The Stability of Rat Liver Ribonucleic Acid *in vivo* after Methylation with Methyl Methanesulphonate or Dimethylnitrosamine

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1. RNA was isolated from rat liver at selected times after the intraperitoneal injection of either [^{14}C]methyl methanesulphonate (50 mg/kg) or [^{14}C]dimethylnitrosamine (2 mg/kg). These doses were chosen to minimize effects due to toxicity. 2. Two methods of extraction and purification of RNA were used and an analysis of the radioactivity present was made by column chromatography of acid hydrolysates of the purified RNA. 3. The extent of methylation of guanine, the principal site of alkylation in rat liver RNA, was determined at times up to 14 days after injection. Although dimethylnitrosamine is a potent liver carcinogen and methyl methanesulphonate is not carcinogenic to rat liver, the rate of disappearance of 7-methylguanine from RNA was similar for both compounds, with a half-life of about 3.5 days. 4. An estimate of the biological half-life of rRNA was made by using [^3H]orotic acid. A half-life of 5 days was obtained and this was not affected by injecting animals with unlabelled methyl methanesulphonate at the same dosage of 50 mg/kg used in the studies of RNA methylation. 5. After administration of labelled orotic acid, reutilization of labelled RNA degradation products probably results in an overestimation of the biological half-life for rRNA. It is suggested that non-toxic doses of methylating agents such as methyl methanesulphonate and dimethylnitrosamine may prove to be a more effective way of accurately estimating the biological turnover of RNA species.

An important aspect of comparative studies with chemical carcinogens and related compounds is the need to distinguish effects caused by toxicity from those relevant to the carcinogenic process. We have examined the methylation of liver RNA in rats injected with low doses of either methyl methanesulphonate or dimethylnitrosamine as part of a study of alkylating agents of different carcinogenic potency. The dose of methyl methanesulphonate used produced no observable toxic effects other than a transient infertility as a result of a selective action on cells in late spermatogenesis (Jackson, Fox & Craig, 1961). It had been shown (Whittle, 1969) that this dose of methyl methanesulphonate produced maximal methylation of rat liver RNA 2 h after injection and that this value remained constant for at least 24 h. This result contrasted with the 40% loss of methylated components observed when the carcinogen dimethylnitrosamine was used (Craddock & Magee, 1963).

In the present study the turnover of methylated components of liver RNA was examined at times up to 14 days after administration of the alkylating agent. After injection of either methyl methane-

sulphonate or dimethylnitrosamine, 7-methylguanine is lost from rat liver RNA at a rate that is only slightly faster than the reported biological turnover time for rat liver RNA (Blobel & Potter, 1968).

The evidence described here and by Craig & O'Connor (1971) suggests that the methylated component, 7-methylguanine, of liver RNA is not labile *in vivo* and that its loss may prove to be a better measure of the biological turnover of rat liver RNA than that obtained by following the decay of radioactivity in RNA after an injection of labelled orotic acid.

MATERIALS AND METHODS

Materials. [$\text{Me-}^{14}\text{C}$]Methyl methanesulphonate (46 mCi/mmol) in ether solution, [^{14}C]dimethylamine hydrochloride (20.6 mCi/mmol) and [$5\text{-}^3\text{H}$]orotic acid (15 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Labelled methyl methanesulphonate was diluted with chemically pure unlabelled methyl methanesulphonate before removal of ether by vacuum evaporation at -78°C and was redissolved in

0.9% NaCl for injection. [^{14}C]Dimethylnitrosamine was prepared from [^{14}C]dimethylamine hydrochloride by the method of Dutton & Heath (1956). The [^{14}C]dimethylamine hydrochloride was diluted with an appropriate amount of unlabelled material at the beginning of the reaction. The yield was 96% from the amine, and the final specific radioactivity of the [^{14}C]dimethylnitrosamine was 2.28 mCi/mmol. Unlabelled orotic acid used to dilute the labelled acid was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Dowex-50 (AG50W-4; H^+ form; 200–400 mesh) ion-exchange resin was purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and Triton X-100 from Lennig Chemicals Ltd., Croydon, Surrey, U.K.

Animal experiments. (a) Injection of methyl methanesulphonate. Male Wistar rats (230–250 g) were allowed food and water throughout the experiments. Single intraperitoneal injections were given between 10.30 and 11.00 a.m. and animals were killed by decapitation without anaesthesia at intervals from 15 min to 14 days later. Methyl methanesulphonate was injected at a dosage of 50 mg/kg in all cases but the specific radioactivity was altered in relation to the sampling interval. Animals killed at times up to 4 days, at 7 and 10 days or at 14 days received methyl methanesulphonate of the following specific radioactivities: 0.915 mCi/mmol, 1.83 mCi/mmol and 4.57 mCi/mmol respectively.

(b) Injection of dimethylnitrosamine. Male Wistar rats weighing 210–220 g were injected with [^{14}C]dimethylnitrosamine at a standard time of 11 a.m. and allowed access to food and water throughout the experiment. Seven rats were given an intraperitoneal injection of [^{14}C]dimethylnitrosamine at a dose of 2 mg/kg and killed after the following times: 5 h, 1, 2, 4, 7, 10 and 14 days.

Determination of the apparent biological half-life of rRNA. This was done by injecting a total of 34 rats (230 g–250 g) intraperitoneally with [^3H]orotic acid (150 μCi /rat; 0.25 mCi/mmol in 0.25 ml of 0.9% NaCl). After the orotic acid injections the animals were kept for 48 h to allow an equilibrium to be established between acid-soluble and acid-insoluble radioactivity (Blobel & Potter, 1968).

Then 20 animals were injected with unlabelled methyl methanesulphonate (50 mg/kg) for comparison with a control group (14 animals), which was not treated further. Four animals were killed immediately, and the others subsequently in groups of four for the methyl methanesulphonate-treated series and in groups of two for the control series at 2, 4, 7, 10 and 14 days. Livers were removed and deep-frozen, and rRNA was prepared by method (ii) described below. Samples of RNA were hydrolysed in acid and the radioactivity per mg of RNA was determined. Samples from the zero-time, 2-, 4-, 10- and 14-day groups were analysed by chromatography on Dowex-50 (H^+ form) to confirm that the radioactivity contained in the UMP and CMP regions of the elution profile accounted for the radioactivity present in RNA.

Preparation of purified RNA. The liver was removed, rinsed in ice-cold NaCl (0.9%), weighed, frozen on solid CO_2 and stored at -15°C for subsequent extraction of RNA in one of two ways.

(i) Liver was homogenized in 5 vol. of 0.5% sodium naphthalenedisulphonate and filtered through no. 14 N nylon cloth (Henry Simon Ltd., Stockport, U.K.). RNA

was extracted and purified by the procedure of Ralph & Bellamy (1964). The yield was 4.6 mg/g of fresh liver.

(ii) Liver was homogenized in 15 vol. of 6% (w/v) 4-aminosalicylate containing 1% (w/v) NaCl and an equal volume of phenol-cresol mixture (500 g of phenol, 70 ml of *m*-cresol, 0.5 g of 8-hydroxyquinoline and 55 ml of water). The rRNA was prepared as described under procedure 2 of Kirby (1965). The yield of rRNA was 4 mg/g of fresh liver. Samples of RNA were checked for the absence of DNA by chromatography on Dowex-1 (formate form) after digestion to the constituent nucleosides with snake venom exonuclease and *Escherichia coli* alkaline phosphatase (Hall, 1967).

Determination of RNA. The amount of purified RNA was calculated from its E_{260} after acid hydrolysis (1 M-HCl for 1 h at 100°C) by using the base ratios for rat liver RNA given by Kirby (1962) and the extinction coefficients given by Burton (1959). An E_{260} of 1.000 for a 1 cm light-path was equivalent to 39 μg of RNA/ml.

Column chromatography of acid hydrolysates of RNA. Columns (1 cm $\times$ 10 cm) of Dowex-50 (H^+ form) were equilibrated with 1 M-HCl. Samples of acid hydrolysates of purified RNA (equivalent to 2–5 mg of RNA) were applied to the column and eluted at a flow rate of 20 ml/h by using a convex gradient of 1–3 M-HCl (Magee & Farber, 1962). The volume of solution in the mixing vessel was 200 ml. Fractions (2.8 ml) were collected and their u.v. absorption was measured with a Unicam SP.3000 spectrophotometer and their radioactivity as described below. The amount of guanine was calculated from the E_{260} of the guanine peak and the amount of 7-methylguanine was calculated from the total radioactivity in the 7-methylguanine peak assuming that the specific radioactivity of the methyl group was the same as that of the methylating agent injected. From this the percentage conversion of guanine into 7-methylguanine could be obtained.

Determination of radioactivity. Samples (2 ml) from the column effluents were blended with 12 ml of a scintillation mixture [2,5-diphenyloxazole, 16 g/l, and 1,4-bis-(5-phenyloxazol-2-yl)benzene, 0.4 g/l, in A.R. toluene mixed with an equal volume of Triton X-100 immediately before counting] and assayed for radioactivity in a Tracerlab spectrometer. A background counting rate was determined for each vial and the counting efficiency was estimated by using an internal standard. The efficiency for tritium counting fell from 9.7% to 5.5% over the gradient but no change in counting efficiency was observed for ^{14}C .

RESULTS

Analysis of the stability of alkylated components in rat liver RNA. Two methods of extracting liver RNA were employed. The procedure described by Ralph & Bellamy (1964) extracts rRNA and tRNA (method i) whereas the procedure described by Kirby (1965) selectively extracts rRNA and a small amount of rapidly labelled RNA (method ii). The loss of alkylated components from these purified RNA preparations was followed by chromatography of acid hydrolysates on columns of Dowex-50 (H^+ form). An example of an elution profile showing the

separation of 7-methylguanine in a 14-day rRNA sample, which had a high content of metabolically labelled radioactive adenine and guanine, is shown in Fig. 1.

Treatment with methyl methanesulphonate. The methylation of guanine at 4h in the rat liver RNA samples (method i) was 0.047%, which agreed with the results of Whittle (1969). The extent of methylation of guanine was measured at 15 and 30 min, and indicated that alkylation was 0.027 and 0.030% of the guanine respectively, showing that the alkylation process was over 50% complete in the first 15 min after the injection. This is consistent with the observation that methyl methanesulphonate was no longer detectable in the circulation after 1.5h (Swann, 1968).

Over a 7-day period the 7-methylguanine component of the combined rRNA + tRNA preparation (method i) was lost, with a decay half-life of about 3.1 days (see Fig. 2a), which suggested that this alkylated component was fairly stable in relation to the 4-day biological half-life reported for rat

liver RNA (Blobel & Potter, 1968). The degree of alkylation and the stability of the alkylated components were then examined for rat liver rRNA (method ii) over a 14-day period. In rRNA the extent of alkylation at 4h was 0.030% of the guanine and the decay half-life for 7-methylguanine over the 14 days was 3.5 days (Fig. 2b).

Apparent half-life of rRNA determined in the presence of methyl methanesulphonate. The half-life for the decay of 7-methylguanine was shorter than the reported biological turnover time of rat liver RNA species (Blobel & Potter, 1968). To assess whether the observed half-life for 7-methylguanine was the result of a slightly accelerated breakdown, the half-life of rRNA was determined by following the loss of radioactivity in rRNA that had been labelled with orotic acid before the injection of unlabelled methyl methanesulphonate (50 mg/kg). The result of this experiment is shown in Fig. 3, which shows that the apparent half-life for rRNA is approx. 5.0 days and that no difference could be detected between the animals that were injected

Fig. 1. Ion-exchange chromatography on Dowex-50 (H^+ form) of an acid hydrolysate of 2.3 mg of rRNA from the liver of a rat treated with [^{14}C]methyl methanesulphonate (50 mg/kg) 14 days previously. Details of the chromatography are given in the text; $\circ$, E_{260}^{1cm} ; $\bullet$, radioactivity; Ade, adenine; Gua, guanine; 7-MeG, 7-methylguanine.

Fig. 2. Semi-logarithmic plot showing the loss of ^{14}C -labelled 7-methylguanine from (a) liver RNA prepared by method (i) (half-life 3.1 days) and (b) rRNA prepared by method (ii) after an injection of [^{14}C]methyl methanesulphonate (50 mg/kg) (half-life 3.5 days). Each point indicates the value obtained for an individual rat liver. Experimental details are given in the text.

Fig. 3. Semi-logarithmic plot showing the decay of radioactivity in rat liver rRNA (method ii) obtained from animals that had been injected with [$5\text{-}^3\text{H}$]orotic acid. At 48 h after the orotic acid injection a group of animals was injected with unlabelled methyl methanesulphonate (50 mg/kg); the control group remained untreated. Each point represents the mean for the time group, and experimental details are given in the text; ●, methyl methanesulphonate-treated; ○, controls. Half-life, 5.0 days.

with methyl methanesulphonate and those that were not.

Metabolic labelling of purines. It has been shown that after the degradation of methyl methanesulphonate, the ^{14}C radioactivity can enter the C-1 pool and label the purine ring (Whittle, 1969). Since liver has a great capacity for purine biosynthesis (Murray, Elliott & Atkinson, 1970), this could

explain why in our experiments the ring labelling of adenine and guanine reached a maximum at about 2 days (Figs. 4a and 4b), long after the labelling by direct methylation had ceased. In the rRNA series where the results cover the 14-day period, the radioactivity associated with the purine peaks decayed slowly with a half-life of about 10 days (Fig. 4b). Since the biological turnover time for rat liver RNA is about 4–5 days, and possibly a little shorter, it is evident that some of this label was reutilized, possibly as preformed purines. The efficiency of reutilization can be calculated to be about 50% from the results for rRNA (Fig. 4b). The larger variations observed with the purine label are consistent with this interpretation and contrast with the much more reproducible values obtained for 7-methylguanine, which is unlikely to undergo reutilization (Craddock, Mattocks & Magee, 1968).

Treatment with dimethylnitrosamine. In this series only rRNA (method ii) was examined. Experiments were carried out with low doses of dimethylnitrosamine (2mg/kg), which did not produce any histologically visible signs of cellular necrosis (Craddock, 1969), although this does not exclude minor ultrastructural and biochemical changes that may be relevant to the carcinogenic process. After an injection of dimethylnitrosamine, methylation of rat liver RNA reached a maximum at 5h, which agreed with the results of Craddock & Magee (1963). The extent of alkylation of guanine residues was 0.083% at 5h and was similar to the value obtained by Craddock (1969), who used rats that were starved overnight before injection,

Fig. 4. Semi-logarithmic plot showing the time-course for the incorporation of radioactivity into adenine (●) and guanine (○). These bases were isolated by chromatography on Dowex-50 (H^+ form) from acid hydrolysates of (a) liver RNA prepared by method (i) and (b) rRNA prepared by method (ii) at selected times after injection of [^{14}C]methyl methanesulphonate at a dose of 50 mg/kg.

although this may have decreased the liver RNA content (see the Discussion section). The decline in the percentage of guanine methylated in rRNA over a 14-day period is shown in Fig. 5 and approximated to first-order kinetics with a half-life of 3.4 days. The ring labelling of adenine and guanine shows a similar pattern to that observed in the study with methyl methanesulphonate, suggesting that the ring-labelled material is reutilized (Fig. 6).

DISCUSSION

In the present paper an attempt has been made to follow the fate of those components of rat liver RNA that are methylated after an injection of dimethylnitrosamine or methylmethanesulphonate. As our first consideration it was decided to study these methylated components by using animals in as stable a metabolic state as possible. Secondly, we have tried to avoid those dosages that might prove toxic to the animals and thereby cause degradative changes. Starvation is known to decrease the RNA content of cells because of an

increased rate of RNA degradation and a decreased rate of RNA synthesis (Davidson, 1947; Hirsch & Hiatt, 1966). In nuclei obtained from the livers of starved rats the Mg^{2+} -dependent RNA synthesis was first to be affected and was decreased by 30% after 1 day of starvation (Rickwood & Klemperer, 1970). Rats that had been adapted to a 48 h feeding cycle of 40 h starvation and 8 h feeding and that had learned to compensate partially for starvation by eating ravenously during the feeding period, nevertheless still showed a net loss of 21% of liver RNA every cycle (Whittle & Potter, 1968). Also, it has been reported that starvation for 1 day will more than double the dimethylnitrosamine demethylase activity of rat liver (Venkatesan, Arcos & Argus, 1970) and this may serve to increase the efficiency of the methylation process after an injection of dimethylnitrosamine. However, this report is in contrast with the work of Swann & McLean (1968) who showed that a protein-deficient diet decreased the rate of metabolism of dimethylnitrosamine to approximately one-half of the normal rate in the intact rat. To maintain a stable

Fig. 5. Semi-logarithmic plot showing the loss of ^{14}C -labelled 7-methylguanine from rat liver rRNA (method ii) after an injection of [^{14}C]dimethylnitrosamine (2 mg/kg). Each point indicates the value obtained for an individual rat liver. Experimental details are given in the text. Half-life, 3.4 days.

Fig. 6. Semi-logarithmic plot showing the time-course for the incorporation of radioactivity into adenine (●) and guanine (○). These bases were isolated by chromatography on Dowex-50 (H^+ form) from acid hydrolysates of rat liver rRNA prepared at selected times after an injection of [^{14}C]dimethylnitrosamine at a dose of 2 mg/kg.

RNA metabolism, animals were allowed unrestricted access to food and water at all times, as was the case in the study by Whittle (1969). After an injection of dimethylnitrosamine or methyl methanesulphonate, the principal methylated component is 7-methylguanine. With dimethylnitrosamine the maximum degree of methylation in liver RNA is achieved at 5h, in agreement with results of Craddock & Magee (1963), resulting in methylation of 0.08% of the guanine in these studies. With methyl methanesulphonate the maximum degree of alkylation for liver RNA prepared by the method of Ralph & Bellamy (1964) was 0.04% of the guanine, which was attained in 2h and was maintained at this value for a further 22h (Whittle, 1969). In our studies the degree of methylation was 0.047% of guanine residues at 4h for liver RNA prepared by the same method, whereas for rRNA prepared by the Kirby (1965) procedure the value was 0.03% of the guanine. The Ralph & Bellamy (1964) preparation (method i) contains both rRNA and tRNA and the Kirby (1965) preparation (method ii) preferentially extracts rRNA; therefore taking into account the fact that 80% of the cellular RNA is rRNA and 15% is tRNA (Davidson, 1969), it can be calculated that the degree of methylation of tRNA is approx. 0.14%, or about four times that obtained for rRNA.

Kriek & Emmelot (1964) showed that the glycosidic link of 7-methylguanine in RNA was stable for at least 100h *in vitro* at neutral pH values. Lawley & Brookes (1963) also showed that this acid-catalysed reaction begins at pH 8.5 for methylated deoxyribose nucleotides but requires a very much

lower pH for the corresponding ribose compounds. There is therefore no reason to expect a significant rate of chemical depurination of RNA methylated *in vivo* at the 7-position of guanine, as would be the case with DNA.

During the time-periods of these experiments, therefore, the loss of 7-methylguanine should be due to the turnover of RNA within the cell, as 7-methylguanine is apparently not reutilized (Craddock *et al.* 1968). Although in these experiments doses of methylating agents have been used that did not show obvious signs of toxicity (Jackson *et al.* 1961; Craddock, 1969) it was thought necessary to check the biological half-life of rRNA as its metabolism is known to be sensitive to dietary changes (see above). This was carried out for the methyl methanesulphonate series, by utilizing an injection of labelled orotic acid. This gave a turnover time of approx. 5.0 days in the treated and control animals, indicating that the low dose of methyl methanesulphonate had not accelerated breakdown of rRNA. This result can be compared with estimates of 4.1 days for rRNA and 3.8 days for tRNA by Blobel & Potter (1968), and to half-life values of 7.2 days for microsomal RNA and 5.7 days for tRNA reported by Gerber, Gerber & Altman (1960). Other reports (Hirsch & Hiatt, 1966; Loeb, Howell & Tomkins, 1965; Wilson & Hoagland, 1967) also indicate a half-life of 4–5 days for rRNA. However, these turnover times are probably overestimated, owing to re-incorporation of labelled RNA degradation products. In fact Blobel & Potter (1968) showed that between 3 and 12 days after the injection of labelled orotic acid the acid-

soluble/acid-insoluble radioactivity ratio is about 0.2, which demonstrates the availability of a constant pool of acid-soluble material. Thus, if the turnover times derived from the orotic acid-labelling experiments are an overestimate as Blobel & Potter (1968) suggested they are, and because the treatment with methyl methanesulphonate had not accelerated the turnover of rRNA, it seems likely that the half-life of 3.5 days for the decay of 7-methylguanine represents the true biological turnover time for rRNA. From this estimate, the slower decay of radioactivity in the orotic acid-labelling experiments (5.0 days) can be calculated to result from a 30% reutilization of labelled materials. This is less than the 50% reutilization calculated for the ring-labelled purines (see the Results section) and this difference is probably due to the greater reutilization of labelled precursors of RNA purines, resulting from the breakdown of nucleic acids within the cell. The precise nature of these intracellular degradation products is not known. Earlier reports of the incorporation of specific bases, nucleosides and nucleotides (Brown & Roll, 1955; Murray *et al.* 1970) are not applicable as they refer to the uptake of labelled compounds when administered to the whole animal or when added to cells in culture.

The dimethylnitrosamine-treated animals showed an identical decay half-life indicating that liver rRNA is equally stable when methylated with the potent liver carcinogen dimethylnitrosamine or with methyl methanesulphonate which is not carcinogenic to rat liver. Clearly there is no intrinsic instability of rRNA after reaction with methyl methanesulphonate or dimethylnitrosamine at these low dosages, whereas Craddock & Magee (1963) found a 40% loss of methylated components by 24h after a dose of dimethylnitrosamine (10mg/kg). Although this dose, the lowest used in their experiments, did not cause overt liver necrosis, it is possible that the initial rapid loss of methyl groups from RNA is a manifestation of the toxicity of the nitrosamine.

The results given in the present paper suggest that a non-toxic dose of a methylating agent such as methyl methanesulphonate or dimethylnitrosamine may prove to be a most effective way of accurately estimating the turnover time for cellular RNA species.

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