- Greenstein, J. P. & Winitz, M. (1961). In *Chemistry of the Amino Acids*, p. 26. New York, London: John Wiley and Sons Inc.
- Gurnani, S. U., Kumta, U. S. & Sahasrabudhe, M. B. (1955). *Biochim. biophys. Acta*, **16**, 553.
- Harms, W. S. & Winnick, T. (1954). *Biochim. biophys. Acta*, **15**, 480.
- Hickinbottom, W. J. (1948). In *Reactions of Organic Compounds*, p. 358. London: Longmans, Green and Co.
- Hirs, C. H. W., Moore, S. & Stein, W. H. (1954). *J. Amer. chem. Soc.* **76**, 6063.
- Hultin, T., Arrhenius, E., Löw, H. & Magee, P. N. (1960). *Biochem. J.* **76**, 109.
- Jacobsen, K. H., Wheelwright, H. J., Clem, J. M. & Shannon, R. N. (1955). *Arch. industr. Health*, **12**, 617.
- Jepson, J. B. & Smith, I. (1953). *Nature, Lond.*, **172**, 1100.
- Koshland, D. E. (1960). *Advanc. Enzymol.* **22**, 45.
- Lawley, P. D. & Wallick, C. A. (1957). *Chem. & Ind.* p. 633.
- MacFadyen, D. A. (1945). *J. biol. Chem.* **158**, 107.
- Magee, P. N. (1956). *Biochem. J.* **64**, 676.
- Magee, P. N. (1962). *Lect. sci. Basis Med.* (in the Press).
- Magee, P. N. & Barnes, J. M. (1956). *Brit. J. Cancer*, **10**, 114.
- Magee, P. N. & Barnes, J. M. (1959). *Acta Un. int. Cancr.* **15**, 187.
- Magee, P. N. & Barnes, J. M. (1962). *J. Path. Bact.* (in the Press).
- Magee, P. N. & Farber, E. (1962). *Biochem. J.* **83**, 114.
- Magee, P. N. & Vandekar, M. (1958). *Biochem. J.* **70**, 600.
- Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
- Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.
- Miller, E. C. & Miller, J. A. (1947). *Cancer Res.* **7**, 468.
- Miller, J. A. & Miller, E. C. (1953). *Advanc. Cancer Res.* **1**, 339.
- Mitoma, C. & Greenberg, D. M. (1952). *J. biol. Chem.* **196**, 599.
- Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 893.
- Nicolet, B. H. & Shinn, L. A. (1939). *J. Amer. chem. Soc.* **61**, 1615.
- Potter, V. R. (1958). *Fed. Proc.* **17**, 691.
- Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
- Reiss, D. & Tayeau, F. (1959). *Bull. Soc. Chim. biol., Paris*, **41**, 1570.
- Rose, F. L. (1958). In *Symp. on the Evaluation of Drug Toxicity*, p. 116. Ed. by Walpole, A. L. & Spinks, A. London: J. and A. Churchill Ltd.
- Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 463.
- Schmährl, D. & Preussmann, R. (1959). *Naturwissenschaften*, **46**, 175.
- Schmährl, D., Preussmann, R. & Hamperl, H. (1960). *Naturwissenschaften*, **47**, 89.
- Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
- Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
- Schoental, R. (1960). *Nature, Lond.*, **188**, 420.
- Schwyzler, R. (1952). *Acta chem. scand.* **6**, 219.
- Searle, J. M. & Westall, R. G. (1951). *Biochem. J.* **48**, 1.
- Sheehan, J. C. & Frank, V. S. (1949). *J. Amer. chem. Soc.* **71**, 1856.
- Siekevitz, P. (1952). *J. biol. Chem.* **195**, 549.
- Stoner, H. B. & Magee, P. N. (1957). *Brit. med. Bull.* **13**, 102.
- Tallan, H. H., Stein, W. H. & Moore, S. (1954). *J. biol. Chem.* **206**, 825.
- Toennies, G. & Kolt, J. J. (1951). *Analyt. Chem.* **23**, 823.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques*, p. 149. Minneapolis: Burgess Publishing Co.
- Vischer, E. & Chargaff, E. (1948). *J. biol. Chem.* **176**, 715.
- Watkins, W. M. & Wormall, A. (1952). *Biochem. J.* **51**, 577.
- Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.
- Zak, F. G., Holzner, J. H. & Popper, H. (1960). *Cancer Res.* **20**, 96.

Biochem. J. (1962) **83**, 114

Toxic Liver Injury and Carcinogenesis

METHYLATION OF RAT-LIVER NUCLEIC ACIDS BY DIMETHYLNITROSAMINE IN VIVO

BY P. N. MAGEE* AND E. FARBER†

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

(Received 10 August 1961)

In the preceding paper (Magee & Hultin, 1962) it was shown that methylation of proteins of rat-liver slices occurred during incubation of the slices

* Present address: The Chicago Medical School, Institute of Medical Research, Division of Oncology, 2020 West Ogden Avenue, Chicago 12, Ill., U.S.A.

† Present address: Department of Pathology, University of Pittsburgh, School of Medicine, Pittsburgh 13, Pa., U.S.A.

with the hepatotoxin and carcinogen dimethylnitrosamine. This finding supports the hypothesis that dimethylnitrosamine undergoes enzymic oxidative *N*-demethylation in the liver with subsequent intracellular formation of a methylating agent, possibly diazomethane. Evidence for reaction with nucleic acids was also obtained. The reactions of nucleic acids and their components with alkylating agents have been studied very extensively *in vitro*

(Timmis, 1960); there is a marked chemical specificity for alkylation on the 7-position of the guanine moieties. This has been shown with dimethyl sulphate (Lawley & Wallick, 1957; Reiner & Zamenhof, 1957), diazomethane (E. Shaw, personal communication) and mustard gas (Brookes & Lawley, 1960). The last-named authors showed further that similar alkylation occurs *in vivo* when ascites tumour cells are exposed to mustard gas.

It seemed probable, therefore, that methylation of nucleic acids might occur in the livers of animals treated with dimethylnitrosamine and in any other organs capable of *N*-demethylation of the compound. This paper describes experiments in which rats were injected with [^{14}C]dimethylnitrosamine and the incorporation of radioactivity into proteins and nucleic acids of liver, kidney and other tissues was measured. The detailed labelling of the liver RNA was studied, after hydrolysis, by ion-exchange and paper chromatography. The dose of dimethylnitrosamine was sufficient to cause acute centrilobular necrosis of the liver in all the injected rats and to induce tumours of the kidney in a small proportion of the animals.

MATERIALS AND METHODS

Chemical reagents

Dimethylnitrosamine. This was obtained commercially and purified by distillation (b.p. 151°).

Isotopic materials. [^{14}C]Dimethylnitrosamine was prepared by Dr D. F. Heath by a previously published method (Dutton & Heath, 1956). Sodium [^{14}C]formate, L-[^{14}C]methionine, L-[^{14}C]leucine and DL-[^{14}C]valine were obtained from The Radiochemical Centre, Amersham, Bucks.

Special chemicals. Samples of 1-methylguanine, 7-methylguanine, 6-hydroxy-2-methylaminopurine, 2-dimethylamino-6-hydroxypurine, 2-amino-6-methoxypurine and 7-methylxanthine were gifts from Dr G. H. Hitchings (Wellcome Research Laboratories, Tuckahoe, N.J., U.S.A.).

Animals. Wistar albino rats of the Porton strain were maintained on M.R.C. diet 41 B (Bruce & Parkes, 1956). Female rats weighing about 200 g. were used.

Conduct of animal experiments

[^{14}C]Dimethylnitrosamine, suitably diluted with the non-radioactive compound, was dissolved in 0.9% (w/v) NaCl and injected intraperitoneally. The dose was 30 mg./kg. body wt., containing 10–35 μC of radioactivity. The other radioactive compounds were given in doses containing the same amount of radioactivity after solution in 0.9% (w/v) NaCl without the addition of non-radioactive carrier. Specific activities were: valine, 1 mc/m-mole; formate 2 mc/m-mole; methionine, 5 mc/m-mole; and leucine 6 mc/m-mole. The rats were kept during the experimental period in a cage placed in a fume cupboard. At 5 hr. after injection of the isotopic material, the rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (Veterinary Nembutal, Abbott), and blood was withdrawn

from the abdominal aorta into a syringe moistened with heparin solution. In most experiments the liver, kidneys, spleen and pancreas were then rapidly dissected out and immediately frozen by immersion in liquid nitrogen.

Preparation of nucleic acid, protein, lipid and acid-soluble fractions

Ribonucleic acid. RNA was prepared by a slight modification (K. S. Kirby, personal communication) of the phenol method of Kirby (1956). The tissues were disintegrated in 0.5% (w/v) naphthalene-1,5-disulphonic acid (disodium salt, pH 6.5), followed by phenol treatment and polysaccharide removal, but dialysis was omitted and the RNA was precipitated from the methylCellosolve-phosphate buffer mixture by the addition of 10 vol. acetic acid. The RNA precipitate was washed with 80% acetic acid, ethanol and ether, twice. In some experiments sodium nucleate was extracted according to Davidson & Smellie (1952), and the RNA nucleotides were separated from the DNA by the modification of the Schmidt & Thannhauser (1945) procedure described previously (Magee & Hultin, 1962). RNA was determined by the method of Webb (1956).

Deoxyribonucleic acid. DNA was prepared essentially by the phenolphthalein diphosphate method of Kirby (1961). After removal of polysaccharide material, the dialysis step was omitted and the DNA was precipitated by adding an equal volume of ethylCellosolve. This precipitate contained a large amount of buffer salts which were removed by repeated solution in water, addition of sodium acetate to a final concentration of 2% and precipitation by 1 vol. of ethylCellosolve. The white fibrous product was washed with ethanol and ether and dried over phosphorus pentoxide *in vacuo*.

Protein. Proteins were isolated by a slight modification of the conventional Schneider (1945) procedure. The tissues were disintegrated in 5% (w/v) trichloroacetic acid containing non-radioactive carrier (10 mg./ml.). After extraction with cold and hot trichloroacetic acid, the tissue residues were washed with water, acetone, ethanol, chloroform-methanol (2:1, v/v), chloroform-methanol-conc. hydrochloric acid (200:100:1, by vol.) (Huggins & Cohn, 1959), ethanol-ether (3:1, v/v) twice and ether twice.

Lipid. The lipid fraction was obtained by evaporation of the pooled lipid extracts to dryness at 60° in an atmosphere of nitrogen, solution of the residue in chloroform-methanol (2:1, v/v) and purification according to Folch, Ascoli, Lees, Meath & LeBaron (1951). The product was dissolved in chloroform and the solution was evaporated to dryness under nitrogen.

Acid-soluble fraction. This was taken as the supernatant obtained by centrifuging the first tissue suspension in 5% trichloroacetic acid. The concentration of [^{14}C]dimethylnitrosamine in this fraction was reduced to negligible levels by repeated extraction with chloroform containing excess of non-radioactive carrier dimethylnitrosamine. Remaining trichloroacetic acid was removed by repeated extraction with ether.

Hydrolysis and chromatography of nucleic acids

Acid hydrolysis. The RNA and DNA preparations were hydrolysed according to Vischer & Chargaff (1948) by heating with *N*-HCl for 1 hr. at 100° . Under these condi-

tions the purine bases and pyrimidine nucleotides are liberated. The acid-soluble fraction of liver was hydrolysed in the same way. Ion-exchange chromatography of the acid hydrolysates was carried out essentially by the method of Cohn (1949) as modified by Weissmann, Bromberg & Gutman (1957). Dowex 50 cation-exchange resin (H^+ form) was used with gradient elution by hydrochloric acid, increasing in concentration from 1 to 4N. For smaller amounts of nucleic acid (up to 50 mg.), the column was 1 cm. $\times$ 10 cm. For larger amounts of nucleic acid (up to 500 mg.) the column was 2 cm. $\times$ 25 cm. The effluent fractions were collected automatically, 10 and 25 ml./hr. from the smaller and larger columns respectively. The extinction of each fraction was measured at 260 m μ against water. The guanine and adenine peaks were identified by their ultraviolet-absorption spectra in each experiment.

Alkaline hydrolysis. This was carried out as described previously (Magee & Hultin, 1962).

Paper chromatography. The products of hydrolysis of nucleic acids were separated on Whatman 3MM paper using the propan-2-ol-ammonia solvent of Markham & Smith (1952) or the propan-2-ol-hydrochloric acid solvent of Wyatt (1951). Ultraviolet-absorbing areas were detected according to Markham & Smith (1949), marked in pencil and eluted with water. Similar areas of paper were cut out and eluted as blanks against which the ultraviolet absorption of the eluates was read at different pH values according to Weissmann *et al.* (1957).

Spectrophotometry and radioactivity measurements

Ultraviolet spectrophotometry was carried out with a Unicam SP. 500 instrument. Solid and liquid samples were assayed for radioactivity as described previously (Magee & Hultin, 1962). Radioautographs were made by exposure of paper chromatograms to Ilford X-ray film.

RESULTS

Preliminary experiments showed that both the protein and the RNA of rat liver became labelled after the animals had been given [^{14}C]dimethylnitrosamine by intraperitoneal injection. As with the system *in vitro* (Magee & Hultin, 1962), the specific radioactivity of the RNA was considerably higher than that of the protein. The ratio of the radioactivity of RNA to that of protein in rats treated with [^{14}C]dimethylnitrosamine was then compared with that in animals receiving various normal metabolites, also labelled (Table 1). Only with dimethylnitrosamine did this ratio exceed unity. The precursors of biological methyl groups, methionine and formate, showed higher ratios than the typical amino acids leucine and valine. The radioactivity of the proteins and RNA of rat liver, kidney, spleen and pancreas was then measured in rats killed 5 hr. after injection of [^{14}C]dimethylnitrosamine (Table 2). The high radioactivity in the liver RNA is in contrast with the lower activity in the kidney and the almost negligible value in the pancreas. This very low activity of pancreatic RNA indicated that the washing and purification pro-

cedures were probably adequate to remove radioactivity not chemically combined or otherwise very firmly bound to the nucleic acid. The high radioactivity of the splenic RNA was unexpected but its probable significance became apparent later (Fig. 7).

The greater specific radioactivity of the RNA compared with that of the protein from the livers of the rats given [^{14}C]dimethylnitrosamine was in agreement with results with rat-liver slices *in vitro* (Magee & Hultin, 1962). Alkaline hydrolysis and ion-exchange chromatography of this RNA gave the elution pattern shown in Fig. 1. Nearly all of the radioactivity appeared just before guanylic acid and merged into it. As with the similar peak of radioactivity obtained *in vitro* (Magee & Hultin, 1962), this radioactivity could be well separated from the guanylic acid by paper chromatography with the propan-2-ol-ammonia solvent system (Markham & Smith, 1952). It appeared therefore that the major radioactive compound *in vivo* was the same as that *in vitro*. In contrast with the results *in vitro*, the bases guanine and adenine were almost unlabelled in the experiments *in vivo*.

Table 1. Incorporation of radioactivity into RNA and proteins of liver in rats treated with [^{14}C]dimethylnitrosamine and with some labelled normal metabolites

All rats received labelled compound containing the same amount of radioactivity (35 μ C) by intraperitoneal injection and were killed 5 hr. later. The no. of rats is given in parentheses.

Labelled compound	Specific radioactivity of RNA/specific radioactivity of proteins
[^{14}C]Dimethylnitrosamine (3)	5.3
L-[Me- ^{14}C]Methionine (3)	0.40
Sodium [^{14}C]formate (4)	0.32
L-[^{14}C]leucine (1)	0.046
DL-[^{14}C]valine (1)	0.040

Table 2. Distribution of radioactivity in RNA and proteins of some organs of rats treated with [^{14}C]dimethylnitrosamine

Female rats (4), of about 200 g. body wt., received [^{14}C]dimethylnitrosamine (30 mg./kg. body wt., 17.5 μ C per rat) and were killed 5 hr. later. Specific radioactivities of proteins and RNA from pooled organs were measured at 'infinite thickness'.

Organ	Specific radioactivity of protein (counts/min./cm. ²)	Specific radioactivity of RNA (counts/min./cm. ²)
Liver	215	1063
Kidney	183	279
Pancreas	535	14
Spleen	106	336

After acid hydrolysis of the RNA under conditions yielding free purine bases and pyrimidine nucleotides (Vischer & Chargaff, 1948), ion-exchange chromatography again gave one major peak of radioactivity, now appearing between guanine and adenine (Fig. 2), and very much smaller peaks in the regions of the pyrimidine nucleotides and adenine respectively. The main radioactive peak corresponded exactly with a very small peak of ultraviolet-absorbing material which had the spectrum of a guanine derivative and, from the work of Lawley & Wallick (1957) and Reiner & Zamenhof (1957), seemed likely to be 7-methylguanine. This material was transferred to paper for chromatography with the propan-2-ol-ammonia solvent and comparison with an authentic sample of 7-methylguanine. The unknown and the authentic materials had nearly the same R_f values (0.28 and 0.26 respectively), and the ultraviolet-absorbing band of the unknown corresponded exactly with the radioactive band as shown by radioautography. 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. 3. The correspondence between the authentic and the unknown is very close and similar results were obtained at pH 6. The ultraviolet-absorption data also agree well with those

given for 7-methylguanine by Weissmann *et al.* (1957). On paper chromatography with the propan-2-ol-hydrochloric acid solvent (Wyatt, 1951), the unknown material had the same R_f as authentic 7-methylguanine, and could be distinguished from

Fig. 2. Ion-exchange chromatography of acid hydrolysate of RNA (30 mg.) from liver of rat treated with [^{14}C]dimethylnitrosamine (30 mg./kg. body wt., 35 μC) 5 hr. earlier. Dowex 50 (H^+ form) column (1 cm. $\times$ 10 cm.) with gradient elution from 1-4 N-HCl. $\bullet$, $E_{1\text{ cm.}}$ at 260 $\text{m}\mu$; $\circ$, radioactivity in counts/min.; G, guanine; A, adenine.

Fig. 1. Ion-exchange chromatography of alkaline hydrolysate of RNA from liver of rat treated with [^{14}C]dimethylnitrosamine (30 mg./kg. body wt., 20 μC) 5 hr. earlier. Dowex 1 (formate form) column (1.5 cm. $\times$ 21 cm.) with gradient elution from 1-4 N-formic acid. $\bullet$, $E_{1\text{ cm.}}$ at 260 $\text{m}\mu$; $\circ$, radioactivity in counts/min.

Fig. 3. 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 [^{14}C]dimethylnitrosamine (Fig. 2). $\bullet$, pH 2; $\circ$, pH 9.

1-methylguanine, 6-hydroxy-2-methylaminopurine, 2-dimethylamino-6-hydroxypurine and 2-amino-6-methoxypurine, which were chromatographed simultaneously on the same paper.

It appeared, therefore, that the unknown major radioactive compound was 7-methylguanine. This was confirmed by conversion of the unknown material into a xanthine derivative by treatment with nitrous acid. Ten rats were injected with non-radioactive dimethylnitrosamine (30 mg./kg. body wt.) in the usual way and RNA was prepared from their combined livers. Acid hydrolysis was followed by ion-exchange chromatography on the larger column and the material appearing between guanine and adenine was collected and added to the acid hydrolysate of the RNA obtained from the liver of one rat treated with [^{14}C]dimethylnitrosamine in the usual way. The combined materials were then applied to the smaller ion-exchange column and eluted as before. The pattern of elution was similar to the previous one (Fig. 2) but there was considerably more ultraviolet-absorbing material corresponding to the main radioactive peak which was derived from the livers of the ten rats treated with non-radioactive dimethylnitrosamine. These fractions were evaporated to dryness and the pooled material was treated with nitrous acid according to Weissman *et al.* (1957). After removal of the nitrous acid, small amounts of guanine and adenine were added as markers and the mixture was rechromatographed on the small column. The radioactivity now emerged considerably earlier, in the expected position of 7-methylxanthine, well before guanine, and was accompanied by an ultraviolet-absorbing peak (Fig. 4). There was now no radioactivity or ultraviolet absorption between guanine and adenine. This material was applied to paper and found to have nearly the same R_f (0.41) as a simultaneously chromatographed authentic sample of 7-methylxanthine (0.40). The unknown ultraviolet-absorbing band again corresponded exactly with the band of radioactivity shown by radioautography. Elution of the unknown and authentic materials and comparison of their ultraviolet spectra showed excellent correspondence between the curves, and the spectral data agreed well with those for 7-methylxanthine given by Weissmann *et al.* (1957). It is concluded that treatment of rats with dimethylnitrosamine under these conditions is followed by the appearance of 7-methylguanine in the RNA of the liver. The amount of 7-methylguanine present corresponds to about 2% of the guanine.

The presence of 7-methylguanine in RNA could be explained by incorporation of an appropriately methylated precursor, by methylation of the polynucleotides or by a combination of the two. If methylated precursors such as nucleosides or

nucleotides were involved these should be detectable in the acid-soluble fraction of the liver. This fraction from the liver of a rat was freed from [^{14}C]dimethylnitrosamine and trichloroacetic acid by repeated extraction with chloroform followed by ether. The whole fraction was then hydrolysed with *N*-hydrochloric acid so that any nucleotide or nucleoside methylated on the 7-position of guanine would be broken down to give 7-methylguanine. The resulting mixture was chromatographed after the addition of small amounts of guanine and adenine as markers. The total amount of radioactivity was not high and several small radioactive peaks appeared as elution proceeded, but there was no significant radioactivity in the expected region of 7-methylguanine between guanine and adenine (Fig. 5).

The labelled RNA from several kidneys was combined and hydrolysed with acid followed by column chromatography. The elution pattern (Fig. 6) was similar to that obtained with liver RNA (Fig. 2) except that the total radioactivity was very much less (Table 2) and the proportion in guanine and adenine was higher. The main radioactive peak occurred in the expected position of 7-methylguanine but insufficient material was present for determination of the ultraviolet-absorption spectrum.

Ion-exchange chromatography of pancreatic RNA, whose total radioactivity was much lower than that of kidney (Table 2), showed only a trace of radioactivity between guanine and adenine and very little activity in these bases.

Fig. 4. Rechromatography of radioactive material appearing between guanine and adenine after it had been treated with nitrous acid (see text). Conditions and symbols as in Fig. 2, with guanine (G) and adenine (A) (500 μg . each) added as markers.

Fig. 5. Ion-exchange chromatography of the acid-soluble fraction of liver from a rat treated with $[^{14}\text{C}]$ dimethylnitrosamine 5 hr. earlier. The fraction was hydrolysed with N-HCl and adenine and guanine markers (500 μg . each) were added before application to the column. Conditions of chromatography and symbols as in Fig. 2.

Fig. 6. Ion-exchange chromatography of pooled RNA from kidneys of rats treated with $[^{14}\text{C}]$ dimethylnitrosamine (30 mg./kg. body wt.) 5 hr. earlier. Conditions and symbols as in Fig. 2.

The RNA prepared from spleen was more radioactive than that from kidney or pancreas (Table 2) and its pattern of elution after acid hydrolysis and chromatography is shown in Fig. 7. Here almost all of the radioactivity corresponds exactly with the major normally occurring bases and there is again only a trace in the expected position of 7-methylguanine. A probable explanation of this finding is that the bases in the splenic RNA had been labelled by normal metabolic processes via the C_1 metabolic pool. Support for this was obtained by examination of RNA prepared from spleens of rats treated with sodium $[^{14}\text{C}]$ formate. After acid hydrolysis and chromatography, the elution pattern was very similar to that obtained after treatment with $[^{14}\text{C}]$ dimethylnitrosamine (Fig. 7) except that there was no detectable radioactivity at all in the position of 7-methylguanine.

The finding of 7-methylguanine in the RNA of rat liver suggested that it might also be present in the DNA. DNA, RNA and protein were isolated from the pooled livers of four rats which had each received 14 μC of $[^{14}\text{C}]$ dimethylnitrosamine 5 hr. before being killed. The specific radioactivities of the RNA and protein were 810 and 160 counts/min./cm.² respectively and that of the DNA was 239 counts/min./cm.² before removal of polysac-

Fig. 7. Ion-exchange chromatography of pooled RNA from spleens of rats treated with $[^{14}\text{C}]$ dimethylnitrosamine 5 hr. earlier. Dose and conditions as in Fig. 6.

charide material. This DNA was hydrolysed with *N*-hydrochloric acid and chromatographed on Dowex 50 (H^+ form) in the same way as the RNA. The elution pattern shown in Fig. 8, where there is a large single peak of radioactivity in the expected region of 7-methylguanine. The total radioactivity in this peak is 411 counts/min. It can be calculated from Fig. 2 that this amount of radioactivity could have been due to the presence of 13 mg. of RNA in the DNA sample. According to Kirby (1961), however, DNA prepared by the phenolphthalein diphosphate method is free from detectable RNA, and moreover determinations of RNA in our DNA samples indicate that not more than 1 mg. of RNA could have been present, and probably less. It appears, therefore, that the liver DNA like the RNA contains 7-methylguanine. DNA was also prepared from the kidneys of two rats which had received $20 \mu C$ of $[^{14}C]$ dimethylnitrosamine 5 hr. earlier. It had low but definite radioactivity (30 counts/min./cm.²) and contained no detectable RNA.

The total lipid material obtained from about 2 g. of liver pooled from four rats killed 5 hr. after receiving $[^{14}C]$ dimethylnitrosamine was assayed for radioactivity after evaporation on a metal planchet. It was found to have 50 counts/min., indi-

cating that the lipid, like the proteins and nucleic acids, contains radioactivity incorporated *in vivo* from dimethylnitrosamine.

DISCUSSION

The results of this investigation clearly show that the hepatotoxin and carcinogen dimethylnitrosamine is capable of methylating both RNA and DNA *in vivo* to produce the abnormal guanine derivative, 7-methylguanine. Although insufficient material was available to enable the identification of this compound by all the classical chemical procedures, the evidence presented leaves very little doubt about its nature. The position of the abnormal guanine in the column and paper chromatograms, its absorption spectrum virtually identical with that of an authentic sample of 7-methylguanine, and its conversion into 7-methylxanthine on deamination all strongly favour its identity with 7-methylguanine.

This alkylated guanine is the only unusual compound found in more than minute amounts in the nucleic acids labelled with dimethylnitrosamine. The other components labelled were guanine and adenine, no doubt due to the incorporation into the purine skeleton of a C_1 fragment derived from dimethylnitrosamine. These findings are chemically analogous to those observed in the protein labelled with dimethylnitrosamine (Magee & Hultin, 1962) where, apart from probable carboxyl methyl esters, histidines methylated in the 1- and 3-positions of the imidazole ring were among the major abnormal components found. It appears, therefore, that there is a special predilection of the methylating agent derived from dimethylnitrosamine for the imidazole ring of histidine or guanine. The similarity of the reaction with nucleic acids *in vivo* with that of alkylating agents *in vitro* (Timmis, 1960) is very striking. The methylation of guanine in liver RNA is considerably more active than is that of histidine in protein.

As discussed in the preceding paper, it was suggested by Schoental (personal communication) and by Rose (1958) that diazomethane may be the methylating agent derived from dimethylnitrosamine. A possible scheme for the metabolism of dimethylnitrosamine by rat liver incorporating this suggestion and based on previous work (Heath & Dutton, 1958; Brouwers & Emmelot, 1960; Magee & Hultin, 1962) and on the present findings is shown in Fig. 9. It should be emphasized that, in this scheme, the initial oxidative *N*-demethylation of dimethylnitrosamine is enzymic. Whether diazomethane is actually formed or whether methylation occurs by some other mechanism is not yet established. The recent demonstration of the existence of the very unstable monomethylnitros-

Fig. 8. Ion-exchange chromatography of pooled DNA from rats treated with $[^{14}C]$ dimethylnitrosamine (30 mg./kg. body wt., $14 \mu C$) 5 hr. earlier. Conditions and symbols as in Fig. 2.

amine and its decomposition to diazomethane and water (Müller, Haiss & Rundel, 1960) supports the concept of the formation of the diazoalkane. On the other hand, Austin (1960) maintains that breakdown of the monomethylnitrosamine may lead to the formation of a carbonium ion, which may be responsible for methylation. Recent work on the relation of chemical structure to biological activity by Druckrey, Preussmann, Schmähl & Müller (1961) and by D. F. Heath (personal communication) is in agreement with the alkylation hypothesis. Heath has shown that *n*-butylmethylnitrosamine induces acute changes in rat liver similar to those induced by dimethylnitrosamine, but that *tert.*-butylmethylnitrosamine, which would not be expected to yield a diazoalkane metabolically, does not.

The occurrence of 7-methylguanine in liver RNA could be explained by chemical methylation of the intact RNA molecule or by incorporation of a methylated precursor. The second possibility is made unlikely by the absence of any radioactive material corresponding to 7-methylguanine in the acid-soluble fraction of the liver after hydrolysis (Fig. 5). The incorporation of a nucleoside or nucleotide methylated in the 7-position is also unlikely because the N-7 atom must become quaternary with the formation of the ribosyl bond on the N-9 atom. In addition to making the mole-

cule unstable, such a change might alter the character of the molecule as a substrate for the enzyme systems involved in the incorporation of precursors into RNA.

The pattern of incorporation of ^{14}C into RNA and protein in the different tissues (Table 2) is interesting in relation to the pathological changes induced by dimethylnitrosamine. At the dose level given the liver is acutely damaged with severe haemorrhagic necrosis (Barnes & Magee, 1954) though severe damage in the kidney, pancreas and spleen is minimal or absent. In some of the rats, however, tumours of the kidney would be expected to appear after about a year (Magee & Barnes, 1959, 1962). There is a clear correlation between pathological action and incorporation into RNA of liver, kidney and pancreas but with spleen it only becomes apparent when it is seen that nearly all of the radioactivity of the RNA is in the commonly occurring bases (Fig. 7). There is no correlation between the radioactivity of the tissue proteins and the distribution of the pathological lesions, but one might become apparent if the relative incorporation into normal and into abnormal protein constituents were known. The high figure in the pancreatic protein probably reflects the very rapid rate of protein synthesis in the pancreas. The radioactivity of the liver protein is rather low in relation to the other tissues, which may be explained by the

Fig. 9. Some postulated metabolic pathways of dimethylnitrosamine.

inhibitory effect of dimethylnitrosamine on amino acid incorporation into the liver (Magee, 1958; Hultin, Arrhenius, Löw & Magee, 1960).

Although insufficient material was available for ultraviolet spectroscopy, the position of the radioactive material in the chromatographic profile of the kidney RNA (Fig. 6), coupled with earlier evidence that dimethylnitrosamine is metabolized to a small extent in the kidney (Magee & Vandekar, 1958), support the conclusion that methylation similar to that in the liver has taken place, though to a much smaller degree. Similarities of the chromatographic profiles of the splenic RNA after administration of [^{14}C]dimethylnitrosamine (Fig. 7) and of [^{14}C]formate respectively are quite consistent with the predicted incorporation from the C_1 metabolic pool of a derivative of dimethylnitrosamine. The very low total radioactivity of pancreatic RNA is also consistent with this (Table 2). The traces of radioactivity in the 7-methylguanine position of the chromatograms of the splenic and pancreatic RNA emphasize the probable necessity of metabolic activation of dimethylnitrosamine before methylation of cell constituents can occur. Even though the compound is distributed in about the same concentration in all the viscera (Magee, 1956), the spleen and pancreas have a negligible capacity to metabolize dimethylnitrosamine *in vitro* (Magee & Vandekar, 1958).

The methylation of DNA in the liver (Fig. 8) seems reasonably well established, since the largest amount of possible RNA contaminant would not have been enough to account for the observed radioactivity unless this RNA was derived from a fraction with very high specific radioactivity. The same probably applies to the kidney DNA, which contained no detectable RNA. However, since the specific radioactivity was low, no firm conclusions on this matter can be drawn.

The possible importance of the methylation of RNA and DNA in carcinogenesis deserves brief consideration. The somatic-mutation hypothesis of the origin of cancer was introduced by Boveri (1914); the frequent occurrence of mitotic abnormalities in tumours led to the idea that neoplasia might originate in cells produced by such abnormal divisions. More recently, it has been suggested that changes in the molecule of DNA, not necessarily accompanied by chromosome changes, may be responsible for the production of mutations and for carcinogenesis (Boyland, 1954; Haddow, 1958). Although this concept has been supported by much work on reactions of carcinogens and mutagens with DNA *in vitro* (Boyland, 1954), evidence of any combination between a carcinogen and DNA occurring *in vivo* has appeared only very recently. Brookes & Lawley (1960) demonstrated alkylation of DNA in Ehrlich ascites-tumour cells by sulphur

mustard but the mustards, although very cytotoxic, are not highly carcinogenic. In a preliminary communication (Farber & Magee, 1960) we reported the probable ethylation of the RNA of liver in rats treated with the hepatic carcinogen [^{14}C]ethionine as well as the methylation of RNA by dimethylnitrosamine. This work with ethionine has been confirmed and extended by Stekol, Mody & Perry (1960) who also reported binding to DNA. The methylation of DNA in rat liver and probably also in kidney which has been demonstrated in the present paper may be relevant to the induction of cancer. As Lawley (1957) has pointed out, alkylation of DNA on the 7-position of guanine leads to instability with the alkylated base splitting out of the molecule. This might lead to breakage of the chain as with the apurinic acids (Brown & Todd, 1955). If the mechanism of carcinogenesis is similar to that of mutation it may also be relevant that Tessman (1959) has demonstrated the production of mutants of the $\phi \times 174$ bacteriophage following the alteration of only one base of the DNA by treatment of the phage with nitrous acid, which may also be a metabolite of dimethylnitrosamine (Heath & Dutton, 1958).

The suggestion that cancer may arise as an extrachromosomal event was made by Lewis (1935) and has received support from Haddow (1944, 1947), Darlington (1948) and Kaplan (1959). The possibility that RNA may be involved in this process is supported by the discovery of infective viral RNA (Fraenkel-Conrat, 1956; Gierer & Schramm, 1956), and by the finding that the nucleic acid of some tumour viruses is RNA (Epstein & Holt, 1958; Bonar & Beard, 1959). The methylation of liver and kidney RNA in rats treated with dimethylnitrosamine suggests the possibility that alterations in this nucleic acid may play a part in the induction of cancer by this compound.

Hems (1958) showed that the 7-position of the imidazole ring of guanosine is particularly vulnerable to ionizing radiation with subsequent ring opening and formation of a 5-formamidopyrimidine derivative. It is probable that a similar pyrimidine derivative is formed on alkaline hydrolysis of rat-liver RNA prepared from rats treated with dimethylnitrosamine (Fig. 1). It seems, therefore, that essentially similar changes in the cellular nucleic acids may occur in radiation injury and in chemical injury by dimethylnitrosamine, and also in viral mutagenesis.

The occurrence of 7-methylguanine in human urine has been known since the work of Krüger & Salomon (1898) and has been confirmed recently by Weissmann *et al.* (1957) and by Wyngaarden, Blair & Hilley (1958). Its presence in dog urine has also been reported (Jackson & Entenman, 1957). The origin of the urinary compound is still not clear but

presumably it is derived from the blood passing through the kidney. It also appears that it can occur naturally in the RNA of *Escherichia coli* (D. B. Dunn, personal communication). It is possible, therefore, that minute quantities of 7-methylguanine may be formed in human nucleic acids by methylating agents in our environment and this, perhaps, might play a part in the induction of cancer in man.

SUMMARY

1. Rats were injected intraperitoneally with the hepatotoxin and carcinogen dimethylnitrosamine labelled with radioactive carbon. The dose was adequate to cause acute necrosis of the liver in all the rats and to induce tumours of the kidney in some of the survivors about a year later. The animals were killed 5 hr. after injection when the liver injury was not far advanced.

2. Incorporation of radioactivity into RNA and protein of liver, kidney, spleen and pancreas was observed and compared with that following similar intraperitoneal injection of labelled methionine, leucine, valine and formate. Incorporation from dimethylnitrosamine occurred into DNA of liver and probably also of kidney. The lipid fraction of liver became labelled.

3. Alkaline hydrolysis of the labelled liver RNA followed by ion-exchange chromatography revealed nearly all of the radioactivity in a peak close to, but distinct from, guanylic acid. This material could be separated from guanylic acid by paper chromatography.

4. Acid hydrolysis of the labelled liver RNA, followed by ion-exchange chromatography, revealed almost all the radioactivity appearing between, and separate from, guanine and adenine, with a corresponding small peak of ultraviolet absorption. This material was identified as 7-methylguanine from its behaviour on ion-exchange and paper chromatography and from its ultraviolet-absorption spectrum. Treatment of this material with nitrous acid gave a product which was identified as 7-methylxanthine by the same criteria.

5. No evidence of 7-methylguanine was found in acid hydrolysates of the acid-soluble fraction of liver from rats treated with radioactive dimethylnitrosamine.

6. Radioactive material in the expected position of 7-methylguanine was found on ion-exchange chromatography of acid hydrolysates of kidney RNA and liver DNA but insufficient material was present for ultraviolet-absorption spectra. Traces only of radioactivity in the position of 7-methylguanine were found in hydrolysates of pancreas and spleen RNA. The latter showed considerable in-

corporation into guanine and adenine. The chromatographic profile of splenic RNA from rats treated with radioactive formate was very similar to that with dimethylnitrosamine but there was no measurable radioactivity in the position of 7-methylguanine.

7. These results are considered to support the hypothesis that an active methylating agent is formed metabolically from dimethylnitrosamine which methylates tissue components *in vivo*. This hypothesis is discussed in relation to current ideas on carcinogenesis, viral mutagenesis and cell injury by alkylating agents and radiation.

We would like to thank Dr D. F. Heath for the [^{14}C]-dimethylnitrosamine and Dr G. H. Hitchings for the methylated purines. We are grateful to Dr G. M. Timmis, Dr Elliot Shaw and many others for helpful discussion, and to Mr J. A. E. Jarvis and Mr R. Price for technical assistance. This work was supported in part by grants from the American Cancer Society and the U.S. Public Health Service (A 610).

REFERENCES

- Austin, A. T. (1960). *Nature, Lond.*, **188**, 1086.
- Barnes, J. M. & Magee, P. N. (1954). *Brit. J. industr. Med.* **11**, 167.
- Bonar, R. A. & Beard, J. W. (1959). *J. nat. Cancer Inst.* **23**, 183.
- Boveri, T. H. (1914). *Zur Frage der Entstehung maligner Tumoren*. Yena: Gustav Fisher.
- Boyland, E. (1954). *Pharmacol. Rev.* **6**, 345.
- Brookes, P. & Lawley, P. D. (1960). *Biochem. J.* **77**, 478.
- Brouwers, J. A. J. & Emmelot, P. (1960). *Exp. Cell Res.* **19**, 467.
- Brown, D. M. & Todd, A. R. (1955). In *The Nucleic Acids*, vol. 1, p. 409. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Bruce, H. M. & Parkes, A. S. (1956). *J. Anim. Tech. Ass.* **7**, 54.
- Cohn, W. E. (1949). *Science*, **109**, 377.
- Darlington, C. D. (1948). *Brit. J. Cancer*, **2**, 118.
- Davidson, J. N. & Smellie, R. M. S. (1952). *Biochem. J.* **52**, 594.
- Druckrey, H., Preussmann, R., Schmähel, D. & Müller, M. (1961). *Naturwissenschaften*, **48**, 134.
- Dutton, A. & Heath, D. F. (1956). *J. chem. Soc.* p. 1892.
- Epstein, M. A. & Holt, S. J. (1958). *Brit. J. Cancer*, **12**, 363.
- Farber, E. & Magee, P. N. (1960). *Biochem. J.* **76**, 58p.
- Folch, J., Ascoli, I., Lees, M., Meath, J. A. & LeBaron, F. N. (1951). *J. biol. Chem.* **191**, 833.
- Fraenkel-Conrat, H. (1956). *J. Amer. chem. Soc.* **78**, 882.
- Gierer, A. & Schramm, G. (1956). *Nature, Lond.*, **177**, 702.
- Haddow, A. (1944). *Nature, Lond.*, **154**, 194.
- Haddow, A. (1947). *Brit. med. Bull.* **4**, 331.
- Haddow, A. (1958). *Brit. med. Bull.* **14**, 79.
- Heath, D. F. & Dutton, A. (1958). *Biochem. J.* **70**, 619.
- Hems, G. (1958). *Nature, Lond.*, **181**, 1721.
- Huggins, C. G. & Cohn, D. V. (1959). *J. biol. Chem.* **234**, 257.
- Hultin, T., Arrhenius, E., Löw, H. & Magee, P. N. (1960). *Biochem. J.* **76**, 109.

- Jackson, K. & Entenman, C. (1957). *Amer. J. Physiol.* **189**, 315.
- Kaplan, H. S. (1959). *Cancer Res.* **19**, 791.
- Kirby, K. S. (1956). *Biochem. J.* **64**, 405.
- Kirby, K. S. (1961). *Biochim. biophys. Acta*, **47**, 18.
- Krüger, M. & Salomon, G. (1898). *Hoppe-Seyl. Z.* **24**, 350.
- Lawley, P. D. (1957). *Proc. chem. Soc., Lond.*, p. 290.
- Lawley, P. D. & Wallick, C. A. (1957). *Chem. & Ind.* p. 633.
- Lewis, W. H. (1935). *Science*, **81**, 545.
- Magee, P. N. (1956). *Biochem. J.* **64**, 676.
- Magee, P. N. (1958). *Biochem. J.* **70**, 606.
- Magee, P. N. & Barnes, J. M. (1959). *Acta Un. int. Cancr.* **15**, 187.
- Magee, P. N. & Barnes, J. M. (1962). *J. Path. Bact.* (in the Press).
- Magee, P. N. & Hultin, T. (1962). *Biochem. J.* **83**, 106.
- Magee, P. N. & Vandekar, M. (1958). *Biochem. J.* **70**, 600.
- Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
- Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.
- Müller, E., Haiss, H. & Rundel, W. (1960). *Chem. Ber.* **93**, 1541.
- Reiner, B. & Zamenhof, S. (1957). *J. biol. Chem.* **228**, 475.
- Rose, F. L. (1958). In *Symposium on the Evaluation of Drug Toxicity*, p. 116. Ed. by Walpole, A. L. & Spinks, A. London: J. and A. Churchill Ltd.
- Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
- Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
- Stekol, J. A., Mody, U. & Perry, J. (1960). *J. biol. Chem.* **235**, PC 59.
- Tessman, I. (1959). *Virology*, **9**, 375.
- Timmis, G. M. (1960). *Biochem. Pharmacol.* **4**, 49.
- Vischer, E. & Chargaff, E. (1948). *J. biol. Chem.* **176**, 715.
- Webb, J. M. (1956). *J. biol. Chem.* **221**, 635.
- Weissman, B., Bromberg, P. A. & Gutman, A. B. (1957). *J. biol. Chem.* **224**, 407.
- Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.
- Wyngaarden, J. B., Blair, A. E. & Hilley, L. (1958). *J. clin. Invest.* **37**, 579.

Biochem. J. (1962) **83**, 124

Citrate-Promoted Helix Formation in Gelatin

THE VISCOSITY-TIME EFFECT

By A. COURTS*

The British Gelatine and Glue Research Association, 2a Dalmeny Avenue, London, N. 7

(Received 7 September 1961)

The changes which take place when native collagen is converted into the water-soluble protein, gelatin, are generally regarded as irreversible reactions. Veis, Anesey & Cohen (1958) describe the essential aspects as: (1) irreversible melting of the polypeptide helical coils; (2) irreversible severance of inter-peptide-chain bonds; (3) irreversible hydrolysis of peptide linkages. Courts (1958) showed that (3) involved only especially-labile peptide bonds and that (1) is likely to be the final stage, resulting from rupture of hydrogen bonds.

In special instances, however, evidence from the application of widely differing techniques shows that the random-coil structure of gelatin molecules appears partially to re-form a collagen-type configuration. Thus Robinson (1953) prepared gelatin films from a 5% solution by hot (55°) and cold (18°) evaporation. The 'cold form' of gelatin gave the characteristic infrared dichroism of collagen. Veis & Cohen (1960) obtained some unusual heat-precipitated gelatin fibres, which revealed in the electron microscope a banded structure with 610 Å spacings. Working with the heat-denatured soluble

fraction of rat-tail-tendon collagen, Flory & Weaver (1960) have demonstrated that a relatively slow transition from a random-coil to a helical configuration seems likely to account for the increases in viscosity in dilute solutions and in optical-rotation values obtained with this material.

Results similar to those of Flory & Weaver (1960) were obtained in the present work, and show that the reversion behaviour is likely to be a general reaction for gelatin. A number of typical gelatins, with a wide variety of values for weight-average molecular weight and rigidity modulus, have been studied as dilute solutions in citrate and other buffers. The increase in viscosity of these solutions with time is clearly enhanced by aliphatic carboxylic acids. Since molecular reorientation of the gelatin is a plausible explanation of this behaviour, the degree to which a gelatin can demonstrate the viscosity-time effect is viewed in relation to some other physical properties, which are governed by the size and shape of the molecule. To examine a possible relationship with molecular weight, it was necessary to fractionate a series of gelatins into units with considerably enhanced weight-average molecular-weight homogeneity. No such relation-

* Present address: Unilever Food Research Department, Colworth House, Sharnbrook, Bedford.