions taking place in liver are similar to those occurring in kidney as far as is known (Craddock & Magee, 1963), but take place to a much greater extent, and so can be more readily investigated. A possible explanation of the absence of liver tumours after a single injection of dimethylnitrosamine is discussed below.

These experiments support the view that 7-methylguanine is the predominating abnormal base formed in DNA *in vivo* after the administration of dimethylnitrosamine. Under the conditions used, approx. 1% of the guanine moieties of liver RNA and 0.5% of those of DNA would have been methylated. Any deamination that might have taken place, within the limit of the experimental error, would have been much less than the extent of alkylation assuming that the deaminated bases, xanthine and hypoxanthine, remained in the nucleic acids. Similarly, any N^6 -hydroxycytosine formed must have been less than approx. 0.1% of the cytosine moieties of DNA. However, there may well be a quantitative difference between the

various reactions of DNA with respect to the extent of the reaction required to induce a biological effect.

Although the experiments were designed specifically to look for the formation of xanthine, hypoxanthine, and N^6 -hydroxycytosine, other abnormal bases formed by reaction with the carcinogen or its metabolites might have been revealed as peaks in the ^{14}C profile on chromatography. It therefore appears that alkylation is the only detectable modification of the nucleic acids. This agrees with the concept of Miller & Miller (1965), that 'all low molecular weight organic chemical carcinogens may initiate the neoplastic change through alkylating or arylating reactions'.

Participation of nucleic acid methylases in carcinogenesis has been suggested by Srinivasan & Borek (1963), and this is supported by the high level of transfer-RNA-methylase activity found in tumours induced by the virus S.V. 40 (Mittelman, Yohn, Hall & Grace, 1965).

If alkylation of nucleic acids is relevant to carcinogenesis, the question arises whether it is the alkylation itself, or the consequent splitting out of the alkylated base, that is the significant reaction. A very similar problem is presented by the mechanism of the mutagenic action of alkylating agents. After a single injection of dimethylnitrosamine there is a slow loss of the methyl group from DNA of kidney, where there is a carcinogenic situation, and a more rapid disappearance of the labelled methyl group from DNA of liver, in which organ dimethylnitrosamine does not act as a carcinogen after a single injection. It may be that the slow loss from kidney represents chemical splitting out of the alkylated base, which is known to occur *in vitro* (Lawley & Wallick, 1957), and that this reaction is carcinogenic. The more rapid loss that takes place in liver may be due to the activity of a 'repair' enzyme (Setlow & Carrier, 1964; Painter & Rasmussen, 1964), which may thus prevent the neoplastic transformation. Study of the mechanism of the rapid loss of 7-methylguanine from liver DNA may help to solve this problem.

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