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Preferential Alkylation of Mitochondrial Deoxyribonucleic Acid by *N*-Methyl-*N*-nitrosourea

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The reaction of the carcinogen *N*-methyl-*N*-nitrosourea with mitochondrial DNA from various rat tissues was examined *in vivo* and *in vitro*. After incubation of isolated mitochondria or cell nuclei with *N*[^{14}C]-methyl-*N*-nitrosourea *in vitro* and subsequent isolation and purification of the DNA the specific radioactivity of the mitochondrial DNA was 3–7 times that of the nuclear DNA. The incorporation of ^{14}C into embryonic mitochondrial DNA *in vitro* was about twice that into the liver mitochondrial DNA. Identical incorporation rates, however, were found for the reaction of isolated mitochondrial DNA or nuclear DNA respectively with *N*[^{14}C]-methyl-*N*-nitrosourea. After intraperitoneal injection of 43.3–58.5mg of *N*[^{14}C]-methyl-*N*-nitrosourea/kg body wt. to adult rats the labelling of the mitochondrial DNA was on average 5 times that of the nuclear DNA. A smaller specific labelling was observed for the ribosomal RNA, transfer RNA, and mitochondrial RNA as well as for the mitochondrial protein as compared with the mitochondrial DNA. After hydrolysis of the alkylated nucleic acids with hydrochloric acid, fractionation was carried out on Dowex 50 cation-exchange columns. In most experiments 70–80% of the input ^{14}C radioactivity was eluted in the 7-methylguanine fraction. The preferential alkylation of the mitochondrial DNA by *N*-methyl-*N*-nitrosourea *in situ* is discussed in connexion with the cytoplasmic-mutation hypothesis of carcinogenesis.

The presence of DNA and RNA in mitochondria of animal cells as well as the ability of these organelles to synthesize DNA, RNA, and protein has now been demonstrated by several investigators (for reviews see S. Nass, 1969; M. M. K. Nass, 1969). Experiments on micro-organisms have given indications of a genetic function of miDNA* (Diacumakos, Garnjobst & Tatum, 1965; Reich & Luck, 1966; Linnane, Saunders, Gingold & Lukins, 1968; Thomas & Wilkie, 1968) and of changes in the miDNA caused by cytoplasmic mutations (Mounolou, Jakob & Slonimski, 1966; Bernardi, Carnevali, Nicolaieff, Piperno & Tecce, 1968; Mehrotra & Mahler, 1968; Wintersberger & Viehhauser, 1968; Fukuhara, Faures & Genin, 1969).

In view of the preferential accumulation of carcinogenic aromatic hydrocarbons in the mitochondria of animal cells Graffi (1940*a*,*b*,*c*) proposed that carcinogenesis might be primarily caused by a mutation of mitochondria. Similar ideas were formulated later as the plasmagene theory of cancer (Darlington, 1948). Recent studies show that the capacity of mitochondria isolated from tumour tissues for the synthesis of proteins (Graffi, Butschak, Schneider & Kuhn, 1965; Graffi, Butschak, Kuhn & Schneider, 1966) and lipids (Butschak, 1967) *in vitro* is lower than that of mitochondria from normal tissues, whereas the DNA/protein ratio is increased in tumour mitochondria (Wunderlich, Schütt & Graffi, 1966). The occurrence of circular molecules of double size was observed in miDNA isolated from human leukaemic leucocytes but not in miDNA of normal leucocytes (Clayton & Vinograd, 1967, 1969).

The binding of a number of carcinogens with different structures (or metabolites derived from them) to DNA and its bases has been shown (cf. Brookes, 1966). This binding is now generally assumed to be a necessary, if not sufficient, condition for carcinogenic action. The investigations so far carried out have considered only the total cell DNA, of which about 99–99.5% is chromosomal. In the present work the reaction of a chemical carcinogen with extrachromosomal DNA *in vitro* and *in vivo* has been studied for the first time.

* Abbreviations: miDNA, mitochondrial DNA; MNU, *N*-methyl-*N*-nitrosourea.

The defined reaction *in vivo* of a carcinogenic *N*-nitroso compound (dimethylnitrosamine) with the DNA of a target organ was first shown by Magee & Farber (1962), and was later demonstrated for numerous other *N*-nitroso compounds (cf. Magee & Barnes, 1967). After administration of dimethylnitrosamine (Magee & Farber, 1962) or MNU (Swann, Craddock & Magee, 1965) to rats 7-methylguanine was detected as the main methylated product in the nucleic acids. This supported the hypothesis that the alkylation of cellular macromolecules is of fundamental importance in carcinogenesis (see Magee & Barnes, 1967; Magee, 1969). However, there were some objections to this hypothesis: non-carcinogenic compounds may also have a relatively strong alkylating effect (Swann & Magee, 1968), and in some cases no alkylation was detected after administration of carcinogenic nitroso compounds (Lijinsky & Ross, 1969). It is also possible that the DNA carbamoylation shown by using [*carboxy*-^{14}C]MNU *in vitro* (Serebriany, Smotriaeva, Krugliakova & Kostianovski, 1969) or the *O*-6 alkylation of deoxyguanosine (Loveless, 1969) have a biological significance.

MNU is a versatile and extremely potent carcinogen that spontaneously decomposes heterolytically and, in contrast with nitrosamines, needs no enzymic activation. After single intravenous (Druckrey, Steinhoff, Preussmann & Ivankovic, 1964) or oral (Leaver, Swann & Magee, 1969) doses tumours can be induced in many organs of the rat. MNU proved to be one of the strongest carcinogens on topical application to the skin (Graffi, Hoffmann & Schütt, 1967). Therefore, in our opinion, MNU was particularly suitable for our experiments.

Preliminary reports on parts of these experiments have been published (Graffi, 1967; Schütt, Wunderlich & Graffi, 1967; Schütt, Wunderlich, Böttger & Graffi, 1968).

MATERIALS AND METHODS

Animals. We used male and female adult rats (150–200g) of our Wistar random-bred colony. Pregnant rats (about day 15 of pregnancy) had a weight of 215–235g. The embryos were used whole, only their heads being rejected.

Administration of MNU in vivo. Animals that had been kept without food for 1 day, but given water *ad lib.*, were injected intraperitoneally with a freshly prepared solution of [*Me*-^{14}C]MNU (specific radioactivity 1.2–2.5mCi/mmol, radiochemical purity >99%; Zentralinstitut für Kernforschung, Dresden, German Democratic Republic) in 0.15M-NaCl. The total dose per animal was 7–12mg, corresponding to 36.5–58.3mg (0.35–0.55mmol) or about 700μCi/kg body wt. This amount was injected every 2h in three single doses each of 1.0ml. At 6h after the first injection (made between 5a.m. and 7a.m.) the animals were killed by cervical dislocation and the organs were removed under sterile conditions.

Isolation of cell fractions. The work was done under sterile conditions at 0–2°C. The tissue was homogenized in medium A (0.25M-sucrose–1mM-EDTA–20mM-tris–HCl, pH7.4) with a Teflon pestle in a Potter–Elvehjem-type homogenizer. The homogenate was diluted with medium A (0.1g fresh wt. of tissue/ml). The cell nuclei were sedimented at 430*g* (15min) and purified by the method of Widnell & Tata (1964). The separation of the nuclei was completed by two centrifugations at 830*g* (each 10min) of the supernatant obtained at 430*g*. The mitochondria were sedimented at 7200*g* (15min) and then washed once with medium A and again with medium A without EDTA. The mitochondrial suspension was then treated with pancreatic deoxyribonuclease and ribonuclease (200μg of each enzyme/ml or about 10μg enzyme/mg of mitochondrial protein, for 40min at 37°C; addition of $MgCl_2$ to final concn. of 5mM). The incubation was stopped by adding an ice-cold solution of EDTA (final concn. 1mM). The mitochondria were centrifuged down and then washed twice with medium A and finally once with 0.79M-sucrose–0.1M-NaCl–0.1M-EDTA–20mM-tris–HCl medium, pH7.0. The degree of purification of the mitochondria was checked by microscopic inspection of smears stained with Feulgen reagent, and the mitochondria were tested for their bacterial content by the plate test. The average yield of purified mitochondria amounted to 6.8mg (liver), 3.4mg (kidney) or 1.1mg (embryo) of mitochondrial protein/g fresh wt.

Incubation in vitro. Because the medium described below had been shown to maintain the structure of mitochondria for long periods of incubation (Graffi, 1967) it was used for incubation of isolated cell fractions with [^{14}C]MNU *in vitro*. It consisted of equal parts (v/v) of Eagle's medium without serum and Kroon's medium (Kroon, 1965). The mixture contained in addition 85mg of sucrose, 200μg of α-oxoglutaric acid, 12μg each of all ribo- and deoxyribo-nucleosides as well as 1mg of streptomycin and 500 i.u. of penicillin per ml. The pH value was 7.4. Volumes containing about 0.1–0.2g wet wt. of mitochondria or nuclei/ml of incubation solution were incubated with [^{14}C]MNU (125 or 250μg/ml, corresponding to 1.5 or 3μCi/ml respectively) for 1–4h at 26°C with continuous shaking in air. Light was excluded during the incubation. An excess of non-radioactive MNU was added to a control portion of the incubation mixture immediately after it had been prepared, and to the remaining samples at the end of incubation. The cell particles were then sedimented for 15min at 1000*g* (nuclei) or 20000*g* (mitochondria). The DNA isolated from the control portion had absolutely no radioactivity.

Isolation of DNA. As described by Böttger, Wunderlich, Schütt, Förster & Graffi (1968), the mitochondria (at least 5–7g wet wt.), suspended in 1–1.5vol. of 0.79M-sucrose–0.1M-NaCl–0.1M-EDTA–20mM-tris–HCl medium, pH7.0, were lysed for 40min at 0°C with sodium dodecyl sulphate (final concn. 2%, w/v). At the end of the lysis 5M-$NaClO_4$ was added dropwise (final concn. 1M). The miDNA was then deproteinized five or six times (each for 15min at 0°C) with phenol (twice-distilled)–chloroform–3-methylbutan-1-ol (25:24:1, by vol.) and precipitated with ethanol. The precipitate was dissolved in 1.0ml of 0.15M-NaCl–15mM-sodium citrate, pH7.0, and successively incubated with heat-treated ribonuclease (180μg/ml for 6h at 0°C), α-amylase (90μg/ml for 6h at 0°C), and

pronase (70 μg/ml for 2h at 20°C). The miDNA was then purified by gel filtration on a column (26cm × 1.2cm) of Sephadex G-200 with 0.15M-NaCl. The excess of ^{14}C radioactivity was removed quantitatively in this way. As shown in separate experiments, the material eluted first from the Sephadex column was sensitive to deoxyribonuclease action and resistant to hydrolysis by alkali (1M-NaOH for 2h at 37°C). Further, it had a typical DNA spectrum ($\lambda_{max.}$ 259nm, $\lambda_{min.}$ 233nm). The yield of purified miDNA was on average 25% of the amount that could be expected after the determinations of total content made by Wunderlich *et al.* (1966). The incubation *in vitro* of mitochondria resulted in a decrease of DNA yield with incubation time (after 2h 14%, after 4h 8.5% of the total content). After incubation for 4h, however, it was still possible to isolate high-molecular-weight miDNA.

The isolation of nuclear DNA was done in the same way; the nuclei, however, were lysed in 20–25vol. of 0.79M-sucrose–0.1M-NaCl–0.1M-EDTA–20mM-tris–HCl medium, pH7.0. After Sephadex chromatography the DNA fractions were dialysed for 10h at 0°C against 1mM-NaCl. Then they were concentrated in a vacuum rotatory evaporator.

Isolation of RNA and protein. (*a*) Mitochondrial RNA. The mitochondrial pellet was taken up in 2vol. of 0.15M-NaCl with 5mg of polyvinyl sulphate/ml. After lysis with sodium dodecyl sulphate (final concn. 2%) five deproteinizations were carried out each for 15min at 20°C with 0.15M-NaCl-saturated phenol. The aqueous upper phase finally obtained was treated with 2.5vol. of ethanol, the precipitate that formed being dissolved in 0.15M-NaCl–5mM-$MgCl_2$ and successively incubated with 25 μg of deoxyribonuclease/ml (1h at 37°C) and 70 μg of pronase/ml (2h at 20°C). The material was then fractionated on a column (34cm × 1.2cm) of Sephadex G-50 with 0.15M-NaCl.

(*b*) rRNA. As with mitochondria, the microsomal fraction (sedimented for 90min at 105000*g*) was lysed and deproteinized with phenol five times. Solid NaCl (final concn. 2M) was added to the aqueous phase to precipitate the RNA. The precipitate was dissolved in water, treated successively with deoxyribonuclease and pronase as mentioned above, and chromatographed on a column (35cm × 3.0cm) of Sephadex G-200 with 0.15M-NaCl.

(*c*) tRNA. The postmicrosomal supernatant was treated with 1vol. of 0.15M-NaCl-saturated phenol five times each for 10min at 20°C, and the aqueous phase was treated with 2.5vol. of ethanol. The precipitate that formed was dissolved in 0.15M-NaCl, and the solution was successively incubated with deoxyribonuclease and pronase and then purified on a column (34cm × 1.2cm) of Sephadex G-50 with 0.15M-NaCl.

After the gel filtration of the various RNA species the same methods as described above for DNA (dialysis etc.) were used.

(*d*) Proteins. The proteins were isolated by a slight modification of the Magee & Hultin (1962) procedure from the interphase protein obtained in the DNA isolation and freed from phenol by washing with ethanol and ethanol/ether. The nucleic acids were removed by twice-repeated extractions with 10% (w/v) NaCl (1h at 100°C) and 5% (w/v) trichloroacetic acid (20min at 90°C). The lipids were extracted from the proteins by washing and then the proteins were dried and dissolved in hot 1M-NaOH. Samples were taken for measurement of radioactivity and for determination of protein.

Analytical determinations. DNA was determined spectrophotometrically ($E^{1\%}_{1cm}$ 220) or by the reaction with diphenylamine (Burton, 1956) on a Unicam SP.700 spectrophotometer; in some cases microcuvettes (0.5ml, 1cm light-path) were used. Deoxyguanine (Fluka A.-G., Buchs, Switzerland), deoxyribose and herring-sperm DNA (both Serva, Heidelberg, Germany) were used as standards. RNA was assayed by the orcinol reaction (Militzer, 1960) with ribose (Serva) as standard. Protein was determined by the method of Itzhaki & Gill (1964) with bovine serum albumin (Forschungsinstitut für Impfstoffe, Dessau, German Democratic Republic) as standard.

Determination of ^{14}C radioactivity. This was carried out in Bray's (1960) solution with a Packard Tri-Carb model 4322 liquid-scintillation spectrometer. Quenching was corrected for by using automatic external standardization. The counting efficiency was 45–75%. If counting rates were low, at least 800 counts (not corrected) were taken twice and the results averaged. The background was 20–30c.p.m. Significant counting rates were always at least twice this background.

Physical characterization of the miDNA. Each miDNA preparation was tested by means of moving-boundary centrifugation on stabilizing D_2O gradients (see Böttger *et al.* 1968) in the Beckman Spinco model E analytical ultracentrifuge. Low-molecular-weight components, which could be expected if there were contamination by degraded nuclear DNA, were not observed. This procedure separates 39S and 27S components, which correspond to the twisted and open circular forms of miDNA. The latter arises from the covalent closed ring by single-strand scission (see M. M. K. Nass, 1969). In experiments with miDNA that had not been treated with MNU (M. Böttger, V. Wunderlich & W. Kuhn, unpublished work) the specific circular nature of these components was proved by interaction with ethidium bromide (Bauer & Vinograd, 1968). In analyses of miDNA from liver and kidney that had been exposed to MNU *in vivo* it was frequently possible to demonstrate only the homogeneous $s_{20,w}$ 27S component but in about a third of the experiments the $s_{20,w}$ 39S component could also be observed. It is conceivable that a part of the 39S component was converted into the 27S component by the action of MNU, because *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Olson & Baird, 1969) and other monoalkylating agents (Boyce & Farley, 1968) can induce single-strand scissions in DNA. Our experiments, however, gave no significant hint of this possibility. The 39S component was not converted into the 27S form by treatment with non-radioactive MNU *in vitro* (2mg/ml in 0.15M-NaCl, pH8.6, 30min incubation at 20°C).

Embryo miDNA was degraded after the deoxyribonuclease incubation at 37°C used in the experiments with MNU. In another connexion, however, Böttger *et al.* (1968) have shown that after deoxyribonuclease treatment at 0°C, but otherwise under the same conditions, high-molecular-weight DNA can be isolated from embryo mitochondria in which no nuclear DNA contamination can be shown.

Hydrolysis and chromatography of the alkylated nucleic

acids. Both DNA and RNA were hydrolysed in 1 M-HCl for 1 h at 100°C. The hydrolysate was fractionated on a column (10 cm × 1 cm) of Dowex 50W (X8; H^+ form; 200–400 mesh) (Magee & Farber, 1962) with a gradient from 1 M- to 4 M-HCl (each 100 ml), the flow rate being 40 ml/h. 7-Methylguanine (Sigma Chemical Co., St Louis, Mo., U.S.A.) was used as carrier in experiments with small amounts of DNA (<80 μg). In all cases a radioactive peak was eluted between guanine and adenine. To minimize the error in determining radioactivity the eluates from several 8 ml fractions were combined in miDNA fractionations. The sum of the counts recovered in the single fractions was 97–101% of the amount placed on the column. The E_{260} value of the combined fractions was measured against water. Guanine and adenine were identified by their u.v. spectrum. The radioactive material that was eluted in the position expected for 7-methylguanine was insufficient for u.v.-spectroscopic identification. For measurement of the radioactivity the eluate was evaporated to dryness and the residue taken up into water.

The radioactive substance eluted between guanine and adenine was examined by paper chromatography in some experiments. Non-radioactive 7-methylguanine was added as carrier. After 6 h ascending chromatography on Schleicher–Schuell no. 2045a paper the position of the base was determined under a u.v. lamp. The chromatogram was cut into pieces, and the radioactivity of the strips obtained was measured in Bray's (1960) solution. The solvents were methanol–concn. HCl–water (7:2:1, by vol.) (R_F of 7-methylguanine 0.28) or butan-1-ol–acetic acid–water (4:1:1, by vol.) (R_F of 7-methylguanine 0.31). Approx. 95% of the radioactivity was found in the 7-methylguanine fraction. There was no ^{14}C radioactivity at the origin.

RESULTS

Experiments in vitro. The action of MNU on miDNA and nuclear DNA was first studied *in vitro* by incubating purified mitochondria or cell nuclei with the ^{14}C-labelled carcinogen. The DNA was then isolated from both cell fractions and its specific radioactivity was determined. The disadvantage of this method was that, owing to the different conditions for incubating the cell fractions, it was not possible to compare directly the incorporation rates for miDNA and nuclear DNA. An advantage, however, was that in this method relatively large amounts of mitochondria could react with a quantity of the expensive carcinogen that was small in comparison with the experiments *in vivo*, so that we had sufficient material for isolating and fractionating the DNA. As shown by electron microscopy, the mitochondria were progressively damaged during the incubation with MNU. Similar changes were previously observed in mitochondria of *Paramaecium* exposed to MNU (Graffi, 1967).

Fig. 1 shows the results obtained after the incubation of mitochondria and cell nuclei from rat liver and rat embryo with [^{14}C]MNU *in vitro*. The absolute extent of the incorporated radioactivity was proportional to the amount of available radioactivity (not shown): on decreasing the concentration of [^{14}C]MNU from 3 to 1.5 μCi/ml the specific radioactivity of DNA was also approximately halved. The relatively smaller increase of

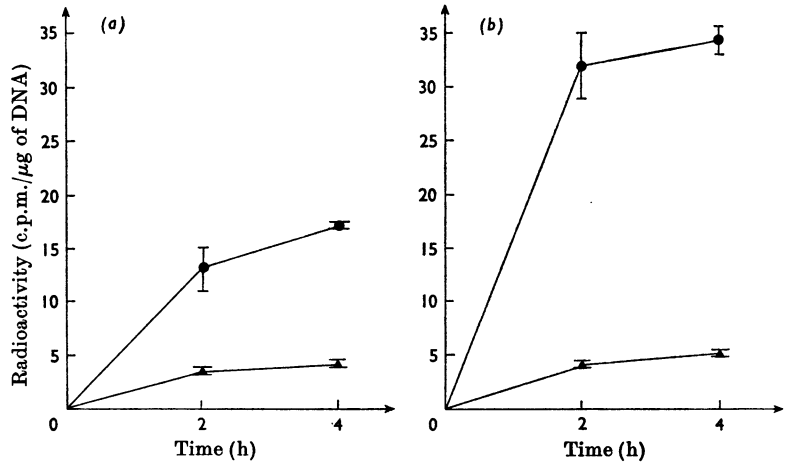

Fig. 1. Dependence of the specific radioactivity of isolated DNA on time of incubation with [^{14}C]MNU. (*a*) DNA of mitochondria (●) and nuclei (▲) from rat liver; (*b*) DNA of mitochondria (●) and nuclei (▲) from rat embryo. Purified mitochondria or nuclei were incubated with [^{14}C]MNU (250 μg or 3 μCi/ml) *in vitro*. The DNA was then isolated from both cell fractions and its specific radioactivity was determined. Each point represents the average of at least two independent experiments. The controls not incubated were completely free of radioactivity. The vertical bars indicate S.E.M.

the labelling after 4h incubation than after 2h can be explained by the instability of MNU in aqueous medium. The significantly higher incorporation of ^{14}C into the DNA of embryo mitochondria than into the miDNA of liver must be emphasized. The DNA of purified nuclei was methylated to an extent one-third to one-seventh that of the miDNA on incubation of these nuclei *in vitro* under the same conditions. The difference between the labelling *in vitro* of embryo DNA and liver DNA, however, was found only with the mitochondria.

Other experiments were performed to fractionate the isolated DNA species after acid hydrolysis on Dowex 50 columns. We had only very small amounts of DNA (on average 70μg). Table 1 presents the analysis of DNA species that were isolated from the mitochondria or nuclei incubated *in vitro* with [^{14}C]MNU. The main amount of the ^{14}C radioactivity added to the column was eluted as a homogeneous fraction between guanine and adenine (see also Fig. 2). Referred to 100μg of DNA the radioactivity determined in this fraction showed an increase proportional to the incubation time for miDNA from 646c.p.m. (1.5h) to 896c.p.m. (3h) and for nuclear DNA from 314c.p.m. (2h) to 408c.p.m. (4h). Paper chromatography revealed that the radioactive material had the same behaviour as authentic 7-methylguanine. The other fractions showed only a small part of the ^{14}C radioactivity. The material eluted in the adenine fraction, probably 1- and 3-methyladenine (Lawley, Brookes, Magee, Craddock & Swann, 1968), was not identified.

The different extents of alkylation of miDNA and nuclear DNA could not be shown when the isolated DNA species were treated with [^{14}C]MNU *in vitro* (Table 2).

Experiments in vivo. After administration of [^{14}C]MNU *in vivo* the preparation of the cell fractions as well as the isolation of DNA were done in the same way as in the experiments *in vitro*. As described in the Materials and Methods section the

Table 1. *Distribution of radioactivity among fractions after ion-exchange chromatography of DNA hydrolysates*

The DNA was isolated from purified rat liver nuclei and mitochondria incubated with [^{14}C]MNU (3μCi/ml) *in vitro*. The DNA was hydrolysed and chromatographed as described in the Materials and Methods section. Results are given as means±average deviation, with numbers of experiments in parentheses. Py, pyrimidine nucleotides; G, guanine; 7-MeG, 7-methylguanine; A, adenine.

	Distribution of radioactivity (% of total)			
Cell fraction	Py	G	7-MeG	A
Nuclei (2); incubation for 2 or 4h	3.2±0.6	1.2±0.3	88.7±0.3	4.6±0.4
Mitochondria (3); incubation for 1.5 or 3h	16.4±5.2	1.3±0.3	77.4±4.3	3.5±0.4

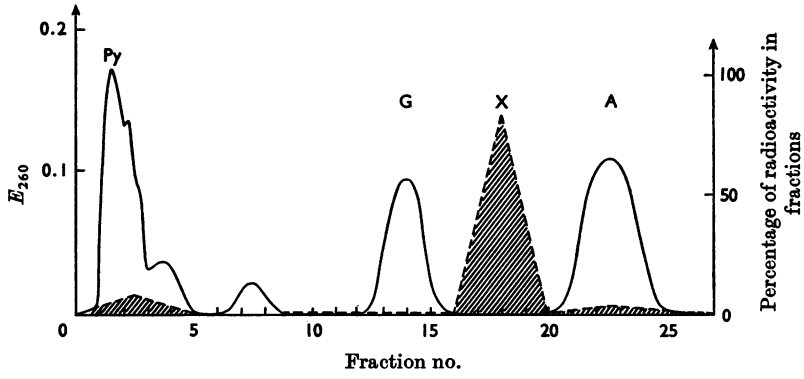

Fig. 2. Ion-exchange chromatography of a hydrolysate of miDNA (100μg) on Dowex 50 (10cm × 1cm column). Elution was with a gradient from 1M- to 4M-HCl. Isolation of DNA from rat liver mitochondria incubated with [^{14}C]MNU *in vitro* was carried out as described in the text. Hatched area, percentage of total radioactivity in combined fractions; ——, E_{260}. The fraction volume was 8ml. Py, pyrimidine nucleotides; G, guanine; A, adenine. The radioactive peak X was shown to be 7-methylguanine.

Table 2. *Reaction of isolated DNA with* [^{14}C]*MNU* in vitro

100 μg of nuclear DNA or miDNA isolated from rat liver, dissolved in 4.0 ml of 0.15 M-NaCl, pH 7.4, were incubated with 3 μCi of [^{14}C]MNU/ml for 4 h at 20°C. After incubation the DNA was separated from the mixture by gel filtration on a column (36 cm × 1.2 cm) of Sephadex G-25 and then precipitated with ethanol followed by acid hydrolysis and chromatography on Dowex 50 columns as described in the Materials and Methods section. Abbreviations are as in Table 1.

	Sp. radioactivity (c.p.m./μg of DNA)	Distribution of radioactivity (%)			
		Py	G	7-MeG	A
Nuclear DNA	27.0	5.3	1.4	87.0	4.7
miDNA	26.0	14.7	0.3	79.6	4.1

Table 3. *Specific radioactivities of DNA species in the experiments with MNU* in vivo

Results are given as means ± S.D., for the numbers of DNA isolations given in parentheses.

Total no. of rats	Dose* (μCi/kg)	Dose* (mg of MNU/kg)	Organ	Sp. radioactivity (c.p.m./μg of DNA) miDNA	Sp. radioactivity (c.p.m./μg of DNA) Nuclear DNA	miDNA/nuclear DNA sp. radioactivity ratio
14	704	58.3	Liver	33.5 (1)	1.98 ± 0.3 (7)	16.9
16	664	52.7	Liver	32.9 (1)	5.3 ± 0.4 (4)	6.2
30	684	55.5	Kidney and heart	22.0 (1)	5.0 ± 1.5 (4)	4.4
32	713	43.3	Liver	5.9 ± 0.4 (3)	1.99 ± 0.1 (6)	2.9
32	713	43.3	Kidney and heart	11.1 (1)	1.47 ± 0.1 (2)	7.5

* The specific radioactivities of batches of MNU were different.

isolated DNA was identified in the analytical ultracentrifuge.

According to experience at our Institute (A. Graffi, & F. Hoffmann, unpublished work) the injected amount of MNU (about 50 mg/kg) is a carcinogenic dose after a single administration. Druckrey, Preussmann, Ivankovic & Schmähl (1967) observed a tumour frequency of 58% after a single, somewhat higher intravenous dose (64 mg/kg body wt.). MNU decomposes very rapidly *in vivo* with the formation of alkylating carbonium ions. According to Swann's (1968) measurements the MNU concentration determined in blood after a single intravenous dose of 100 mg of MNU/kg decreased to less than 5% of the initial amount within 15 min.

The specific labelling of the miDNA and nuclear DNA was determined after administration of MNU *in vivo* in several experiments. Despite some variation the specific radioactivity of the miDNA was greater than of the nuclear DNA in all cases (Table 3). The difference in alkylation seemed to be more pronounced when higher doses were used. The DNA from mitochondria of heart and kidney was isolated together because of the lack of material, although tumours can be induced by MNU in the kidney but not in the heart. The samples of nuclear DNA from both organs had nearly identical specific radioactivities. After administration of [^{14}C]MNU to pregnant rats the nuclear DNA of the embryo was alkylated to an extent about three times that of the nuclear DNA of the maternal liver (Wunderlich & Tetzlaff, 1970).

The separation of the DNA hydrolysates on Dowex 50 columns gave results (Table 4, see also Fig. 3) similar to those of the experiments *in vitro*. Material was used from experiments where there was only a relatively small difference in the extent of alkylation of miDNA and nuclear DNA when the animals were given a dose of 43.3 mg/kg body wt. The amounts of DNA or radioactivity taken for fractionation were predetermined on samples. For separation of the nuclear DNA we used an amount of radioactivity in Expt. IV that corresponded to the experiments with miDNA. A reproducible separation of the DNA hydrolysates was obtained even with the relatively small amounts used, and most of the radioactivity was found in the 7-methylguanine fraction. As in the experiments *in vitro* (Table 1), the proportion of radioactivity obtained in the pyrimidine nucleotide fraction was considerably higher in the miDNA than in the

Table 4. *Fractionation of DNA hydrolysates from mitochondria and nuclei of the liver of rats given* [^{14}C]*MNU* in vivo

Each rat received 43.3mg MNU/kg body wt. Procedures for hydrolysis and chromatography of the DNA species and for determination of small amounts of radioactivity are described in the Materials and Methods section. Abbreviations are as in Table 1.

Cell fraction	Expt. no.	DNA applied to column (μg)	Radioactivity applied to column (c.p.m.)	Distribution of radioactivity (%) Py	G	7-MeG	A	Remainder
Mitochondria	I	41.3	235	24.3	3.0	55.7	17.0	—
	II	51.3	328	18.4	4.9	60.4	12.9	3.3
	III	55.1	305	39.4	2.9	47.0	8.4	2.2
			Average:	27.3	3.6	54.4	12.8	
Nuclei	IV	112.1	228	7.5	6.1	69.4	12.7	4.4
	V	238.0	438	8.4	6.3	73.5	8.6	3.3
	VI	1364	2712	6.3	7.9	73.3	11.8	0.8
			Average:	7.4	6.8	72.1	11.0	

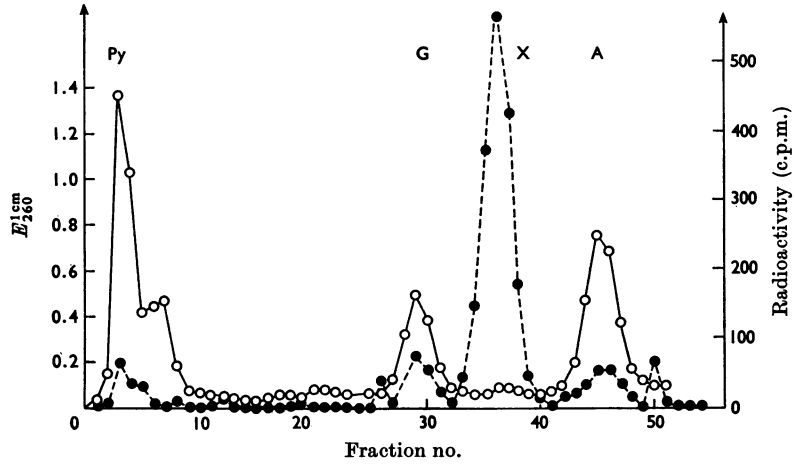

Fig. 3. Ion-exchange chromatography of nuclear DNA (1.36mg) from the liver of rats given 43.3mg of [^{14}C]MNU/kg body wt. intraperitoneally. The isolated DNA was hydrolysed in 1M-HCl at 100°C for 1h and chromatographed on Dowex 50 (10cm × 1cm column) with 1M- to 4M-HCl gradient elution. Fractions (4ml) were collected. ----, Radioactivity; ——, E^{1cm}_{260}. The radioactive peak X was shown to be 7-methylguanine. Other abbreviations are as in Fig. 2.

nuclear DNA. It must be assumed that this miDNA fraction included some ^{14}C-labelled contaminants, but their nature was not further examined. These contaminants might have caused erroneous results in the specific radioactivities of DNA shown in Table 3. The higher specific labelling, however, is also significant when the amount of radioactivity found in the pyrimidine nucleotide fraction (27.3% and 7.4% for miDNA and nuclear DNA respectively; see Table 4) is subtracted from the total radioactivity.

The preferential alkylation of the miDNA *in vivo* and in the isolated cell fraction *in vitro* is also shown by a comparison of the degrees of methylation of guanine (Table 5). The extent of alkylation of guanine, however, in isolated miDNA and nuclear DNA incubated with MNU is practically the same. The number of altered base triplets can be estimated from the degree of alkylation of guanine. One in 3000 and one in 1280 of the triplets was methylated *in vivo* by a dose of 43.3mg of MNU/kg body wt. in the nuclear DNA and the miDNA respectively. The extent of alkylation of the nuclear DNA found in the experiments *in vivo* corresponds approximately to the results obtained by Swann & Magee (1968), according to which in the nuclear DNA of

Table 5. *Extent of methylation of guanine of the miDNA and nuclear DNA from rat liver after reaction with* [^{14}C]*MNU* in vitro *and* in vivo

The extent of methylation of guanine was calculated from the base composition of the DNA species. The values of the proportions of guanine (miDNA, 19.4% G; nuclear DNA, 21.0% G) were taken from Schneider & Kuff (1965).

Experimental conditions	Extent of methylation of guanine (%)		miDNA/nuclear DNA guanine methylation ratio
	miDNA	Nuclear DNA	
Isolated DNA, 4h incubation *in vitro*, 3μCi/ml	0.69	0.76	0.85
Mitochondria or cell nuclei, 3h incubation *in vitro*, 3μCi/ml	0.378	0.141	2.7
In vivo, 43.3mg/kg body wt. intraperitoneally	0.134	0.053	2.5

Table 6. *Labelling of RNA and protein of rat liver after reaction with* [^{14}C]*MNU* in vitro *and* in vivo

	No. of expts.	Dose	Sp. radioactivity (c.p.m./μg)	miDNA/RNA sp. radioactivity ratio
RNA *in vitro*				
Mitochondrial RNA, 2h incubation	2	3μCi/ml	7.3	1.7
Mitochondrial RNA, 4h incubation	2	3μCi/ml	13.2	1.3
RNA *in vivo*				
Mitochondrial RNA	3	58.3mg/kg	17.1	2.0
rRNA	4	58.3mg/kg	4.5	7.4
tRNA	3	58.3mg/kg	7.0	4.8
				miDNA/protein sp. radioactivity ratio
Mitochondrial protein				
in vitro				
2h incubation	1	3μCi/ml	5.7	2.2
4h incubation	1	3μCi/ml	4.2	4.0
in vivo	1	58.3mg/kg	9.0	3.7
	2	43.3mg/kg	3.8	1.5

Table 7. *Ion-exchange chromatography of rat liver mitochondrial RNA after reaction with* [^{14}C]*MNU*

The RNA was isolated from purified mitochondria incubated with [^{14}C]MNU (3μCi/ml) *in vitro* and from rats given 36.5mg of [^{14}C]MNU/kg body wt. intraperitoneally. Acid hydrolysis and ion-exchange chromatography were as described in the Materials and Methods section.

Material	Sp. radioactivity (c.p.m./μg of RNA)	Distribution of radioactivity (%)			
		Py	G	7-MeG	A
Mitochondrial RNA *in vitro*	7.3	4.5	2.3	82.4	9.3
Mitochondrial RNA *in vivo*	9.4	10.2	3.2	75.8	8.5

liver 0.12% and of the kidney 0.11 or 0.075% respectively of the guanine residues were methylated 4h after single intravenous or oral administration of 90mg of MNU/kg body wt. to rats. The dose given in the experiment *in vivo* shown in Table 5 amounted to about half the dose in the experiments of Swann & Magee (1968). Because we have found an alteration of 0.05% of the guanine a proportionality also exists between the amount of MNU given and the extent of alkylation of guanine *in vivo*. The extent of alkylation seems to be independent of the route of administration at least in the liver and kidney.

The small amount of miDNA obtained did not allow determination of the content of RNA and protein. Therefore we cannot exclude the possibility

that the miDNA was still contaminated by proteins or RNA of high specific radioactivity. RNA contaminants in particular could introduce errors into the measured amount of radioactivity found in the 7-methylguanine fraction of the miDNA hydrolysates. We therefore studied the alkylation of RNA and protein from mitochondria as well as of tRNA and rRNA (Table 6). A smaller specific labelling of the non-DNA fractions, in contrast with the miDNA, was found in all cases. Thus it is practically impossible that the finding of a preferential alkylation of the miDNA is falsified by contamination. It is of note that the mitochondrial RNA was the most strongly alkylated of the RNA species investigated. The ^{14}C radioactivity incorporated into the RNA was again found mainly in the 7-methylguanine fraction (Table 7).

DISCUSSION

The purpose of the studies presented here was to test whether the action of the carcinogen MNU causes an alkylation of the miDNA *in vitro* and *in vivo*. The experiments showed that *in situ* the miDNA is alkylated to a higher extent than the nuclear DNA whereas DNA isolated from mitochondria and nuclei was alkylated to nearly the same extent.

Experiments *in vivo* on the action of a chemical carcinogen with miDNA can be performed virtually only on liver, because the yield of mitochondria from other organs is so small that the amounts of DNA isolated are not sufficient for exact determination of the specific radioactivity and for subsequent fractionation.

In general, MNU does not induce liver tumours (Druckrey *et al.* 1967; Leaver *et al.* 1969); in some cases, however, after intravenous injection of MNU, liver tumours were observed (A. Graffi & F. Hoffmann, unpublished work). The relevance of the results described here for the process of carcinogenesis is therefore not yet understood. The miDNA isolated from kidney, in which MNU easily causes tumours, was also preferentially alkylated; but because of lack of material we had to restrict our investigations to some experiments with a mixture of miDNA from kidney and heart. In preliminary experiments with [^{14}C]dimethylnitrosamine (single intraperitoneal dose of 30mg/kg body wt.), a typical carcinogen for liver, we also found a preferential alkylation of the miDNA from rat liver *in vivo* (V. Wunderlich, I. Tetzlaff & A. Graffi, unpublished work).

Recently it was shown (Alexandrov, 1969; S. Ivankovic, personal communication) that MNU induces malignant tumours after transplacental administration. Thus, the finding that alkylation of embryo miDNA *in vitro* was about twice that of liver miDNA could be related to the susceptibility of embryonic tissues to MNU. In experiments with MNU *in vivo* also a high extent of alkylation of embryonic nuclear DNA was found (Wunderlich & Tetzlaff, 1970).

There are several possible explanations for the preferential alkylation of miDNA by MNU. (1) The histone-free miDNA could be attacked by MNU more easily than nuclear DNA covered with histones. (2) It is conceivable that DNA molecules involved in synthesis are specifically sensitive towards alkylation. The rate of DNA synthesis in rat liver was found to be two- to 14-fold higher in mitochondria than in nuclei (Schneider & Kuff, 1965; Neubert, 1966; Chang & Looney, 1967). This ratio seems to vary with the age of the animals (Neubert, 1966). The ratios of alkylation *in vivo* of miDNA to nuclear DNA were found to be very similar in our experiments with rats of corresponding age. A proportionality between the extent of alkylation of nuclear DNA in various organs of rats and the growth rate of these tissues was also observed after administration of [^{14}C]MNU *in vivo* (Wunderlich & Tetzlaff, 1970). (3) As with carcinogenic hydrocarbons (Graffi, 1940*a*,*b*,*c*), it is possible that the lipophile MNU accumulates in mitochondria, particularly in the membranes rich in lipids, and a concentration gradient is formed within the cell. Specific conditions of permeability of the mitochondrial membranes might also be important for the accumulation of MNU. Since miDNA is attached to the mitochondrial membrane system and since this membrane association seems to be important for replication of miDNA (M. M. K. Nass, 1969) the concentration of MNU could be high in the regions in which replication of miDNA takes place. (4) It is possible that the extent of alkylation is influenced by specific conditions as was shown for the concentrations of cysteine (Schoental, 1967) or phosphate ions (McCalla, 1968) *in vitro*. Possibly such factors operate within cells.

It seems possible that different rates of loss of methyl groups (cf. Magee & Barnes, 1967) in both DNA species are important for the effect of alkylation. Although the reaction between MNU and nuclear DNA or miDNA respectively is fundamentally the same it might have different consequences for the cell independent of the extent of alkylation. Genetic studies with yeast cells (Schwaier, 1969) showed that it is not possible to draw direct conclusions from the karyotic effect of a mutagenic agent to its cytoplasmic action. By using haploid yeast strains it was shown that the carcinogenic nitrosamides *N*-methyl-*N*-nitrosourethane (Schwaier, Nashed & Zimmermann, 1968) as well as 1-nitroso-imidazolidin-2-one (Schwaier, 1969) frequently induce cytoplasmic mutants deficient in respiration apart from karyotic mutants.

The former agent showed a higher cytoplasmic specificity. In contrast, nitrous acid, which is probably non-carcinogenic, did not cause a deficiency of respiration in mitochondria (Schwaier *et al.* 1968). A cytoplasmic mutagenic effect has also been reported for MNU on sunflower plastids (Beletskii, Razoriteleva & Zhdanov, 1969) as well as for the carcinogenic *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine on yeast (Nordström, 1967) and on *Neurospora crassa* (Bertrand & Pittenger, 1968). Likewise, 4-nitroquinoline 1-oxide and several of its carcinogenic derivatives induce respiration deficiency in yeast cells (Mifuchi, Morita, Yamagihara, Hosoi & Nishida, 1963; Nagai, 1969).

It is well known that numerous analogies exist between cytoplasmic mutants of micro-organisms deficient in respiration and cancer cells in animals (cf. Gause, 1966). Further, Schwaier (1969) suggested that mitochondrial mutations have a particular chance of becoming manifest in diploid cells, which are generally present in somatic tissues. Therefore we tend to regard the preferential alkylation of the miDNA in mammalian tissues by MNU described in this paper as a further support for the mitochondrial mutation hypothesis of carcinogenesis presented earlier by Graffi (1940*a*,*b*,*c*, 1967, 1968).

At present, our knowledge of cytoplasmic mutations as well as of the gene products of miDNA in animal cells is still extremely small. In micro-organisms, however, the miDNA seems to contain the genetic information at least for the structural protein of the inner mitochondrial membrane and for the mitochondrial rRNA and tRNA (see M. M. K. Nass, 1969). As compared with wild-type cells, cytoplasmically mutated yeast cells show changes in the miDNA (Mounolou *et al.* 1966; Bernardi *et al.* 1968; Mehrotra & Mahler, 1968) as well as the mitochondrial structural protein (Tuppy, Swetly & Wolff, 1968). These findings and the preferential binding of cytoplasmic mutagens to the miDNA of yeast (Tewari, Vötsch, Mahler & Mackler, 1966) or to the kinetoplastic DNA of trypanosomes (Newton & Le Page, 1967; Simpson, 1968) support the hypothesis that the miDNA is the target for these agents. It is likely that an attack on the miDNA causes an alteration of the whole cell similar to effects obtained with *Neurospora* in which mutated mitochondria can determine the phenotype of a cell (Diacumakos *et al.* 1965). Recently K.-H. von Wangenheim (personal communication) has shown that in plant cells extrachromosomal mutations influence the activity of mitosis and differentiation. Future experiments will show if similar events are important for carcinogenesis in animal cells.

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