Methylation of Nucleic Acids by $N[^{14}C]$-Methyl-N-nitrosourethane *in vitro* and *in vivo*

By R. Schoental
Medical Research Council Laboratories, Toxicology Research Unit, Woodmansterne Road, Carshalton, Surrey

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MNU* is a very effective carcinogen that can induce malignant tumours of the oesophagus, lung and stomach (squamous and glandular) with one or a few doses given by stomach tube (Schoental, 1960, 1963, 1966; Schoental & Magee, 1962). As yet, no liver tumours have been seen in rats given MNU, either intragastrically (one or several doses) or in drinking water throughout the life-span of the animals (Druckrey, Preussmann, Schmähl & Müller, 1961).

MNU interacts with free thiols at neutral pH, no heating being necessary, and releases nitrogen gas (Schoental & Rive, 1965). The course of the reaction can be followed manometrically in a Warburg apparatus. When commercial DNA was tested under such conditions, no significant gas evolution could be seen (Schoental, 1966).

However, when $[Me\text{-}^{14}C]$MNU was used (prepared by nitrosylation of $N[^{14}C]$-methylurethane, obtained from The Radiochemical Centre, Amersham, Bucks.) and the reactants were left to interact for several hours, the recovered DNA was radioactive. The reactions were performed as in the following example. A 10μl. sample of $[Me\text{-}^{14}C]$-MNU in 0·5ml. of 50% (v/v) ethanol was added to a solution of the sodium salt of DNA (50mg.) in phosphate buffer, pH 7·2 (1ml.), and the mixed solution was left in a fume cupboard for several hours. The solution was then divided into two parts; to one of these cysteine was added (5mg. in 0·5ml. of water), and both reaction mixtures were left to react for a further 16hr. The nucleic acids were obtained from each of the reaction mixtures separately by repeatedly (three or four times) precipitating with ethanol, centrifuging, redissolving in water and washing the precipitate with ethanol. The radioactivity of the nucleic acids thus obtained was $1{\cdot}3\times10^3$ counts/min./mg. in the absence of cysteine and $2{\cdot}5\times10^3$ counts/min./mg. in its presence.

Both preparations of DNA were then hydrolysed with N-HCl at 90° for 1hr. and the hydrolysis products were subjected to column chromatography on Dowex 50 (H^+ form) and eluted with a gradient of 1–4N–HCl in an automatic fraction collector. Consecutive 10ml. fractions were then assayed for $E_{260m\mu}$ and for radioactivity (samples of the concentrated fractions were counted in Diotol in a Packard automatic scintillation counter). The values plotted against the fraction numbers gave the chromatographic 'profiles' (Magee & Faber, 1962) shown in Fig. 1.

Several radioactive peaks are present; the largest between the large absorbing peaks of guanine and adenine represents 7-methylguanine. This was confirmed by comparing the chromatographic behaviour and the absorption spectrum of this material (obtained by concentrating the combined fractions 35–43) with authentic 7-methylguanine (given by Dr P. N. Magee). The next largest radioactive peak is present in fractions 2–3 (Fig. 1*a*). These fractions contain among other constituents pyrimidine nucleotides, which are not hydrolysed under the conditions of the acid hydrolysis used. To separate the radioactive components, fractions 1–9 were combined and concentrated to dryness in a stream of air at room temperature, and the brownish residue was rechromatographed on a column of Dowex 1 and eluted with a gradient of 1–4N-formic acid as described by Gilbert & Yemm (1958), an automatic fraction collector being used. The 'profile' obtained from this column showed three large u.v.-absorbing peaks; the peak present in fractions 2–3 showed the highest radioactivity, the peaks corresponding to the pyrimidines being only slightly radioactive. Fractions 2–3 contain several components, including the sugar moiety, which under the conditions of the hydrolysis used appears to undergo dehydration to a furfural derivative (hence the strong u.v. absorption of these fractions).

Very similar profiles were obtained from total nucleic acids isolated from the livers (Fig. 1*b*) and from the stomachs of rats killed 2, 24 and 48hr. after a single intragastric dose of $[Me\text{-}^{14}C]$MNU (5mg./0·5ml. of 50% ethanol; 10^6 counts/min./rat). High radioactivities were present in the fractions corresponding to 7-methylguanine in the nucleic acids of both the liver (the organ in which no tumours have been induced by MNU) and the stomach (the organ in which this compound induces readily malignant tumours). In both organs the radioactive 7-methylguanine decreases rapidly with time.

* Abbreviation: MNU, N-methyl-N-nitrosourethane.

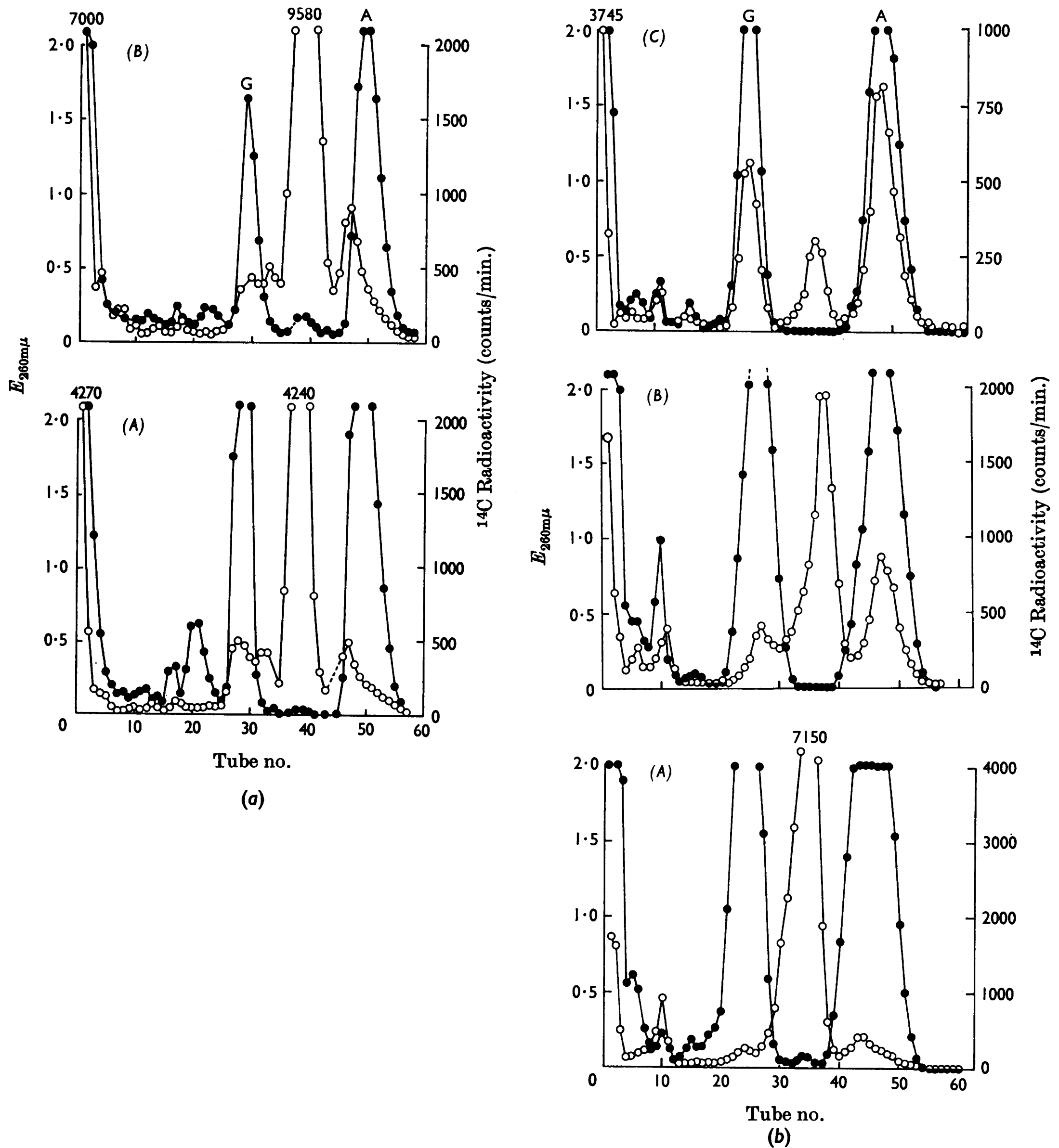

Fig. 1. Chromatographic profiles, obtained by gradient elution with 1–4N-HCl from Dowex 50 (H^+ form), of acid hydrolysates of: (*a*) DNA (thymus DNA, sodium salt; British Drug Houses Ltd., Poole, Dorset) treated *in vitro* with [*Me*-^{14}C]MNU in the absence (*A*) or in the presence (*B*) of cysteine; (*b*) total nucleic acids from livers of rats killed 2hr. (*A*), 24hr. (*B*) or 48hr. (*C*) after a single intragastric dose of [*Me*-^{14}C]MNU. ●, $E_{260m\mu}$; ○, ^{14}C radioactivity. Markers: A, adenine; G, guanine.

The next highest radioactive peak in the chromatographic profiles of the total nucleic acids from both organs was again present in fractions 2–3, as in the profiles obtained from methylation of DNA *in vitro* (the minor peaks to the right and to the left of the 7-methylguanine peak were similarly present). This suggests that the radioactivity of this peak is likely to be mainly due to methylated compounds, and only to a minor extent to the incorporation of ^{14}C from the C_1 pool.

Rechromatography of fractions 1–9 obtained from livers of three rats on Dowex 1 gave chromato-

graphic profiles with radioactivity distribution again similar to that obtained from methylation of DNA *in vitro*.

Fractions 2–3 obtained from the Dowex 1 column were subjected to paper chromatography in different solvent systems. By using the appropriate spraying reagents evidence was obtained for the presence in these fractions of sugars (aniline phthalate), amino acids (ninhydrin) and sulphur (platinic iodide).

The results obtained show that:

(1) MNU can act *in vitro* at neutral pH as a direct methylating agent for DNA, and methylates mainly the 7-position of guanine and to a smaller extent also other positions of the purines.

(2) MNU methylates in addition some constituents of DNA that are present in the fractions containing the sugar moiety.

(3) The degree of methylation of these constituents of DNA increases when free thiols (cysteine) are present in the reaction mixture.

(4) MNU methylates *in vivo* the nucleic acids of the liver and of the stomach to give chromatographic profiles very similar to those obtained *in vitro*.

(5) The degree of methylation of guanine at the 7-position is higher in the liver nucleic acids than in the stomach nucleic acids, although these two organs strikingly differ in their susceptibility to the carcinogenic action of MNU. In both organs it decreases rapidly with time, whereas that of fractions 2–3 is retained during the same period.

The conventional procedure for the extraction of total nucleic acids from tissues (Schneider, 1945) yields preparations that, though not as 'pure' as some obtained by more recently introduced methods, are consistently reproducible. The proteinaceous material associated with these nucleic acids may possibly have special biological significance and deserves to be studied, particularly when the action of carcinogens such as MNU is being investigated.

It remains to be shown whether the methylation of nucleic acids by MNU is due to the presence of free thiols in the proteinaceous material with which they appear associated, or to thiopyrimidines. The latter have been detected in certain transfer-RNA molecules (Lipsett, 1965; Carbon, Hung & Jones, 1965).

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Concurrent Bromoacetate Reaction at Histidine and Methionine Residues in Ribonuclease

By D. M. Glick*, H. J. Goren and E. A. Barnard
Department of Biochemistry, State University of New York, Buffalo 14, N.Y., U.S.A.

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The reaction of bromoacetate or, more slowly, iodoacetate with pancreatic ribonuclease inactivates at pH 5–7, with the carboxymethylation mainly of histidine-119 but with a small extent of reaction at histidine-12, these being mutually exclusive sites (Barnard & Stein, 1959; Stein & Barnard, 1959; Gundlach, Stein & Moore, 1959; Crestfield, Stein & Moore, 1963). In contrast, an inactivating reaction occurring only at several methionine residues was reported at or below pH 2·8 (40°), this being ascribed to the unfolding of the protein in acid (Gundlach *et al.* 1959; Neumann, Moore & Stein,

*Present address: Department of Biochemistry, Marquette University, Milwaukee, Wis., U.S.A.