Biochem. J. (1968) **107**, 179
Printed in Great Britain

Occurrence of 7-Methylguanine in Nucleic Acids of Rat Liver

BY VALDA M. CRADDOCK, S. VILLA-TREVINO* AND P. N. MAGEE
Toxicology Research Unit, Medical Research Council Laboratories, Carshalton, Surrey

(*Received* 6 *October* 1967)

1. Microsomal and soluble RNA of rat liver have been studied by column and paper chromatography after administration of [*Me*-^{14}C]methionine; evidence was obtained for the occurrence of 7-methylguanine, the methyl group being derived from methionine. 2. No evidence was obtained for the occurrence of 7-methylguanine in DNA.

Though nine or more methylated bases are known to be minor components of RNA isolated from various sources, there is less information about the RNA of rat liver. Special interest in the methylated bases in this tissue arose during a study of the biochemical reactions of those carcinogens that have been shown to alkylate nucleic acids. Certain of these compounds, including dimethylnitrosamine and nitrosomethylurea, methylate rat liver nucleic acids to form predominantly 7-methylguanine, and it is conceivable that this reaction is involved in their carcinogenic actions (Magee & Barnes, 1967). To help assess this possibility, it was decided to test whether 7-methylguanine occurs in normal rat liver RNA or DNA. This methylated base is of special interest for the additional reason that it appears to have a turnover rate higher than that of other methylated purines in the intact animal (Mandel, Srinivasan & Borek, 1966).

The extensive work of Smith and Dunn had shown that a number of methylated bases are present in rat liver RNA (Dunn, 1959; Smith & Dunn, 1959*a*), including thymine (Price, Hinds & Brown, 1963), but 7-methylguanine had been shown to occur only in pig liver soluble RNA (Dunn, 1963) and in calf liver soluble RNA (D. B. Dunn, personal communication), although there was suggestive evidence for its occurrence in rat liver soluble RNA (Sluyser & Bosch, 1962). The occurrence of this base in DNA does not appear to have been reported. However, methylation of guanine in DNA in the 7-position is known to labilize the glycosidic bond, and the methylated base is fairly rapidly split out of the nucleic acid (Lawley & Wallick, 1957). It therefore seemed possible that 7-methylguanine might have a temporary existence in DNA and have escaped detection. Also, if it is present at a very low concentration, it would be advantageous to use isotopic methods for its investigation. The effect of methylation on the stability of RNA is in dispute (Kriek & Emmelot, 1963; Lawley & Brookes, 1963).

There is much evidence that the methylated bases occurring in nucleic acids *in vivo* are formed by enzymic methylation of the preformed polymer, rather than by incorporation of the methylated base itself, and that the reactions are catalysed by specific methylases that use *S*-adenosylmethionine as the methyl donor (Mandel & Borek, 1963; Borek & Srinivasan, 1966). An approach to the problem was therefore to administer [*Me*-^{14}C]methionine to rats, and after a suitable time to kill the animals, isolate the nucleic acids and analyse the radioactive bases present. A preliminary account of some of this work has been published (Villa-Trevino & Magee, 1966).

There is evidence (Bergquist & Matthews, 1962) that there is an increased proportion of methylated bases in nucleic acids of tumour tissues. The experiments described below are the first stage in a more detailed study of the effect of carcinogens and other toxic agents on the methylated bases of nucleic acids.

EXPERIMENTAL

Animals and materials

Wistar albino rats of the Porton strain were maintained on M.R.C. diet 41B (Bruce & Parkes, 1956). Female rats, 200g. body wt., were used. [*Me*-^{14}C]Methionine was purchased from The Radiochemical Centre, Amersham, Bucks.

Treatment of animals and separation of nucleic acids

Expt. 1. Two rats, starved overnight, were placed in metabolism units for the separate collection of urine and faeces. Each animal was given a series of five intraperitoneal injections of 20μc (0·1mg.) of [^{14}C]methionine, each in 1ml. of 0·9M-NaCl, at hourly intervals. One hour after the final injection the rats were killed by a blow on the head and

* Present address: Departamento Biologia Celular, Centro de Investigacion y de Estudios Avanzados del Instituto Politecnico Nacional, Apartado Postal 14–740, Mexico 14, D.F., Mexico.

decapitation, the livers removed and RNA and DNA isolated by a modification of the method of Kirby (1962) as suggested by Professor K. S. Kirby (personal communication). The tissue was homogenized in a solution of 0·5% naphthalene-1,5-disulphonic acid (sodium salt), containing 0·1ml. of 2·5M-K_2HPO_4 (pH 7·3)/100ml. of solution, adjusted to pH 6·5 with dilute H_3PO_4. The RNA was extracted with phenol–*m*-cresol–8-hydroxyquinoline–water (500:70:0·5:55, by wt.) mixture. DNA was freed of RNA by treatment with ribonuclease, and polysaccharide was removed from both nucleic acids by the methoxyethanol–phosphate procedure (Kirby, 1956). DNA and RNA were precipitated by addition of an equal volume of 1% cetyltrimethylammonium bromide, and after the precipitates had been washed the nucleic acids were converted into the sodium salts by treatment with 2% (w/v) sodium acetate in 70% (v/v) ethanol, and washed with ethanol, ethanol–ether (1:1, v/v) and ether.

Expt. 2. Two rats, starved overnight, were given a series of four intraperitoneal injections of [^{14}C]methionine at hourly intervals, the first of 40μc (0·2mg.) in 0·8ml. of 0·9M-NaCl, the remaining three injections of 20μc (0·1mg.) in 0·4ml. of 0·9M-NaCl. One hour after the final injection the rats were killed by a blow, the livers removed, and microsomal and pH 5 RNA (referred to below as 'soluble RNA') isolated by a modification of the method of Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958). The microsomal and soluble fractions were resuspended in 15ml. of solution *A* (0·25M-sucrose–4mM-$MgCl_2$–25mM-KCl–50mM-tris–HCl buffer, pH 7·6) and 15ml. of water, 0·3ml. of 10% sodium dodecyl sulphate solution and an equal volume of 90% phenol were added, and the mixture was shaken for 1hr. at 0–5°. After centrifugation at 17000*g* for 25min. the supernatant was aspirated and the phenol layer was washed with 0·25vol. of the solution *A*–water–sodium dodecyl sulphate solution and recentrifuged. To the pooled aqueous extracts were added 0·1vol. of 2M-potassium acetate and 2·5vol. of ethanol, and the mixtures were left overnight at −25°. The RNA precipitates that formed were dried in ethanol–ether and ether.

Analysis of nucleic acids

Expt. 1. Samples of liver RNA (32mg.) and DNA (28·5mg.) were hydrolysed with $HClO_4$ by the method of Wyatt (1952). The hydrolysates were diluted to 3ml. with water and centrifuged, carriers were added as appropriate, and the supernatant solutions were chromatographed on columns (1cm.×10cm.) of Dowex 50 at a flow rate of 12·5ml./hr., with N-HCl for the first 300ml. and then a linear gradient approaching 4N-HCl. The extinction at 260mμ was recorded.

Expt. 2. Microsomal RNA (*a*). To act as an internal marker for any 7[^{14}C]-methylguanine in the RNA, a sample of RNA containing 7[^{3}H]-methylguanine was added to the preparation. The tritiated material had been prepared previously from rats given [^{3}H]dimethylnitrosamine, and was known to contain more than 85% of the radioactivity as 7-methylguanine (Lee, Lijinsky & Magee, 1964). The microsomal RNA (14mg.) from the rat treated with [^{14}C]methionine was mixed with the tritium-labelled methylated RNA (0·7mg.), and the mixture was hydrolysed with 4ml. of N-HCl for 60min. at 100° and analysed on a column (20cm.×2cm.) of Dowex 50 at a flow rate of 25ml./hr. with an exponential gradient of 0·25–3N-HCl.

Microsomal RNA (*b*). A sample of microsomal RNA (14mg.) was mixed with total liver RNA (0·65mg.) prepared from a rat treated with [^{3}H]dimethylnitrosamine as described above. The mixture was hydrolysed with 0·3N-KOH (1ml.) for 18hr. at 37°, adjusted to pH 1 with $HClO_4$ and centrifuged, the precipitate was washed, and the first supernatant and washings were pooled, adjusted to pH 7 with N-KOH and analysed on a column (20cm.× 2cm.) of Dowex 1 (formate form). For the elution procedure, 250ml. of water was placed in the mixing chamber of the gradient device. In the reservoir were water (tubes 1–8), N-formic acid (tubes 9–28) and then 4N-formic acid.

Soluble RNA. The preparation (4·5mg.) was treated as for the acid hydrolysis of microsomal RNA (*a*) described above.

Determination of radioactivity

Samples of fractions from column chromatography were evaporated to dryness in a stream of air, and radioactivity was determined as described previously (Craddock & Magee, 1966), 9ml. of the 10ml. fractions being used in Expt. 1, and 5ml. of the 10ml. fractions in Expt. 2.

Paper chromatography

The remainders of the fractions from column chromatography were pooled, corresponding to peaks of radioactivity, and were concentrated by the use of a rotary evaporator. Paper chromatography was carried out in methanol–conc. HCl–water (7:2:1, by vol.), butan-1-ol–aq. NH_3 (sp.gr. 0·88)–water (85:2:13, by vol.) and propan-2-ol–water (7:3, v/v) with NH_3 in the gaseous phase (Markham & Smith, 1952). The chromatograms were cut into narrow strips crosswise, the pieces eluted with N-HCl and the eluates evaporated to dryness, and radioactivity was determined as described above.

Treatment of urine

The urine collected during Expt. 1 was fractionated as described by Craddock & Magee (1967) and the purine fraction analysed by column chromatography on a column (1cm.×10cm.) of Dowex 50 with a linear gradient of 1–4N-HCl.

RESULTS

The result of column chromatography of liver RNA, Expt. 1, is shown in Fig. 1. The compounds responsible for the extinction profile are uracil, xanthine, hypoxanthine, guanine, 7-methylguanine and adenine. The relative positions of the radioactive peaks, and the results of paper chromatography, are consistent with peak 1 being thymine, peak 2 5-methylcytosine, peak 3 N^2-methylguanine and N^2-dimethylguanine, peak 4 1-methylguanine, peak 5 7-methylguanine and peak 6 1-methyladenine. Some of the radioactivity in peaks 3 and 6 is, as expected, in guanine and adenine as a result of purine biosynthesis. The result of column chromatography of DNA is shown in Fig. 2. The radioactive peak 1 is presumably thymine and peak 2 is 5-methylcytosine.

The result of column chromatography of the

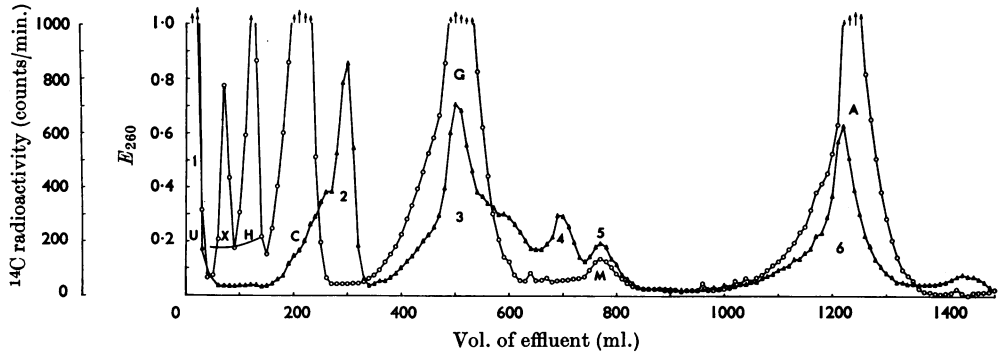

Fig. 1. Ion-exchange chromatography of an $HClO_4$ hydrolysate of total RNA of rat liver, after administration of a total of 100 μc of [^{14}C]methionine/rat. Details are given in the text. Carrier xanthine (X), hypoxanthine (H) and 7-methylguanine (M) were added. Other peaks shown by E_{260} readings are uracil (U), cytosine (C), guanine (G) and adenine (A). ○, E_{260}; △, radioactivity.

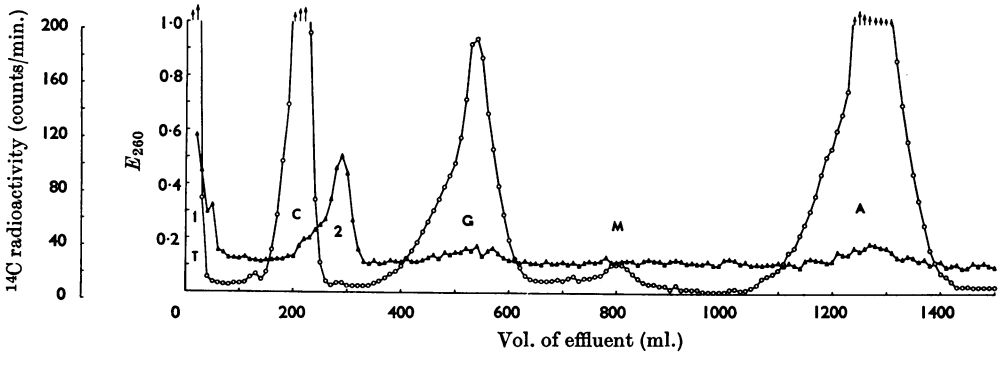

Fig. 2. Ion-exchange chromatography of an $HClO_4$ hydrolysate of rat liver DNA, after administration of a total of 100 μc of [^{14}C]methionine/rat. Details are given in the text. Carrier 7-methylguanine (M) was added. Other peaks shown by E_{260} readings are thymine (T), cytosine (C), guanine (G) and adenine (A). ○, E_{260}; △, radioactivity.

acid hydrolysates of microsomal RNA (Fig. 3) and soluble RNA (Fig. 4) shows a peak of ^{14}C radioactivity in the position of 7-methylguanine, the profile of which corresponds to the profile of 7[^{3}H]-methylguanine originating in the RNA added to act as a marker. Paper chromatography of samples from the pooled samples of this peak from the microsomal RNA shows that all the ^{14}C and ^{3}H radioactivity moves with the same R_F as authentic 7-methylguanine.

Column chromatography of the alkaline hydrolysate of microsomal RNA with added RNA from a rat treated with [^{3}H]dimethylnitrosamine is shown in Fig. 5. The ^{3}H radioactivity profile shows only one major peak, which is presumably 2,4-diamino-6-hydroxy-5-methylformamidopyrimidine, formed from 7-methylguanine in RNA by treatment with alkali (Haines, Rees & Todd, 1962). The ^{14}C radioactivity profile also shows a peak in this position. The increased number of radioactive peaks compared with the column chromatography of bases resulting from acid hydrolysis is possibly due to the presence of 2'[^{14}C]-*O*-methyl-ribonucleotides (Smith & Dunn, 1959*b*).

The urine analysis (Fig. 6) showed a peak of radioactivity in the position of 7-methylguanine. The initial high peak of radioactivity is presumably due to ^{14}C incorporated in the mixture of uric acid, xanthine, hypoxanthine and 1-methylhypoxanthine.

DISCUSSION

The experiments shown in Figs. 1–5 give evidence that rat liver RNA, including microsomal and soluble RNA, contains 7-methylguanine, although

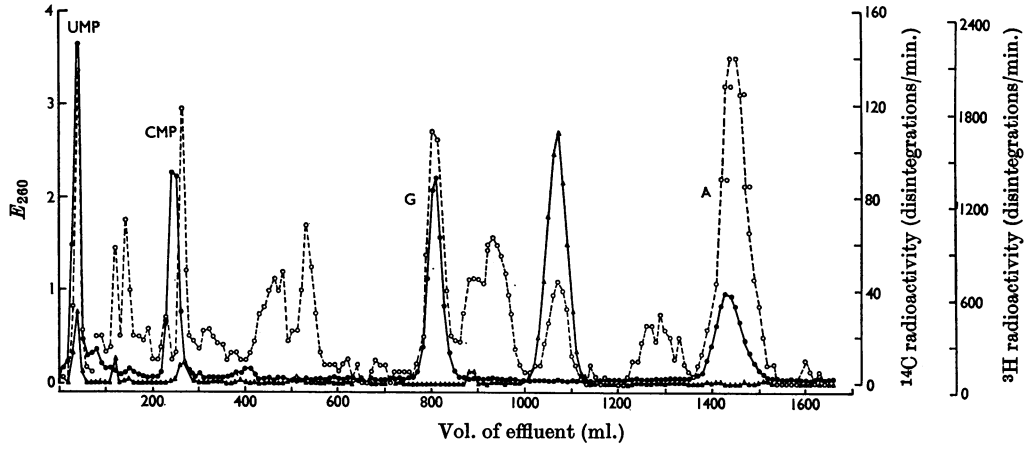

Fig. 3. Ion-exchange chromatography of an HCl hydrolysate of a mixture of liver microsomal RNA from a rat treated with [^{14}C]methionine and total liver RNA from animals treated with [H^{3}]dimethylnitrosamine, the latter having been added to act as a marker for 7-methylguanine. For details see the text. Peaks shown by E_{260} readings are UMP, CMP, guanine (G) and adenine (A). ●, E_{260}, ○, ^{14}C radioactivity; △, ^{3}H radioactivity.

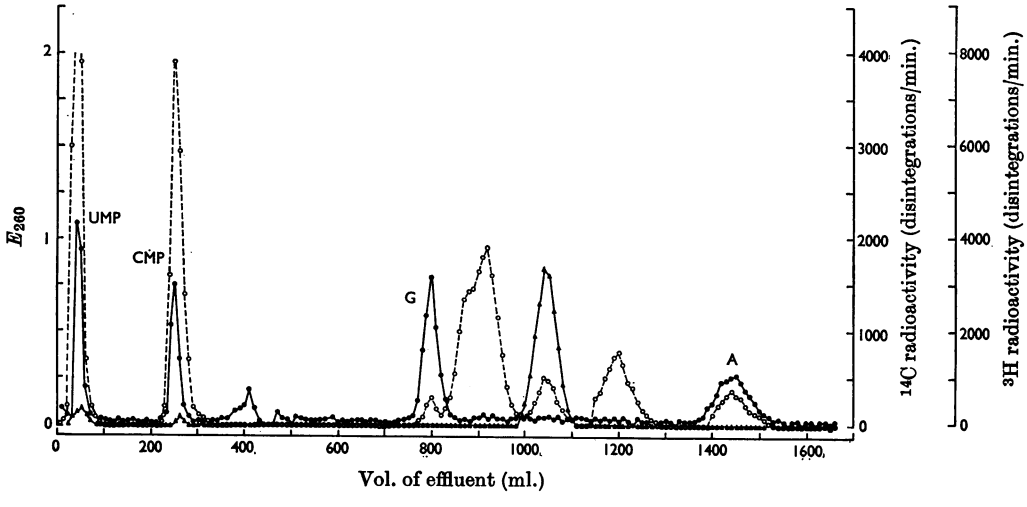

Fig. 4. Ion-exchange chromatography of an HCl hydrolysate of a mixture of liver soluble RNA from a rat treated with [^{14}C]methionine and total liver RNA from animals treated with [^{3}H]dimethylnitrosamine, the latter having been added to act as a marker for 7-methylguanine. For details see the text. Peaks shown by E_{260} readings are UMP, CMP, guanine (G) and adenine (A).

it is not excluded that the much smaller amount of the base found in microsomal RNA occurs in soluble RNA bound to it. However, the difference in the relative heights of the radioactive peaks in the two profiles argues against this. Messenger RNA probably occurs in the microsomal fraction, but there is evidence that messenger RNA does not contain methylated bases (Moore, 1966). The results show also that methionine is a methyl donor during the biosynthesis of 7-methylguanine and of certain other methylated bases in rat liver *in vivo*. It has been shown by Rodek, Feldman & Littauer (1967) that an RNA methylase preparation from rat liver methylates *Escherichia coli* soluble RNA to form, among other methylated bases, 7-methylguanine. However, this does not necessarily imply

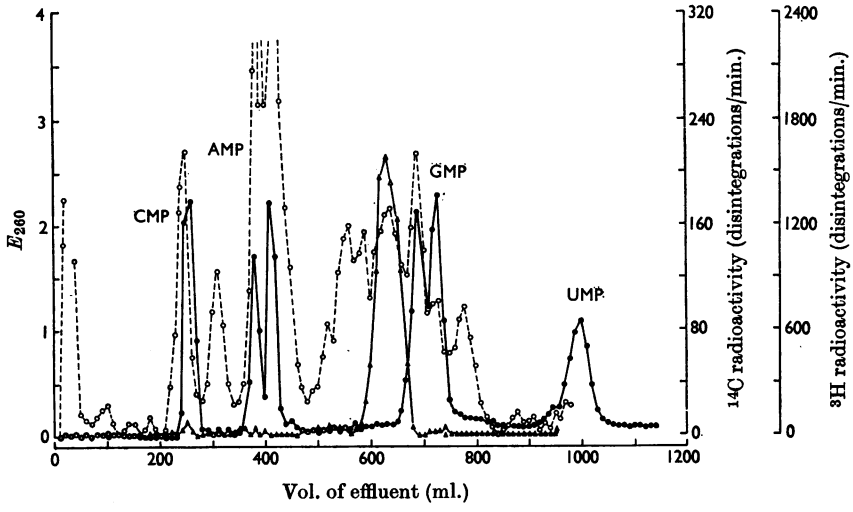

Fig. 5. Ion-exchange chromatography of alkaline hydrolysate of a mixture of liver microsomal RNA from a rat treated with [^{14}C]methionine and total liver RNA from animals treated with [^{3}H]dimethylnitrosamine, the latter having been added to act as a marker for 7-methylguanine. For details see the text. Peaks shown by E_{260} readings are CMP, AMP, GMP and UMP. ●, E_{260}; ○, ^{14}C radioactivity; △, ^{3}H radioactivity.

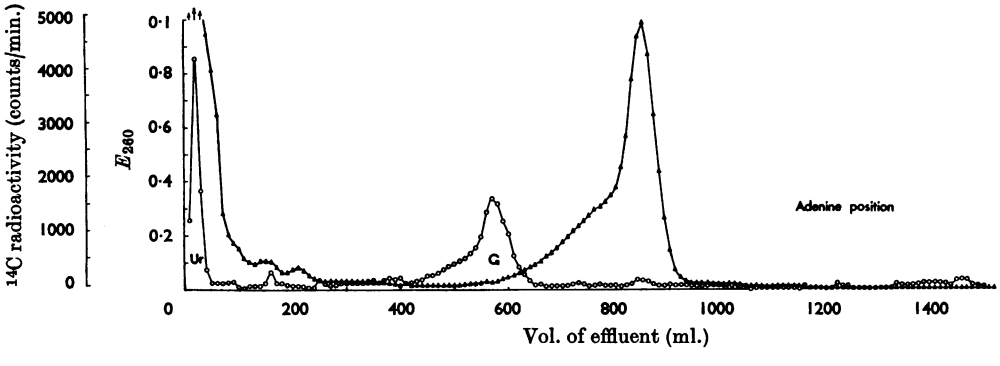

Fig. 6. Ion-exchange chromatography of purine fraction of urine collected for 5 hr. during administration of [^{14}C]methionine. Carrier guanine (G) was added. Peaks shown by E_{260} readings are the initial mixture of purine metabolites, chiefly uric acid (Ur), and guanine (G). ○, E_{260}; △, radioactivity.

that 7-methylguanine is present in rat liver RNA, as methylation is believed to depend on the presence of the appropriate base sequence in the RNA substrate, which may have been present in *E. coli* RNA but not in rat liver RNA.

Normal urine is known to contain 7-methylguanine (Krüger & Salomon, 1898*a*,*b*; Weissmann, Bromberg & Gutman, 1957), and there is evidence, based on the excretion of radioactive 7-methylguanine by rats whose nucleic acids had been prelabelled in the neonatal period with [^{14}C]formate, that the methylated base originates in the nucleic acids (Craddock & Magee, 1967). Mandel *et al.* (1966) showed that methionine is the methyl donor of the 7-methylguanine excreted in urine. Thus it seems reasonable to assume that catabolism of RNA, or release of the methylated base by depurination, accounts for some 7-methylguanine in urine. The other methylated bases found in RNA occur to a much smaller extent, if at all, in the 5 hr. urine sample analysed, and therefore either are further metabolized or have a slower turnover rate than

7-methylguanine. The short time period of the experiment, designed primarily to detect methylation of RNA, probably explains why no labelled 1-methyladenine was detected in urine, although it had been found by Mandel *et al.* (1966). These authors found that most of the radioactivity of the urinary purines occurred in 7-methylguanine after administration of [^{14}C]methionine to mice; as this base also had a comparatively higher specific radioactivity, it was suggested that it has a higher rate of turnover than other methylated bases. The possible functions of the methylated bases remain obscure, although a role in protein synthesis and in cell differentiation has often been postulated (Borek & Srinivasan, 1966).

The experiments give no evidence for the occurrence of 7-methylguanine in DNA, although conceivably the methyl group could be derived from a methyl donor other than methionine. Alkylation on the 7-position of guanine labilizes the glycosidic bond, and the methylated base splits out of the DNA spontaneously (Lawley & Wallick, 1957). However, the rate of this non-enzymic excision under physiological conditions *in vitro* (Lawley & Brookes, 1963) and when 7-methylguanine is formed by the action of a low dose of dimethylnitrosamine *in vivo* (V. M. Craddock, unpublished work) is such that loss of 7-methylguanine would be unlikely to occur to a considerable extent during the 5hr. period of the experiments described here. In addition, column chromatography of a large preparation of rat liver DNA (386mg.) gave no evidence for the presence of 7-methylguanine from extinction determinations (P. F. Swann, personal communication; Shank & Magee, 1967). Thus rat liver DNA differs from mammalian sperm DNA, where 1-methylguanine has been reported in human sperm DNA and N^2-dimethylguanine in bull sperm DNA (Unger & Venner, 1966). When 7-methylguanine is formed in rat liver DNA after administration of certain carcinogens, it can therefore be regarded as being an abnormal base, whose presence might be expected to exert an effect on the biological functioning of DNA.

The authors thank Mr J. A. E. Jarvis, Mr R. Hunt and Miss A. Taylor for skilled technical assistance.

REFERENCES

Bergquist, P. L. & Matthews, R. E. F. (1962). *Biochem. J.* **85**, 305.
Borek, E. & Srinivasan, P. R. (1966). *Annu. Rev. Biochem.* **35**, 275.
Bruce, H. M. & Parkes, A. S. (1956). *J. Anim. Tech. Ass.* **7**, 54.
Craddock, V. M. & Magee, P. N. (1966). *Biochem. J.* **100**, 724.
Craddock, V. M. & Magee, P. N. (1967). *Biochem. J.* **104**, 435.
Dunn, D. B. (1959). *Biochim. biophys. Acta*, **34**, 286.
Dunn, D. B. (1963). *Biochem. J.* **86**, 14P.
Haines, J. A., Reese, C. B. & Todd (Lord) (1962). *J. chem. Soc.* p. 5281.
Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. I. & Zamecnik, P. C. (1958). *J. biol. Chem.* **231**, 241.
Kirby, K. S. (1956). *Biochem. J.* **64**, 405.
Kirby, K. S. (1962). *Biochim. biophys. Acta*, **55**, 545.
Kriek, E. & Emmelot, P. (1963). *Biochemistry*, **2**, 733.
Krüger, M. & Salomon, G. Z. (1898*a*). *Hoppe-Seyl. Z.* **24**, 364.
Krüger, M. & Salomon, G. Z. (1898*b*). *Hoppe-Seyl. Z.* **26**, 350.
Lawley, P. D. & Brookes, P. (1963). *Biochem. J.* **89**, 127.
Lawley, P. D. & Wallick, C. A. (1957). *Chem. & Ind.* p. 633.
Lee, K. Y., Lijinsky, W. & Magee, P. N. (1964). *J. nat. Cancer Inst.* **32**, 65.
Magee, P. N. & Barnes, J. M. (1967). *Advanc. Cancer Res.* **10**, 164.
Mandel, L. & Borek, E. (1963). *Biochemistry*, **2**, 555.
Mandel, L., Srinivasan, P. R. & Borek, E. (1966). *Nature, Lond.*, **209**, 586.
Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.
Moore, P. (1966). *J. molec. Biol.* **18**, 38.
Price, T. D., Hinds, H. A. & Brown, R. S. (1963). *J. biol. Chem.* **238**, 311.
Rodek, R., Feldman, M. & Littauer, U. Z. (1967). *Biochemistry*, **6**, 451.
Shank, R. C. & Magee, P. N. (1967). *Biochem. J.* **105**, 521.
Sluyser, M. & Bosch, L. (1962). *Biochim. biophys. Acta*, **55**, 479.
Smith, J. D. & Dunn, D. B. (1959*a*). *Biochem. J.* **72**, 294.
Smith, J. D. & Dunn, D. B. (1959*b*). *Biochim. biophys. Acta*, **31**, 573.
Unger, G. & Venner, H. (1966). *Hoppe-Seyl. Z.* **344**, 280.
Villa-Trevino, S. & Magee, P. N. (1966). *Biochem. J.* **100**, 36P.
Weissmann, B., Bromberg, P. A. & Gutman, A. B. (1957). *J. biol. Chem.* **224**, 407.
Wyatt, G. R. (1952). *J. gen. Physiol.* **36**, 201.