Effect of a Single Dose of Dimethylnitrosamine on Biosynthesis of Nucleic Acid and Protein in Rat Liver and Kidney

By B. W. STEWART AND P. N. MAGEE

*Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School,
London W1P 5PR, U.K.*

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1. Administration of a single dose of dimethylnitrosamine to rats temporarily fed on a protein-deficient diet causes a high incidence of kidney tumours. The effect of such a dose of dimethylnitrosamine (40 mg/kg body wt.) on metabolism of nucleic acids and protein in rat liver and kidneys was examined during the week immediately after administration. 2. Incorporation of [^{14}C]leucine and [^{14}C]orotate into hepatic macromolecules was inhibited within 5 h of injection of dimethylnitrosamine, and did not recover for at least 5 days. Interpretation of these results is complicated by the concomitant extensive hepatic necrosis. 3. Renal RNA synthesis was assayed by incorporation of [^{14}C]orotate *in vivo* and measurement of DNA-dependent RNA polymerase activity *in vitro*. Both systems indicate biphasic inhibition; minimal activity was recorded 9 h and 3 days after treatment. Changes in incorporation of [^{14}C]leucine into renal protein were similar but less marked. 4. Sucrose-density-gradient analysis of renal cytoplasmic RNA indicated increased synthesis of rRNA 24 h after injection of the nitrosamine. The rate of loss of radioactivity from kidney ribosomes pre-labelled with [^{14}C]orotate was not modified by dimethylnitrosamine. 5. Dimethylnitrosamine increased incorporation of [^3H]thymidine into renal DNA. The three distinct periods of stimulated synthesis observed are discussed, with particular reference to recently published morphological studies of the sequential development of kidney tumours induced by dimethylnitrosamine in protein-depleted rats.

Administration of a single LD_{50} dose of dimethylnitrosamine to rats causes severe liver damage (Barnes & Magee, 1954) and subsequent development of kidney tumours in about 20% of surviving animals (Magee & Barnes, 1962). The balance of the available evidence suggests that the toxic and carcinogenic effect of nitroso compounds is not a property of the compounds themselves but is due to a chemical reaction during their decomposition, but this is not established conclusively (Magee & Barnes, 1967).

Rats fed on a protein-deficient diet become more resistant to the acute toxic effects of dimethylnitrosamine and the median lethal dose is doubled (Swann & McLean, 1968, 1971). When such an increased dose is administered, all surviving animals develop kidney tumours (McLean & Magee, 1970). The rate of hepatic metabolism of dimethylnitrosamine in the protein-depleted rat is halved and the increased incidence of renal tumours may be attributed to longer persistence of the carcinogen in the body, and hence longer exposure of the susceptible kidney cells.

Observations by Hard & Butler (1970*a,b*, 1971*a,b,c*) on the structure and ultrastructure of the kidney tumours induced in protein-depleted rats by a single dose of dimethylnitrosamine are relevant to the work described below. They have confirmed earlier observations by Magee & Barnes (1962) that there are two distinct types of renal tumour, of epithelial and mesenchymal origin respectively, and claimed that cells indistinguishable ultrastructurally from those of the mesenchymal tumours can be detected within the first week (Hard & Butler, 1971*a*).

Biochemical change produced by dimethylnitrosamine in protein-depleted rats during the week immediately after administration is the subject of the present report. In view of Hard & Butler's (1970*b*) findings and also of the rapid degradation and elimination of dimethylnitrosamine within 24 h of administration it seems that molecular changes essential for the induction of renal tumours occur during this time. In seeking phenomena relevant to the carcinogenic process, dimethylnitrosamine-induced changes in liver and

kidney have been contrasted, since the nitrosamine is metabolized by both tissues but a single exposure to dimethylnitrosamine in the adult rat does not produce liver tumours (Magee & Barnes, 1962). Subsequently, certain modifications of kidney metabolism were subjected to closer examination.

MATERIALS AND METHODS

Chemicals. Dimethylnitrosamine was purchased from Eastman Organic Chemicals, Rochester, N.Y., U.S.A., and purified by distillation (b.p. 151°C). The following radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.: [*Me*-³H]thymidine, 23 Ci/mmol; [6-¹⁴C]orotic acid, 60.8 mCi/mmol; L-[U-¹⁴C]leucine, 312 mCi/mmol; and [8-³H]guanosine 5'-triphosphate, 11.8 Ci/mmol. Nucleoside triphosphates (sodium salts) were purchased from Sigma (London) Chemical Co., London S.W.6, U.K., and laboratory reagents (A.R. grade) from BDH Chemicals Ltd., Poole, Dorset, U.K., or Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Animals. Female Wistar rats of the Courtauld Institute colony were maintained on Rowett Research Institute diet 86 until 5–7 weeks old (body wt. 125–150 g), when they were placed in grid-bottom cages and fed on the protein-deficient diet described by McLean & McLean (1966). Dimethylnitrosamine dissolved in 0.9% NaCl was administered (40 mg/kg body wt.) by intraperitoneal injection 1 week later. Control animals received an equal volume of 0.9% NaCl by the same route. The rats were fed on the protein-deficient diet for a further week before being returned to the complete diet.

Synthesis of nucleic acid and protein in vivo. The rate of synthesis of DNA, RNA or protein was measured by administration of [³H]thymidine (80 μ Ci/kg body wt.), [¹⁴C]orotic acid (8.0 μ Ci/kg) or L-[¹⁴C]leucine (6.4 μ Ci/kg) respectively in 0.9% NaCl by subcutaneous injection. Under sodium phenobarbitone anaesthesia (Nembutal; Abbott Laboratories Ltd., Queenborough, Kent, U.K.), liver and kidneys were rapidly excised 30 min later, frozen in liquid N₂ and stored at -20°C for up to 1 week before analysis. To measure the specific radioactivity of nucleic acids, tissues were treated by the modified Schmidt-Thannhauser method described by Munro & Fleck (1966). After extraction of the hydrolysed RNA, the precipitate remaining was treated as described by Threlfall, Taylor & Black (1966) to extract DNA, and the radioactivities of the respective hydrolysates were determined. RNA was assayed by its *E*₂₆₀ (Munro & Fleck, 1966) and DNA by the Giles & Myers (1965) modification of Burton's (1956) method, with calf thymus DNA (Worthington Biochemical Corp., from Cambrian Chemicals Ltd., Croydon, Surrey, U.K.) as standard.

The specific radioactivity of liver and kidney protein was measured in frozen tissue (about 1.0 g) homogenized in 10 vol. of water. A sample (2.5 ml) of this homogenate was added to an equal volume of 10% (w/v) trichloroacetic acid. The preparation was left for 30 min then centrifuged and the precipitate washed twice at room temperature and then incubated for 20 min at 90°C with separate portions (5 ml) of 5% trichloroacetic acid. The

precipitate was washed with water, extracted successively with 95% (v/v) ethanol, ethanol-diethyl ether (3:1, v/v) and diethyl ether, and finally dissolved in 0.06 M-NaOH (5 ml) by incubation at 40°C for 20 min. Portions of this solution were used for the determination of protein by the biuret method of Gornall, Bardawill & David (1949), and of radioactivity as described below.

DNA-dependent RNA polymerase activity in kidney. DNA-dependent RNA polymerase activity was determined in kidney nuclei isolated by a modification of the method of Blobel & Potter (1966). Kidneys from three rats were pooled and homogenized in 4 vol. of 0.25 M-sucrose–2 mM-MgCl₂ by using 10 strokes of a Teflon-glass homogenizer (pestle speed 1400 rev./min). The preparation was maintained at 2°C throughout the procedure. The homogenate (about 15 ml) was filtered through eight layers of gauze, diluted by addition of 2 vol. of 2.3 M-sucrose–1.0 mM-MgCl₂, and layered over 10 ml of 2.3 M-sucrose–1.0 mM-MgCl₂ in a Spinco SW 25.1 centrifuge tube. This discontinuous gradient was centrifuged for 60 min at 63 500g. The supernatant fluid was decanted, the tube inverted to drain and the nuclear pellet resuspended in 0.25 M-sucrose–1.0 mM-MgCl₂–10 mM-tris-HCl buffer, pH 8.0, at 20°C by using a vortex mixer. This suspension contained 65% of the DNA detected in the crude homogenate. The preparation was not contaminated by whole cells or cell fragments as determined by light microscopy. Incorporation of GMP into RNA, with labelled GTP as substrate, was measured in a reaction mixture that contained in a final volume of 0.25 ml: nuclei containing up to 50 μ g of DNA, 50 mM-tris-HCl buffer (pH 8.0), 1.0 mM-ATP, -CTP and -UTP and 0.12 mM-[³H]GTP (20.9 mCi/mmol). In addition either 5 mM-MgCl₂ or 400 mM-(NH₄)₂SO₄ and 5 mM-MnCl₂ were added (Smuckler & Koplititz, 1969). Incubation was carried out at 37°C. At various times for up to 20 min, samples were deposited on Whatman 3MM filter-paper discs, which were then placed in cold 5% trichloroacetic acid with 1% (w/v) sodium pyrophosphate. The discs were washed twice in 5% trichloroacetic acid, twice in 95% ethanol, twice in diethyl ether and then air-dried (Bollum, 1966) before assay for radioactivity. The time-course of GMP incorporation was determined and the initial velocity expressed as pmol of GMP incorporated/min per mg of DNA.

Sucrose-density-gradient analysis of RNA. Cytoplasmic RNA from the pooled kidneys of four rats was analysed by sucrose-density-gradient centrifugation. Each animal received 32 μ Ci of [¹⁴C]orotate/kg body wt. intravenously followed 30 min later by 25 times this quantity of unlabelled orotate intraperitoneally. At 3 h after the first injection, the kidneys were removed and homogenized in 3 vol. of TKM-sucrose buffer (0.25 M-sucrose–50 mM-tris-HCl–25 mM-KCl–5 mM-MgCl₂, pH 7.5 at 20°C) by using five strokes of a Teflon-glass homogenizer (pestle speed 1400 rev./min). After centrifugation at 10 000g for 15 min, 0.1 vol. of freshly prepared 10% (w/v) sodium dodecyl sulphate was added to the postmitochondrial supernatant. The preparation was stirred for 1 min, then mixed with an equal volume of phenol reagent (phenol, 500 g; *m*-cresol, 70 ml; water, 55 ml; 8-hydroxyquinoline, 0.5 g) and shaken in the cold for 30 min. After centrifugation (10 000g, 20 min) the aqueous phase was re-extracted with half the volume of phenol reagent in the

same way. Then 3 vol. of 70% (v/v) ethanol containing 2% (w/v) sodium acetate was added to the aqueous phase and RNA precipitated by keeping at 2°C for 2h. The RNA was isolated by centrifugation, washed successively with 70% ethanol and then ethanol and dissolved in acetate buffer as used for the sucrose density gradients. The E_{260} was determined and 20–25 E_{260} units were layered on a linear 0.4–1.0M sucrose density gradient (32ml total volume) prepared with ribonuclease-free sucrose (Mann Research Laboratories, New York, N.Y., U.S.A.) in acetate buffer (0.01M-sodium acetate–0.1M-NaCl buffer, pH5.1). Centrifugation was carried out in a Spinco SW 25.1 rotor at 25 000 rev./min (2°C) for 24h. The centrifuge tubes were emptied by means of a narrow-gauge metal tube which extended through the solution to the base of the tube and the contents were analysed by pumping through a Uvicord II photometer. Transmission was converted into absorption with a Unicam SP.45 Concentration Readout Unit. Radioactivity in collected fractions (1.0ml) was measured by mixing the complete fractions with the Triton–toluene scintillation solution. Alternatively, samples (0.1ml) were deposited on Whatman 3MM filter-paper discs; the discs were washed twice in 5% trichloroacetic acid and dried. Radioactivity values detected were independent of the method used.

Turnover of kidney rRNA. After being fed on the protein-deficient diet for 5 days, each of 12 rats was given [^{14}C]orotate (24 $\mu\text{Ci/kg}$ body wt.). Then 2 days later six of these animals were treated with dimethylnitrosamine, the remainder serving as controls. At 9h and 24h after injection of the nitrosamine the animals were killed in groups of three, and the kidneys excised. Right kidneys were individually frozen in liquid N_2 . The pooled left kidneys of each group were homogenized in 10ml of TKM–sucrose buffer by using ten strokes of a Teflon–glass homogenizer, centrifuged (10 000g, 15min) and the postmitochondrial supernatant was centrifuged at 105 000g for 1h (Spinco type 40 rotor). The microsomal pellet was completely resuspended in 2.5ml of water and precipitated by addition of 0.3M- HClO_4 (5ml). RNA contained in this preparation was considered to be rRNA. The radioactivity of this RNA and that of the RNA in the individual whole kidneys ('total' RNA) was determined by the method of Munro & Fleck (1966).

Measurement of radioactivity. A Packard Tri-Carb model 3320 liquid-scintillation spectrometer was used. Filter-paper discs carrying radioactive material were immersed in 0.6% 2,5-diphenyloxazole in toluene, the radioactivity was counted and the results were expressed in c.p.m. This scintillation solution mixed with Triton X-100 (5:1, by vol.) was used for solutions of 1.0M- HClO_4 and those containing sucrose (Patterson & Greene, 1965). Otherwise aqueous samples were mixed with Bray's (1960) scintillation solution. With these systems, corrections from c.p.m. to d.p.m. were made by the channels-ratio method (Baillie, 1960). Efficiency was in the range 50–60% with ^{14}C and 25–30% with ^3H .

Presentation of results. The results are presented as means $\pm$ s.d. with Student's t test for significance of difference between means where appropriate.

Histology. For histological work animals were killed with ether and tissues fixed in 1% CaCl_2 –10% (w/v) formaldehyde. Paraffin sections cut at 7 μm were stained with haematoxylin and eosin.

RESULTS

In contrast with rats fed on a complete diet (Whittle & Potter, 1968; Lebouton & Handler, 1970), diurnal variation of RNA and protein synthesis in liver and kidneys of protein-depleted rats was not detected. Incorporation of labelled orotate and leucine in groups of five rats was measured at 0900, 1100, 1500 and 2000 hours during the day; no significant differences were found. In such control animals these radioactivities remained constant during the period corresponding to the week after dimethylnitrosamine administration. Lack of diurnal variation in leucine and orotate incorporation is consistent with results of Fishman, Wurtman & Munro (1969). They reported that hepatic polyribosome profiles and tyrosine transaminase activity show diurnal rhythms among rats given access *ad libitum* to Purina Chow or to a synthetic dry diet containing 24% protein; both rhythms were absent in rats maintained on a protein-deficient diet.

As expected from earlier work with rats receiving a complete diet (Villa-Treviño, 1967), dimethylnitrosamine caused a marked decrease in amino acid incorporation into liver protein, reaching its lowest point at 9h after the injection (Fig. 1). This decrease was preceded by a transitory rise in incorporation at 2h. The inhibition was followed by a return almost to control values of incorporation

Fig. 1. Specific radioactivity of liver protein labelled with [^{14}C]leucine administered at various times after dimethylnitrosamine treatment. Each point is the mean $\pm$ s.d. of five determinations and is expressed as a percentage of the mean specific radioactivity of hepatic protein from control animals (223 ± 27 d.p.m./mg). This value was determined from 30 experiments and the control range of 1 s.d. is indicated (----).

from 19 to 48h and then by a second marked decrease at 3 days. Inhibition of incorporation of [^{14}C]orotate in the liver also occurred rapidly after injection of dimethylnitrosamine (Fig. 2). Again the maximal depression occurred at 9h, followed by a partial recovery between 12 and 24h and then a second period of decline, reaching its lowest point at 2 days.

Cell division in adult liver and kidney is infrequent. The mean specific radioactivity of DNA labelled with [^3H]thymidine did not vary from day to day during the period under study. There was no change in hepatic DNA synthesis during the first 19h after dimethylnitrosamine. Between 19 and 24h the mean specific radioactivity of DNA increased more than tenfold (Fig. 3) and remained at a high value for the next six days; wide variation between individual dimethylnitrosamine-treated animals was encountered throughout this period, though values in all were severalfold higher than controls.

In contrast with previous observations on rats maintained on complete diets (Magee, 1958), administration of dimethylnitrosamine induced a definite decrease in protein synthesis in the kidney, reaching its lowest value, as in liver, at 9h but returning to control values at 14h and remaining there until the 7th day, apart from a slight depression on the 3rd day (Fig. 4). Dimethylnitrosamine caused a biphasic decrease in RNA synthesis in the kidney (Fig. 5). The earlier depression,

to about 55% of the control value, occurred at the same time as the maximal depression in protein synthesis, but the second and greater decrease occurred between the 2nd and 5th days during which time protein synthesis was minimally affected.

Decreased labelling of macromolecules by radioactive precursors will occur if transport of precursor to the tissue concerned is inhibited by the treatment applied. Since dimethylnitrosamine toxicity characterized by extensive liver necrosis and intraperitoneal haemorrhage (Barnes & Magee, 1954) acid-soluble radioactivity in extracts of liver and kidney was measured when low incorporations were recorded (Table 1). The amount of acid-soluble radioactivity after administration of [^{14}C]leucine 9h or 2 days after dimethylnitrosamine injection was equal to, or slightly greater than that in the liver or kidney of control saline-treated rats. Measurement of acid-soluble radioactivity after injection of [^{14}C]orotate at corresponding times of decreased incorporation indicates a possible decrease in transport of [^{14}C]orotate to the liver as contributing to the lowered incorporation 2 days after administration of the carcinogen. Confirmation of such an effect would require determination of the specific radioactivity of each of the radioactive acid-soluble compounds in preparations from treated and control rats.

The nature of the biphasic inhibition of renal RNA synthesis was investigated further by assay of DNA-dependent RNA polymerase activity in

Fig. 2. Effect of dimethylnitrosamine on hepatic RNA synthesis. [^{14}C]Orotate was administered at various times after dimethylnitrosamine treatment and the specific radioactivity of RNA from liver determined as described in the text. Each point is the mean $\pm$ 1 s.d. of five experiments and is expressed as a percentage of the mean specific radioactivity from 30 control rats (4.49 ± 0.547 d.p.m./ μg of RNA). The control range of 1 s.d. is indicated (----).

Fig. 3. Stimulation by dimethylnitrosamine of DNA synthesis in liver. [^3H]Thymidine was administered at various times to control and dimethylnitrosamine-treated rats. The mean specific radioactivity $\pm$ 1 s.d. from 30 determinations of liver DNA from saline-treated rats was 1934 ± 477 d.p.m./mg, and that from the dimethylnitrosamine-treated rats is expressed as multiples of this value. Each point is the mean of five determinations and the control range of 1 s.d. is indicated (----).

nuclei prepared from the kidneys of control and dimethylnitrosamine-treated rats. The polymerase activity was measured in media of both high and low ionic strength, and control activity was greater in the high-ionic strength medium, as had been found with nuclei from liver (Tata & Widnell, 1966) and kidney (Threlfall, Taylor, Mandel & Ramuz, 1967) tissue of rats fed on complete diets. RNA polymerase activity, assayed in either the presence or the absence of ammonium sulphate, was decreased in animals given dimethylnitrosamine 8h before being killed (Table 2). Thereafter the polymerase activities varied as the orotate incorporation (Fig. 5), showing the same biphasic inhibition and also the recovery 1 and 7 days after dimethylnitrosamine injection.

Two attempts were made to isolate nuclei from the pooled kidneys of three rats 3 days after dimethylnitrosamine treatment. In both experiments the nuclear pellet was almost imperceptible and the amount of DNA contained (0.24 and 0.18mg) was very small compared with that from equal weights of control (15.0mg) and dimethylnitrosamine-treated (9.5mg after 2 days, 8.5mg after 5 days) kidneys. The failure of kidney nuclei obtained from rats 3 days after dimethylnitrosamine treatment to sediment through 2.3M-sucrose may be the result of a decrease in the DNA content and consequently the density of these nuclei. This would be consistent with the work of Fisher, Holbrook & Irwin (1963) who fractionated nuclei from normal and regenerating rat liver by isopycnic

Fig. 4. Specific radioactivity of kidney protein labelled with [¹⁴C]leucine administered at various times after dimethylnitrosamine treatment. Each point is the mean ± 1 s.d. of five experiments and is expressed as a percentage of the mean specific radioactivity of protein from the kidneys of control animals (141 ± 24 d.p.m./mg). This value was determined from 30 experiments and the control range of 1 s.d. is shown (----).

Fig. 5. Effect of dimethylnitrosamine on renal RNA synthesis. [¹⁴C]orotate was administered at various times after dimethylnitrosamine treatment and the specific radioactivity of renal RNA determined. Each point is the mean ± 1 s.d. of five experiments and is expressed as a percentage of the mean specific radioactivity from 30 control rats (16.47 ± 2.20 d.p.m./μg of RNA). The control range of 1 s.d. is indicated by (----).

Table 1. Effect of dimethylnitrosamine on the acid-soluble radioactivity in liver and kidney

[¹⁴C]Leucine and [¹⁴C]orotic acid were administered and tissue was treated as described for determination of nucleic acid and protein synthesis *in vivo*. After centrifugation of the homogenate, portions (0.5 ml) of supernatant were added to Bray's (1960) scintillation solution and radioactivity was determined. The results below are the mean of five determinations and are expressed as d.p.m./0.5 ml of supernatant.

	[¹⁴ C]Leucine			[¹⁴ C]Orotic acid		
	Control	+ Dimethylnitrosamine		Control	+ Dimethylnitrosamine	
		9 h	2 days		9 h	2 days
Liver	69	81	105	2855	2720	1723
Kidney	158	150	208	6318	5857	5714

Fig. 6. Sucrose-density-gradient analysis of kidney cytoplasmic RNA from control (a) and dimethylnitrosamine-treated (b-e) rats. Each sample was prepared from the pooled kidneys of four rats killed 3 h after administration of [^{14}C]orotate. E_{254} , —; ●, radioactivity in 1.0 ml fractions. The labelled precursor was injected 6 h (b), 1 day (c), 2 days (d) and 3 days (e) after dimethylnitrosamine.

sucrose-density-gradient centrifugation and found that the increased density of the nuclei of each fraction reflected the DNA content. Conversely a decrease in kidney nuclear density observed 3 days after dimethylnitrosamine treatment would be consistent with the decrease in DNA content of the

kidney at this time (Table 3). This loss of DNA from kidney is coincident with minimal RNA synthesis.

Both incorporation of [^{14}C]orotate and DNA-dependent RNA polymerase activity *in vitro* indicate little, if any, quantitative modification of

total renal RNA synthesis 24h after injection of dimethylnitrosamine. To detect possible effects of dimethylnitrosamine on different RNA species the pattern of incorporation of [¹⁴C]orotate into cytoplasmic RNA was examined. Sucrose-density-gradient analysis of kidney cytoplasmic RNA indicated no dimethylnitrosamine-induced decrease in the incorporation of labelled precursor. However, during the period from 9h to 3 days after dimethylnitrosamine treatment there were major changes in the pattern of labelling, the most marked effect being observed at 24h (Fig. 6). At this point the radioactivity associated with the rRNA (18S+28S) is three times that detected in the preparation from saline-treated control rats.

A possible result of the administration of dimethylnitrosamine is the degradation of pre-existing kidney ribosomes, perhaps as the result of alkylation of rRNA and probably protein by dimethylnitrosamine, as demonstrated by Villa-Treviño (1967). The results in Table 4 suggest, however, that this does not occur. When rats previously treated with [¹⁴C]orotate were injected with dimethylnitrosamine the rate of loss of radioactivity from the

Table 2. *Effect of dimethylnitrosamine on kidney nuclear DNA-dependent RNA polymerase*

Nuclei were isolated from groups of control and dimethylnitrosamine-treated rats. In a cell-free system, the time-course of incorporation of [³H]GMP in the presence of both low (5mM-MgCl₂) and high [5mM-MnCl₂ + 400mM-(NH₄)₂SO₄] ionic strength was determined by using at least two series of reactions with differing concentrations of nuclei. The mean initial velocity of each activity is expressed in pmol of GMP incorporated/min per mg of DNA. The relative values are expressed as percentages.

Time after dimethylnitrosamine	Polymerase activity	
	Low ionic strength	High ionic strength
0 (Control)	306 (100)	581 (100)
8 h	156 (51)	434 (75)
1 day	216 (70)	581 (100)
2 days	115 (38)	340 (58)
5 days	69 (23)	300 (52)
7 days	259 (85)	565 (97)

Table 3. *Influence of dimethylnitrosamine on rat kidney DNA content*

Kidneys from control and dimethylnitrosamine-treated rats were homogenized and the DNA content determined as described in the Materials and Methods section. Each value given is the mean ± 1 s.d. of five determinations, with Student's *t* test for significance of difference where appropriate.

Time after dimethylnitrosamine (days)	DNA content (mg/g wet wt.)	
	Control	+ Dimethylnitrosamine
1	7.30 ± 0.54	7.41 ± 1.00
3	7.60 ± 0.54	6.43 ± 0.15 (<i>P</i> < 0.025)
4	7.62 ± 0.32	7.41 ± 0.54
5	7.45 ± 0.37	7.30 ± 0.64
7	7.53 ± 0.24	7.84 ± 0.16 (<i>P</i> < 0.05)

Table 4. *Effect of dimethylnitrosamine on the stability of kidney RNA*

Kidneys were pre-labelled with [^{14}C]orotic acid as described in the text. At intervals after injection of dimethylnitrosamine the radioactivity associated with the total RNA of each kidney, as well as that with the microsomal RNA, was determined. Radioactivity is expressed as $10^{-3} \times \text{c.p.m./kidney}$; that of 'total' RNA is the mean $\pm$ 1 s.d. of three determinations and the microsomal results are those from the pooled kidneys of three individuals.

Time after dimethylnitrosamine (h)	'Total' RNA		Microsomal RNA	
	Control	+ Dimethylnitrosamine	Control	+ Dimethylnitrosamine
9	40.2 $\pm$ 2.0	44.9 $\pm$ 4.0	13.2	15.6
24	38.5 $\pm$ 1.2	37.8 $\pm$ 4.0	12.2	11.5

Fig. 7. Stimulation by dimethylnitrosamine of DNA synthesis in kidney. [^3H]Thymidine was administered at various times to control and dimethylnitrosamine-treated rats. The mean specific radioactivity $\pm$ 1 s.d. from 30 determinations with saline-treated rats was 1230 ± 290 d.p.m./mg of DNA and that from the dimethylnitrosamine-treated rats is expressed as multiples of this value. Each point is the mean $\pm$ 1 s.d. of at least five determinations and the control range of 1 s.d. is indicated (----).

kidneys in the ensuing 24 h was the same as that from control animals (Table 4).

Dimethylnitrosamine-induced changes in renal DNA synthesis were uniform and separable into distinct phases (Fig. 7). Within 2 h of administration of the nitrosamine the amount of thymidine incorporated into kidney DNA doubled, after which it fell gradually to reach the control value at 19 h. A second and more sustained increase immediately followed, culminating in a peak of synthesis 3 days after injection of the carcinogen and returning to the control value at 4 days. Incorporation increased again to a maximum rate of about tenfold that in control rats, which was reached 6 days after treatment, to be followed by a return almost to the base-line on the 7th and subsequent days.

DISCUSSION

Dimethylnitrosamine under the conditions of the present experiments induces severe liver damage, and biochemical changes observed in this tissue are difficult to interpret. In contrast, the acute structural changes induced in the kidney by dimethylnitrosamine are far less severe and, with the dose used, they are difficult to detect by light-microscopy in conventional histological preparations. Consequently biochemical changes detected in the kidney may be specific responses to dimethylnitrosamine.

Villa-Treviño (1967) recorded maximal inhibition of hepatic protein synthesis 6 h after administration of dimethylnitrosamine to rats fed with a complete diet; a comparable dose produced the same effect in the protein-depleted rat in 9 h (Fig. 1). Such a result is consistent with Villa-Treviño's (1967) hypothesis that inhibition of protein synthesis is dependent on metabolism of dimethylnitrosamine to a reactive methylating intermediate, the rate of production of such an intermediate being decreased in the protein-depleted animal. The same author concluded that no inhibition of RNA synthesis occurs during the first 2 h, based on determination of radioactivity in RNA after administration of [^{14}C]orotate to the phenobarbitone-anaesthetized rat. Incorporation of this precursor is decreased by phenobarbitone anaesthesia (B. W. Stewart, unpublished work) and the consequent loss in sensitivity may have obscured early dimethylnitrosamine-induced changes in RNA synthesis. Immediate inhibition as described in Fig. 2 has been detected in the liver of rats fed on a complete diet (B. W. Stewart, unpublished work). Such a decrease may be directly dependent on dimethylnitrosamine degradation and not a consequence of a prior decrease in protein synthesis.

After the inhibition at 9 h (Figs. 1 and 2) there is a partial recovery of synthesis of RNA and protein by the liver. Incorporation of orotate reached 70% of the control value before a second, more sustained phase of inhibition was initiated, and during this

interval hepatic necrosis reached its maximum severity. Some decrease in labelling is attributable to decreased amounts of precursor reaching the tissue. Nevertheless, this fall in orotate incorporation was followed by a second inhibition of protein synthesis 3 days after injection of dimethylnitrosamine.

A similar series of changes in orotate incorporation by kidney was paralleled by variation of DNA-dependent RNA polymerase activity of isolated kidney nuclei (Table 2). Therefore the results *in vivo* are more likely to reflect the rate of RNA synthesis rather than variation of the specific activity of the precursor pool. Both polymerase activities, as well as the results in Fig. 5, indicate apparent recovery of RNA synthesis in the kidney after the earlier inhibition. Sucrose-density-gradient analysis of kidney cytoplasmic RNA 24 h after injection of dimethylnitrosamine (Fig. 6) indicates considerable increase in the synthesis of rRNA. However, there is no evidence that dimethylnitrosamine causes degradation of pre-existing ribosomes. Craig & O'Connor (1971) have examined the effect of methyl methanesulphonate on the half-life of hepatic rRNA. The dose used (50 mg/kg body wt.) was sufficient to convert 0.046% of guanine into the 7-methyl derivative, an amount similar to that expected in the kidney RNA in the present conditions (Villa-Treviño, 1967; Swann & Magee, 1968). Methyl methanesulphonate treatment also had no detectable effect on the loss of label after a single injection of [^3H]orotate.

Fig. 6 revealed no decrease in [^{14}C]orotate labelling of kidney cytoplasmic RNA at various times up to 3 days after dimethylnitrosamine treatment. This result is not necessarily inconsistent with that in Fig. 5 and Table 6 which indicate a net decrease in synthesis of RNA after administration of dimethylnitrosamine. The latter assays are measurements of rates of nRNA synthesis which may not correspond to the rate of incorporation of [^{14}C]orotate into RNA isolated from the cytoplasm. At 2 days after injection of the nitrosamine, the second phase of inhibition of kidney RNA synthesis was easily demonstrable; at the same time leucine incorporation was unaffected. The decrease in protein synthesis at 3 days may be the result of the earlier and greater inhibition of RNA synthesis.

The biphasic inhibition of synthesis of RNA may be an expression of the complex composition of preparations from whole kidney, the net result of a single and sustained inhibition of RNA synthesis in one cell population and a concomitant and short period of stimulated synthesis in another. This explanation is considered unlikely, since the recovery in synthetic activity 9–24 h after administration of the nitrosamine was also detected in liver

(Fig. 2), a result that probably expresses the reaction of one cell population to dimethylnitrosamine poisoning. It seems therefore that the initial inhibition of renal RNA synthesis is reversible. Under the present conditions the nitrosamine (40 mg/kg body wt.) could not be polarographically detected in the bloodstream later than 12 h after injection (Swann & McLean, 1971). Hence the second inhibition of RNA synthesis, initiated 12 h after the disappearance of dimethylnitrosamine from the blood, cannot be attributed to a further increase in alkylation of cell constituents by the nitrosamine.

The burst of hepatic DNA synthesis 19–24 h after dimethylnitrosamine treatment (Fig. 3) is similar to that observed after partial hepatectomy (Bucher, 1963). After this synchronous increase in activity, the wide variations between individual rates of synthesis were presumably dependent on the ratio of necrotic to regenerating cells. In contrast, the changes in kidney DNA synthesis during the week were uniform (Fig. 7). The biological significance of the prompt doubling of incorporation of thymidine 2–4 h after treatment is difficult to assess. Threlfall (1968) found no change in the extent of incorporation of [^3H]thymidine into kidney DNA after a single injection of folic acid until 18 h after treatment; an approximately linear increase followed, reaching about 15 times normal by 32 h. This result is similar to that obtained from other model systems for studying biochemical changes associated with cellular proliferation, e.g., the isoproterenol-stimulated salivary gland (Baserga, 1966) and regenerating rat liver (Bucher, 1963). The interval of at least 15 h before DNA synthesis is initiated by a variety of procedures (Baserga, 1968) argues against a sudden increase in cell division as being responsible for the rapid response to dimethylnitrosamine, and Hard & Butler (1970b) did not report an increase in renal cell division immediately after administration of the compound. The first phase of increased DNA synthesis in the kidney may perhaps represent 'repair' synthesis. Roberts, Crathorn & Brent (1968) demonstrated non-semiconservative DNA synthesis by mammalian cells as a result of alkylation and suggested that such synthesis constitutes a mechanism capable of 'repairing' alkylated lesions in DNA.

Hard & Butler (1970b) concluded that the nitrosamine-induced renal mesenchymal tumours originate from interstitial lesions which persist from hypercellular aggregations within the kidney interstitium first seen 2–4 days after administration of the carcinogen. The incidence of this early interstitial reaction correlated with tumour incidence. At 7 days after dimethylnitrosamine Hard & Butler (1970b) observed cell necrosis in tubules throughout the cortex for the first time. These

findings may be directly related to the results shown in Fig. 7; the second phase of increased thymidine incorporation (1–3 days) would correspond to proliferation of the interstitial cells and the high incorporation on day 6 to regenerating tubule epithelium. Thus evidence of some necrotic cells in kidney is manifest 5 days after initiation of hepatic regeneration. Similarity of initial responses of RNA and protein synthesis to the administration of dimethylnitrosamine in both liver and kidney suggests that a catabolic pathway producing similar reactive intermediates may be common to both tissues. Initial interaction with cellular constituents after hepatic metabolism may be responsible for the centrilobular necrosis. However, the renal regeneration might be correlated with either the second inhibition of RNA synthesis or some subsequent event.

The present report describes various responses to dimethylnitrosamine that are initiated days after any reaction between tissue constituents and the nitrosamine. The most marked kidney changes are detected 3 days after administration. Failure of nuclei to sediment through 2.3M-sucrose, loss in DNA content and inhibition of RNA synthesis all suggest that the nucleus is severely affected at this time. The effects described may be related to the toxic and/or to the carcinogenic activity of dimethylnitrosamine. However, foci of kidney interstitial cells appearing within 1 week of dimethylnitrosamine treatment have been implicated by Hard & Butler (1970b) in the development of kidney tumours. Further biochemical investigation of the present system may clarify this hypothesis.

The induction of kidney tumours by a single pulse of dimethylnitrosamine with its relatively short biological half-life may be compared with malignant transformation of cultured cells. According to Sachs (1966), the acquisition of fixation of the transformation state requires cell replication, and this may have its counterpart in the second and third peaks of increased DNA synthesis in the kidney. Finally, the various changes in RNA and DNA biosynthesis occurring in the early and perhaps crucial phase of renal carcinogenesis by dimethylnitrosamine should be considered in the light of possible activation of oncogenic viruses by chemical carcinogens (Huebner & Todaro, 1969; Temin, 1971).

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