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Toxic Liver Injury

INHIBITION BY DIMETHYLNITROSAMINE OF INCORPORATION OF LABELLED AMINO ACIDS INTO PROTEINS OF RAT-LIVER PREPARATIONS *IN VITRO*

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Dimethylnitrosamine (*N*-nitrosodimethylamine) has been shown to cause acute centrilobular necrosis of the liver in several mammalian species, including the rat (Barnes & Magee, 1954). It is carcinogenic in the rat, producing tumours of the liver (Magee & Barnes, 1956; Schmähl & Preussmann, 1959) and of the kidney (Magee & Barnes, 1959; Zak & Holzner, 1959). The compound is rapidly metabolized *in vivo* (Magee, 1956; Dutton & Heath, 1956; Heath & Dutton, 1958) and *in vitro* by liver slices and broken-cell preparations (Magee & Vandekar, 1958). When given to rats in doses sufficient to cause necrosis of the liver (50 mg./kg. body wt.) incorporation of isotopically labelled amino acids into liver proteins was greatly reduced 3 hr. after the administration of the poison, but no effect was observed in other organs. At this time, histological evidence of damage to the liver was minimal or absent. A smaller inhibition of incorporation of ^{32}P into the liver ribonucleic acid also occurred, but labelling of the acid-soluble and the lipid phosphorus was not affected nor was the concentration of liver glycogen. It was concluded that the structures, giving rise to the microsomes, may be particularly vulnerable to the damaging action of dimethylnitrosamine (Magee, 1958).

The present paper describes the effect of dimethylnitrosamine on the incorporation of labelled precursors into proteins and nucleic acids of rat-liver slices *in vitro*, and the effect of pretreatment of the animals with the compound on the capacity of cell-free preparations of liver to incorporate

labelled amino acids into proteins. Experiments with isolated mitochondria are also described. A preliminary account of this work has already been given (Hultin, Magee & Arrhenius, 1958).

MATERIALS AND METHODS

Chemical reagents

Dimethylnitrosamine. This was obtained commercially and purified by distillation (b.p. 151°). After suitable dilution with water it was given to rats by stomach-tube. The dose was 50 mg./kg. body wt. unless otherwise stated. It was estimated in tissue samples by the polarographic method of Heath & Jarvis (1955).

Radioactive compounds. DL-[4- ^{14}C]Valine, L-[^{14}C]leucine (generally labelled), [8- ^{14}C]adenine sulphate and [^{32}P]phosphate were supplied by The Radiochemical Centre, Amersham, Bucks., England. [^{32}P]Pyrophosphate was prepared by prolonged heating of $\text{K}_2\text{H}^{32}\text{P}_4$ at 325°.

Special reagents. Yeast hexokinase was prepared essentially according to Berger, Slein, Colowick & Cori (1946). Phosphoenolpyruvate was synthesized by the method of Ohlmeyer (1951). Adenosine 5'-phosphate (AMP) and adenosine triphosphate (ATP) were obtained from the Sigma Chemical Company, St Louis, Mo., U.S.A., and crystalline bovine-plasma albumin from Armour Laboratories, Chicago, Ill., U.S.A.

Experiments on tissue slices

Albino rats of the Wistar strain were maintained on a diet of vitamin-enriched rat-bread (Lindberg, Älvsjö, Sweden), and were killed by decapitation.

Male rats, usually weighing about 250 g., were starved overnight before use. Their livers or kidneys were rapidly removed and placed in ice-cold Krebs-Henseleit (1932) medium. Thin slices were cut with a razor blade fixed in a holder, with a device not previously described (Fig. 1).

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This device consisted of an aluminium plate, *A* (5 cm. $\times$ 6 cm.), in which grooves of varying depth (0.25–0.5 mm.) and 0.3 cm. in width had been cut. The organs were gently pressed with the finger on to the plate over the appropriate groove (in these experiments 0.25 mm. deep). In this way the tissue could be sliced off with the razor blade to give a slice corresponding in thickness to the depth of the groove. The organ was then moved aside and the thin slice was picked up with fine forceps and placed in ice-cold incubation medium. The plate was so constructed that it would fit exactly into an accurately machined depression (*B*) in the top of a cylindrical aluminium block (*C*). The top of the block was machined to have a peripheral flange 2 mm. high, so that the tissues could be kept in contact with cooled incubation medium while they were cut; cooling was maintained by surrounding the cylinder with ice. The dimensions of the aluminium block were such that the presence of the flange did not interfere with the movement of the razor blade. The slices were blotted and weighed on a torsion balance before transfer to the chilled incubation tubes. Each tube contained 80 mg. of tissue (usually five or six slices), 1.7 ml. of medium and 0.2 ml. of 0.25 M-glucose. The gas phase was $\text{CO}_2 + \text{O}_2$ (5:95) supplied to each tube by a loosely fitting glass manifold. The temperature of the bath was 37° and the tubes were shaken in a mechanically driven rack. Radioactive compounds, dissolved in 0.1 ml. of water, were added at the appropriate time with a 0.1 ml. micropipette. The amounts added to each tube were: [^{14}C]valine (1.34 μmoles) containing 0.19 μC ; [^{14}C]adenine (0.21 μmole) containing 1 μC . At the end of the incubation, the tubes were plunged into ice-cold water and the medium was removed from the slices and discarded. The slices were washed twice with ice-cold medium containing a large excess of non-radioactive valine or adenine, and were

disintegrated in water, again containing excess of the unlabelled compound, by an all-glass homogenizer (Potter & Elvehjem, 1936). An equal volume of 10% (w/v) trichloroacetic acid was then added to the tissue suspension with stirring.

Experiments on cell-free preparations of rat liver

The incorporation of L-[^{14}C]leucine into protein of cell-free systems *in vitro* was studied in preparations from animals given dimethylnitrosamine at various times before killing. Each experiment included one or two control and one or two treated rats. The livers were chilled with ice and thoroughly perfused *in situ* with cold 0.155 M-KCl. They were then taken out, rapidly weighed, minced and repeatedly washed in an ice-cold medium containing 0.25 M-sucrose, 25 mM-KCl, 0.01 M-MgCl₂ and 35 mM-2-amino-2-hydroxymethylpropane-1,3-diol (tris) -HCl buffer pH 7.8. The minced liver was disintegrated in a glass homogenizer with the same medium and was centrifuged for 6 min. at 14 000 g at 0°. The fat-free portion of the supernatant ('14 000 g supernatant') was transferred to the tubes of a Spinco preparative ultracentrifuge, rotor 40. The samples were centrifuged for 45 min. at 105 000 g, and the clear supernatants constituting the cell-sap fraction were collected by suction. The microsome pellets were drained and repeatedly washed with homogenizing medium. A small fluffy layer, which covered the translucent microsome pellets, was completely removed by these washings. To each tube 2.5 ml. of medium was added, and the microsome sediments were dispersed with a homogenizer. With the unseparated '14 000 g supernatants' 0.7 ml. was used in the incubation mixtures. With the recombined fractions 0.6 ml. of cell sap and 0.1 or 0.2 ml. of the dispersed microsomes was used. The complete incubation system contained 0.6 ml. of cell sap, 0.1 or 0.2 ml. of microsome suspension, 10 μmoles of phosphoenolpyruvate, 1 μmole of ATP and 0.08 μmole of L-[^{14}C]leucine (1.5 mc/m-mole), the final volume being brought to 1.1 ml. by the addition of homogenizing medium. The samples were incubated for 15 min. at 35°. After the incubation the tubes were chilled in ice and unlabelled L-leucine was added in large excess for isotope dilution. In order that the specific radioactivities of the proteins would be directly comparable, corrections for the protein content of each tube were made according to Decken & Hultin (1958). To each tube containing 0.1 ml. of the microsome suspension, 0.1 ml. of the same suspension was added, thus equalizing the volumes. After the determination of microsome-protein content, the final correction was made by adding the calculated amount of the appropriate microsome suspension to those tubes with the lower protein concentration; the proteins were then precipitated with trichloroacetic acid.

Amino acid-activating enzymes were determined by the method of Hoagland, Keller & Zamecnik (1956). The amino acid-dependent pyrophosphate-ATP exchange was measured as described previously by Hultin & Decken (1958).

Liver mitochondria were isolated by the method of Schneider & Hogeboom (1950) as modified by Ernster & Löw (1955). Oxygen consumption was measured by the conventional Warburg technique. Each Warburg flask contained mitochondrial suspensions from 200 mg. of liver (wet wt.), 20 μmoles of substrate, 50 μmoles of [^{32}P]phosphate, 10 μmoles of Mg^{2+} ions, 3 μmoles of AMP and

Fig. 1. Tissue-slicing device. For a description see the Materials and Methods section.

150 μ moles of sucrose. Where indicated, hexokinase in excess and 60 μ moles of glucose were added. The final volume was 2 ml., the gas phase air, the temperature 30° and the time of incubation 20 min. Oxidative phosphorylation was measured according to Ernster & Löw (1955) and adenosine-triphosphatase activity according to Löw (1959).

Analytical methods

Phosphorus. This was determined by the method of Martin & Doty (1949) as described by Lindberg & Ernster (1956).

Protein. This was determined according to Lowry, Rosebrough, Farr & Randall (1951).

Nucleotides from ribonucleic acid. After chromatographic separations, these were determined by means of ultra-violet-absorption spectra obtained by a Beckman DK-2 spectrophotometer.

Radioactivity of protein and ribonucleic acid. Proteins were isolated according to Decken & Hultin (1958). The dry protein powders were assayed on aluminium planchets (area 1 cm.²) and corrected to 'infinite thickness' with an empirical correction curve. In some cases incorporation of [¹⁴C]adenine into nucleic acid was measured by preparing 'crude nucleoproteins', i.e. omitting to extract the nucleic acids from the residue after extraction of acid soluble material and lipids. These 'nucleoprotein' powders were plated and assayed as above. In most experiments the powders were afterwards treated with 0.5 M-KOH for 20 hr. at 32°—a modified procedure of Schmidt & Thannhauser (1945). The hydrolysates were then acidified with 0.3 N-perchloric acid to pH 1-2 and kept in the refrigerator for 3 hr. before centrifuging. The soluble RNA nucleotides, which remained in the supernatant, were separated by ion-exchange chromatography on columns of Dowex-50 resin (Cohn, 1949). The specific activities were determined on 'infinitely thin' samples evaporated on glass planchets, after determinations of the nucleotide contents. All radioactivity assays were made with an end-window counter with an automatic sample changer (Tracerlab, Boston, Mass., U.S.A.).

Histological methods

Samples of liver were fixed in 10% formalin-1% CaCl₂ and in Helly's, Rossman's and Carnoy's fixatives before embedding in paraffin; they were sectioned at 5 μ . Sections were stained by haematoxylin and eosin, methyl green and pyronin, galloxyanin and Altmann's method for mitochondria. The periodic acid-Schiff and Feulgen methods were done according to Pearse (1953). Fat was stained with Sudan IV in frozen sections of material fixed in 10% formalin-1% CaCl₂.

RESULTS

Experiments with tissue slices

Incorporation of [¹⁴C]valine into proteins of slices. Preliminary experiments showed that dimethylnitrosamine, even in concentrations as high as 10 mM, had no significant effect on the incorporation of [¹⁴C]valine into the proteins of rat-liver slices during the standard incubation period of 45 min. Slices were then incubated in the presence of dimethylnitrosamine for varying periods before

the addition of the isotope, after which incubation was continued for a further period of 45 min. The results of such an experiment are shown in Fig. 2. It will be seen that pre-incubation with dimethylnitrosamine in a concentration of 1 mM caused a

Fig. 2. Effect of pre-incubation with dimethylnitrosamine on incorporation of [¹⁴C]valine into proteins of rat-liver slices *in vitro*. The incubation was continued for 45 min. after the addition of [¹⁴C]valine. ●, Control slices; ○, slices pre-incubated with dimethylnitrosamine (1 mM).

Fig. 3. Effect of pre-incubation for 3 hr. with various concentrations of dimethylnitrosamine on incorporation of [¹⁴C]valine into proteins of rat-liver and rat-kidney slices *in vitro*. The time of further incubation after addition of [¹⁴C]valine was 45 min. ●, Liver; ○, kidney.

decrease in the incorporation of the labelled amino acid; this decrease became greater with time. A characteristic feature of these experiments was the increased incorporation by the control slices with longer pre-incubation. A similar effect has been recorded by Ziegler & Melchior (1955).

The effect of varying the concentration of dimethylnitrosamine with a fixed period of pre-incubation is shown in Fig. 3. In this experiment some inhibition was seen at 0.01 mM, which became marked at 0.1 mM and 1 mM. The slight decrease in incorporation at 1 μ M was probably insignificant. Inhibition did not always occur at 0.01 mM, but there was always some effect at 0.1 mM and it was usually 50% or more at 1 mM, with a 3 hr. pre-incubation period.

Similar experiments were done with rat-kidney slices. The effect of pre-incubation for 3 hr. with different concentrations of dimethylnitrosamine is shown in Fig. 3. In contrast with the results with liver, no inhibition of incorporation occurred in the concentration range 0.1–10 mM. If anything, a slight increase in incorporation occurred at 10 mM. This increase may be insignificant but was seen again when the experiment was repeated.

Incorporation of [14 C]adenine into nucleic acids of tissue slices in vitro. Liver slices were pre-incubated with dimethylnitrosamine as before, followed by incubation for a further 45 min. in the presence of labelled adenine. In preliminary experiments, a measure of the incorporation into the nucleic acids was obtained by determination of the specific

activities of the 'nucleoprotein' powders (see Methods section). A similar type of effect was obtained to the incorporation of [14 C]valine into the tissue proteins, but the decrease on pre-incubation was less. This effect was confirmed in further experiments in which the specific radioactivities of the chromatographically isolated adenylic acid and guanylic acid were determined as well as those of the 'nucleoprotein' powders. The effect of pre-incubation of the slices for varying times in the presence of 1 mM-dimethylnitrosamine is shown in Fig. 4. There was reasonably good correlation between the results with the 'nucleoprotein' powders and with the isolated constituents of RNA (Table 1). The inhibitory effect of pre-incubation was again present at a concentration of 0.1 mM-dimethylnitrosamine and rather greater at 1 mM, as shown in Fig. 5.

Table 1. *Effect of pre-incubation with dimethylnitrosamine on the incorporation of [14 C]adenine into ribonucleic acid of rat-liver slices in vitro*

The incubation was continued for a further 45 min. after the addition of [14 C]adenine. The nucleotides were isolated chromatographically.

Time of pre-incubation (min.)	Radioactivity (counts/min./ μ mole)			
	Adenylic acid		Guanylic acid	
	Untreated	Treated	Untreated	Treated
0	1450	1350	81	83
50	1670	1400	98	64
100	—	—	76	37
150	1590	1020	55	24

Fig. 4. Effect of pre-incubation with dimethylnitrosamine on incorporation of [14 C]adenine into 'nucleoprotein' powders of rat-liver slices *in vitro*. The incubation continued for a further 45 min. after the addition of [14 C]adenine. ●, Control slices; ○, slices pre-incubated with dimethylnitrosamine (1 mM).

Fig. 5. Effect of pre-incubation for 2.5 hr. with various concentrations of dimethylnitrosamine on incorporation of [14 C]adenine into 'nucleoprotein' powders of rat-liver slices *in vitro*. The time of further incubation after the addition of [14 C]adenine was 45 min.

Table 2. *Penetration of dimethylnitrosamine into rat-liver slices in vitro*

Rat-liver slices were incubated with 1 mM-dimethylnitrosamine and the compound was estimated in the slices and surrounding medium after varying periods of incubation. Each concentration is the mean value from three incubations.

Time of incubation (min.)	Concn. of dimethylnitrosamine	
	In slices ($\mu\text{g./g. of slice water}$)	In medium ($\mu\text{g./ml.}$)
10	49	52
30	51	53
60	43	44

Table 3. *Concentrations of dimethylnitrosamine in rat liver in vivo*

Male rats of 150–200 g. body wt. were used. Dimethylnitrosamine was estimated in the liver at intervals, after oral administration, and the concentration in the liver water was calculated. Each value is the mean value from two rats.

Dose of dimethylnitrosamine (mg./kg. body wt.)	Interval (hr.)	Concn. of dimethylnitrosamine in liver water (μM)
30	4	34
20	4	12
15	4	0
20	1	162
20	2	15
20	3	1
20	4	0
30	1	330
30	2	210
30	3	64
30	4	66

Penetration of dimethylnitrosamine into tissue slices. The delayed inhibition of incorporation of both precursors could have been due to slow penetration of the slices by dimethylnitrosamine during incubation. Experiments were done to test this possibility. Liver slices were incubated with 1 mM-dimethylnitrosamine for varying times and the concentration of the inhibitor was measured in the slices and in the medium. The water content of the slices was also measured, and the concentration of dimethylnitrosamine in the slice water was calculated. As shown in Table 2, equilibrium with the medium with respect to dimethylnitrosamine was obtained after incubation for 10 min. and was maintained after the longer periods of incubation. The fall in concentration of dimethylnitrosamine is consistent with its metabolism by the slices (Magee & Vandekar, 1958). These results suggest that the delayed inhibitory effects of dimethylnitrosamine are not due to its failure to penetrate the liver slices.

Experiments on the intact rat

Since the main purpose of this work is the elucidation of the mechanism of action of dimethylnitrosamine in damaging the liver cell, it is clearly important to know what relation the inhibitory concentrations, recorded above, bear to the concentration of the poison which is required in the liver *in vivo* in order to produce morphological evidence of hepatic-cell damage. In these experiments, rats were given varying doses of dimethylnitrosamine and their livers were examined histologically 24 hr. after the administration. No definite morphological evidence of hepatic-cell necrosis was observed with doses less than 20 mg./kg. body wt. In another series of rats, the concentration of dimethylnitrosamine in the liver during the first 4 hr. after administration in various doses was determined. The calculated concentrations in liver water are shown in Table 3. At the critical dose of 20 mg./kg. body wt. concentrations of the order of 0.1 mM were obtained during the first 2 hr., which are of the same order as those needed to produce inhibitory effects *in vitro*. These experiments also confirm the rapid metabolism of dimethylnitrosamine by the intact animal.

Experiments with cell-free liver preparations

Incorporation of [^{14}C]leucine into protein by liver fractions in vitro. In these experiments the rats were given dimethylnitrosamine orally and killed after various time intervals. The dose, 50 mg./kg. body wt., was sufficient to cause acute necrosis of the liver. A preliminary test, with the 14 000 g supernatant from the liver of a rat treated 6 hr. previously with dimethylnitrosamine, showed a considerable decrease in the uptake of the labelled amino acid compared with a similar preparation from an untreated control animal. The specific activity of the control protein was 363 counts/min./cm.² and of the treated 190 counts/min./cm.². Similar experiments were made at 3 and 2 hr. after giving the dimethylnitrosamine. In addition to measuring the incorporation by the '14 000 g supernatant', further fractionation was done and control cell sap was combined with treated and with control microsomes. In each case two amounts of microsome suspensions were used, 0.1 and 0.2 ml. respectively. The results of two experiments at 3 and 2 hr. after giving dimethylnitrosamine are shown in Table 4. There is clearly a definite decrease in incorporation of the labelled amino acid into the preparations from treated rats, and it appears that there is a defect in the microsomes.

Measurements of amino acid-dependent pyrophosphate-ATP exchange by the soluble liver fractions gave no indication of any decreased capacity for amino acid-activation in livers from

Table 4. *Effect of treatment of rats with dimethylnitrosamine on the incorporation of [¹⁴C]leucine into protein by cell-free liver preparations*

Male rats of 200–250 g. body wt. were used. The treated rats received 50 mg. of dimethylnitrosamine/kg. body wt. orally and were killed after varying intervals. Incorporations of [¹⁴C]leucine into protein by '14 000 g supernatant' and by separated and recombined microsome and cell-sap fractions were measured.

Interval after dimethyl-nitrosamine (hr.)	Whole '14 000 g supernatant' (counts/min./cm. ²)		Microsomes combined with untreated cell sap (0.6 ml.) (counts/min./mg. of microsomal protein)	
	Untreated rats	Treated rats	Untreated microsomes	Treated microsomes
3	359	176	130	56
3	501	195	100	54
2	614	359	80	52
2	501	266	100	70

Table 5. *Effect of treatment of rats with dimethylnitrosamine on oxidative phosphorylation in isolated liver mitochondria*

Male rats of 200–250 g. body wt. were used. The treated rats received 50 mg. of dimethylnitrosamine/kg. body wt. orally and were killed after varying intervals. Oxidative phosphorylation of mitochondria equivalent to 200 mg. of liver (wet wt.) was measured with glutamate and succinate as substrates.

Interval after treatment with dimethylnitrosamine (hr.)	Substrate	Oxygen uptake in incubation period (μg.atoms)		P/O ratio
		Hexokinase absent	Hexokinase added	
Untreated	Glutamate	1.7	6.6	2.5
2	Glutamate	0.7	7.5	2.7
3	Glutamate	1.8	9.8	2.4
Untreated	Succinate	—	10.6	1.7
2	Succinate	—	10.9	1.9
3	Succinate	—	12.1	2.1

rats treated with dimethylnitrosamine 2–3 hr. previously.

Mitochondrial activity in vitro. The condition of the mitochondria was examined after dimethylnitrosamine was given. No effect was found on oxidative phosphorylation or on induction of adenosine triphosphatase at 2 and 3 hr. in several experiments. Typical results are shown in Table 5. Microsome preparations from the same animals, on the other hand, showed definite decrease in incorporation of [¹⁴C]leucine into protein *in vitro*, 47% at 2 hr. and 61% at 3 hr. The addition of dimethylnitrosamine to isolated mitochondria had no effect in concentrations up to 10 mM, and even at 0.1M inhibition was only about 50%. The functions tested were the oxidation of citrate, fumarate, pyruvate, glutamate and octanoate with malate as primer, and oxidative phosphorylation with glutamate as substrate.

DISCUSSION

The decreased capacity of cell-free liver preparations from rats treated with dimethylnitrosamine to incorporate [¹⁴C]L-leucine into protein is in agreement with earlier work where a similar reduc-

tion in incorporation was observed *in vivo* (Magee, 1958). The experiments where control cell sap was incubated with microsomes from treated rats, as well as the study of the amino acid-activating enzymes, suggest that the main defect may be microsomal. The absence of effect on oxidative phosphorylation and other mitochondrial activities in livers showing marked defect in amino acid incorporation strengthens the hypothesis that the microsomes may be specially vulnerable to dimethylnitrosamine or a metabolic product. Our results with isolated mitochondria are in agreement with recent work by Bailie & Christie (1959). These workers found no defect in the oxidation of pyruvate and octanoate until 12 hr. and none in that of the tricarboxylic acid-cycle intermediates until even later by isolated liver mitochondria from rats given 100 mg./kg. body wt. of dimethylnitrosamine.

The inhibition of amino acid and adenine incorporation into liver slices is also consistent with the findings *in vivo*. There is reasonably good correlation between the concentration of dimethylnitrosamine needed to cause this inhibition and that necessary in the liver *in vivo* for definite histological evidence of hepatic-cell necrosis. Incuba-

tion of liver slices with dimethylnitrosamine does not cause a general inhibition of all synthetic processes since there is no decrease in the concentration or synthesis of ATP in slices after incubation for 3 hr. at a concentration of 1 mM (C. J. Threllfall, personal communication).

The possibility that some metabolite other than dimethylnitrosamine may be the toxic substance has been suggested before (Magee, 1956) and recent work by D. F. Heath (unpublished work) leads to the same conclusion. The present work gives some support for this as a period of pre-incubation for rat-liver slices is required before inhibitory effects on [14 C]valine and [14 C]adenine incorporation can be detected. Under the conditions of incubation in these experiments metabolism of dimethylnitrosamine would certainly occur (Magee & Vandekar, 1958). Failure of penetration of the nitrosamine to the active site in the liver cell is unlikely in view of the rapid attainment of concentrations within the slice equal to those in the surrounding medium. It is uncertain whether the kidney can metabolize dimethylnitrosamine or not. If it can, the rate of metabolism is much less than that of the liver (Magee, 1956; Magee & Vandekar, 1958). If a toxic metabolite is involved, this difference in metabolism might explain the difference in behaviour of kidney and liver slices (Fig. 3).

The nature of the toxic metabolite can only be a matter of speculation. Formaldehyde is formed on incubating dimethylnitrosamine with liver slices *in vitro* (Magee & Hultin, 1958) and has several properties which make it a possible candidate, such as its great reactivity and its possible mutagenicity (Rapoport, 1948; Boyland, 1954). On the other hand, it is probably a normal metabolite with a central role in the metabolism of active C_1 intermediates and this makes it less likely. Further, many drugs are known to undergo oxidative demethylation and these reactions have been studied in great detail by Brodie and his colleagues (Brodie *et al.* 1955). Among these drugs are Pyrimidon (*N*-dimethylaminoantipyrine), its monomethyl derivative and ephedrine. The metabolism of these compounds *in vitro* resembles that of dimethylnitrosamine in several features (Magee & Vandekar, 1958), but they are not known to be toxic or carcinogenic to the liver. On the whole, therefore, formaldehyde seems unlikely to be the toxic molecule unless it is formed at a metabolic site in the cell that is different from that of the above-mentioned drugs and from that of sarcosine and dimethylaminoethanol, which also give rise to formaldehyde in the liver (Frisell & Mackenzie, 1955). Another possible toxic metabolite is nitrite, as suggested by Heath & Dutton (1958). This could be formed, together with methylamine, by hydrolysis of the postulated unstable monomethylnitros-

amine. These authors demonstrated methylamine derived from dimethylnitrosamine in liver and urine of rats treated with the latter compound. The possible formation of diazomethane has been suggested by Dr Regina Schoental (personal communication) and independently by Rose (1958). This suggestion is based on the assumption that the enzymic demethylation of dimethylnitrosamine is a stepwise reaction in which there could be momentary formation of the highly unstable monomethylnitrosamine. By analogy with a postulated mechanism for the hydrolysis of *N*-nitrosomethylurethane (Sidgwick, 1937) the monomethyl compound would immediately break down to diazomethane and water. There are several factors in favour of this compound being the active molecule. It is a very active alkylating agent and highly toxic, and it has been shown to be mutagenic (Rapoport, 1948; Jensen, Kirk, Kølmark & Westergaard, 1951).

With this possibility in mind our results can be compared with those of Clark, Goodlad & Munro (1958), who studied the action of the alkylating agent di-(2-chloroethyl)methylamine hydrochloride (the nitrogen mustard HN2) on amino acid incorporation into protein and other synthetic processes in rat-liver slices *in vitro*. Uptake of [14 C]glycine was inhibited but to a smaller extent than that of 32 P into RNA nucleotides. Other synthetic processes were also inhibited. These authors concluded that the inhibition of amino acid incorporation was due to a general inhibition of synthetic reactions rather than to a specific action on the mechanism of protein synthesis. The apparently more selective action of dimethylnitrosamine can be explained if a toxic metabolite, perhaps also an alkylating agent is released in close spatial proximity to sites of active amino acid incorporation (cf. Hultin, 1956, 1957). Since the intracellular structures which give rise to the microsome fraction are the most active in protein synthesis *in vivo* (Hultin, 1950) and microsomes are involved in amino acid incorporation *in vitro* (Zamecnik & Keller, 1954) and in the metabolism of dimethylnitrosamine (Magee & Vandekar, 1958) it seems reasonable that these structures might bear the brunt of the damage caused by a non-specific toxic metabolite.

SUMMARY

1. Incorporation of [14 C]valine into proteins of rat-liver slices *in vitro* was inhibited by pre-incubation of the slices with dimethylnitrosamine (0.1 mM). No inhibition of incorporation was observed with kidney slices.

2. Incorporation of [14 C]adenine into ribonucleic acid of rat-liver slices *in vitro* was also inhibited on pre-incubation with dimethylnitrosamine.

3. The concentration of dimethylnitrosamine in rat-liver slices rapidly approached that of the incubation medium.

4. The smallest dose of dimethylnitrosamine necessary to produce definite necrosis of the liver in the rat was about 20 mg./kg. body wt. Concentrations of dimethylnitrosamine in the livers of rats given this dose reached levels of the order of 0.1 mM, calculated in terms of liver water.

5. Incorporation *in vitro* of [¹⁴C]leucine into proteins of microsome plus cell-sap preparations of rat liver was measured at 2 and 3 hr. after the animals had been given a hepatic-necrotizing dose of dimethylnitrosamine (50 mg./kg. body wt.) and compared with that in similar preparations from untreated rats. Incorporation was considerably less in the preparations from treated rats than in those from untreated controls and the decrease was also present when treated microsomes were combined with control cell sap.

6. Amino acid-activation as measured by the amino acid-dependent pyrophosphate-adenosine triphosphate exchange was unimpaired in treated animals at 2-3 hr. after the necrotizing dose of dimethylnitrosamine.

7. Oxidative phosphorylation and the induction of adenosine triphosphatase were unimpaired in liver mitochondria isolated from rats 2 and 3 hr. after administration of dimethylnitrosamine. Incorporation of [¹⁴C]leucine into microsome plus cell-sap preparations from the same animals was greatly impaired. The addition of dimethylnitrosamine to isolated mitochondria respiring *in vitro* had no effect on the oxidation of several substrates in concentrations up to 10 mM.

8. The results are discussed in relation to the mechanism of liver damage by dimethylnitrosamine. They are thought to support the idea of a toxic metabolite as the tissue-damaging agent and its possible identity is discussed. They are also thought consistent with the hypothesis that the intracellular structures which give rise to microsomes on homogenizing are particularly vulnerable to the damaging action of dimethylnitrosamine.

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