Toxic Liver Injury

THE METABOLISM OF DIMETHYLNITROSAMINE *IN VITRO*

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Dimethylnitrosamine has been shown to cause acute centrilobular haemorrhagic necrosis of the liver in rats, rabbits and other animals when administered in doses of the order of 50 mg./kg. body wt. Damage to other tissues was minimal or absent and may be related to the liver injury (Barnes & Magee, 1954). When added to the diet of rats at a level of 50 p.p.m. it was shown to cause a high incidence of malignant hepatic tumours (Magee & Barnes, 1956). The compound is rapidly metabolized *in vivo* by rats and mice, as shown by Dutton & Heath (1956), who administered [^{14}C]-dimethylnitrosamine and recovered a high proportion of the radioactivity in the expired carbon dioxide during the first few hours after injection.

Magee (1956) studied the metabolism of dimethylnitrosamine *in vivo*, the polarographic method of Heath & Jarvis (1955) being used for its determination in tissues and body fluids. The compound was found in about the same concentration in most of the tissues of the body and appeared to be distributed uniformly throughout the body water. Evidence from hepatectomized rats indicated that the liver is the main organ concerned in the metabolism of dimethylnitrosamine.

The present paper deals with the metabolism of dimethylnitrosamine *in vitro* by tissue slices and broken-cell preparations.

MATERIALS AND METHODS

Dimethylnitrosamine. This was obtained commercially and was purified by distillation (b.p. 151°). It was estimated in tissue samples by the polarographic method of Heath & Jarvis (1955).

Coenzymes. Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) were prepared by Dr W. N. Aldridge and their purity was assayed chromatographically by Mr C. J. Threlfall. The DPN was 90% pure with no contamination by TPN and the TPN was 69% pure with no DPN. Reduced TPN (TPNH) was prepared by the method of Kaplan, Colowick & Neufeld (1952). Adenosine triphosphate (ATP) was obtained from Sigma Chemical Co.

Inhibitor. SKF 525A (2-diethylaminoethyldiphenyl-propylacetate hydrochloride) was a gift from Dr K. Carter, of Smith, Klyne and French Ltd.

Animals. Albino rats of the Porton strain maintained on M.R.C. diet no. 41 (Bruce & Parkes, 1949) and cross-bred rabbits maintained on M.R.C. diet no. 18 (Bruce, 1947) were used.

Experiments on tissue slices. Rats of either sex, usually weighing about 200 g., were killed by decapitation and their livers or other organs rapidly removed and placed in ice-cold Krebs-Ringer phosphate (Umbreit, Burris & Stauffer, 1951), which was the incubation medium. After blotting, slices were cut with a slicer (Stadie & Riggs, 1944), and weighed on a torsion balance and transferred to Warburg flasks containing ice-cold medium. The water content of representative samples of the tissue slices was determined by drying overnight in an electric oven at 105°. Solutions of dimethylnitrosamine were freshly prepared by dilution with medium and added to the Warburg flasks. Sometimes glucose was added to a final concentration of 0.01 M, and the final volume was 3 ml. Incubation was in a conventional Warburg apparatus at 37°, with gas phase either O_2 or N_2 , gassing taking 5 min. In most experiments the O_2 uptake was followed manometrically. After incubation the slices were disintegrated with a Watson blade homogenizer and the resulting suspension was transferred quantitatively to a measuring cylinder. Proteins were precipitated with sulphosalicylic acid. The contents of the cylinder were made up to a known volume (20 or 25 ml.) and filtered. Dimethylnitrosamine was determined in the filtrate.

Experiments on broken-cell preparations. Rabbit liver, and sometimes other organs, were disintegrated in ice-cold potassium phosphate buffer, pH 7.4, usually containing 0.1% (w/v) of nicotinamide, by means of a homogenizer (Potter & Elvehjem, 1936) with a plastic (Perspex) pestle. Centrifugal fractionation of the suspensions was carried out in a refrigerated centrifuge (Measuring and Scientific Equipment Ltd., Angle 13) at 0°. The standard incubation medium was similar to that of La Du, Gaudette, Trousof & Brodie (1955) and contained 100 μmoles of nicotinamide, 75 μmoles of MgCl_2 and 0.1 M-phosphate buffer (pH 7.4) to give a final volume of 5 ml. Known volumes of tissue suspension, usually 2 ml., were incubated at 37° with this medium in 25 ml. conical flasks in a Dubnoff metabolic shaking incubator (Precision Scientific, Chicago, Ill., U.S.A.). Dimethylnitrosamine was diluted with the standard medium before adding it to the flasks, and other additions were as described in the text. The gas phase was either O_2 or N_2 , obtained by passing a stream of gas over the surface of the liquid for 2 min. through glass tubes and then closing the flasks with rubber bungs.

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RESULTS

Experiments with rat-tissue slices

Rat liver, kidney and brain slices, and blood were incubated for 75 min. with dimethylnitrosamine in an atmosphere of O₂. Control flasks contained boiled tissue slices or no tissue. The results, shown in Table 1, Expt. A, suggested that liver was the only tissue among those tested which could metabolize dimethylnitrosamine *in vitro*. Liver slices were incubated in atmospheres of O₂, air or N₂ with added dimethylnitrosamine. As shown in Table 1, Expt. B, complete breakdown of added dimethylnitrosamine was obtained in the presence of O₂, and breakdown was considerably reduced in air. When N₂ was the gas phase, no metabolism of dimethylnitrosamine occurred.

Approximately the same weights of liver slices were incubated with dimethylnitrosamine for different periods. The amount of the compound broken down was proportional to the time of incubation (Fig. 1, A). In a similar experiment different weights of liver slices were incubated with the same amount of dimethylnitrosamine, and an approximately linear relationship between the degree of breakdown of dimethylnitrosamine and the amount of liver tissue was found. Different quantities of dimethylnitrosamine were incubated with roughly the same weights of liver slices (429–483 mg.) for 75 min. The results (Table 2) indicated that, at all concentrations used, similar amounts of dimethylnitrosamine were broken down.

The O₂ uptake of liver slices respiring in a

medium containing glucose was measured in the presence of 13.5 mm-dimethylnitrosamine, and in its absence, during a 5 hr. incubation period. At first the O₂ consumption was not affected by the dimethylnitrosamine, but as incubation proceeded the slices incubated with dimethylnitrosamine showed a greater decrease in O₂ consumption than the control slices (Fig. 2), suggesting the possible formation of an inhibitory metabolite.

Fig. 1. Breakdown of dimethylnitrosamine with different times of incubation. A, Approximately equal weights of rat-liver slices (352–366 mg.) were incubated with 50 µg. of dimethylnitrosamine. B, Equal volumes (2 ml.) of rabbit-liver microsomes + cell-sap fraction were incubated with 40 µg. of dimethylnitrosamine.

Table 1. Recovery of dimethylnitrosamine after incubation with rat-tissue slices

Expt. A. Tissue slices or blood were incubated with 60 µg. of dimethylnitrosamine for 75 min. at 37° in an atmosphere of O₂. Expt. B. Liver slices were incubated with 30 µg. of dimethylnitrosamine under various conditions, as indicated.

Expt. A			Expt. B		
Tissue	Wt. of tissue (mg.)	Dimethyl-nitrosamine recovered (µg.)	Tissue	Wt. of tissue (mg.)	Dimethyl-nitrosamine recovered (µg.)
Liver slices	294	33.4	Liver slices	424	O ₂ 0.0
	306	35.6		457	O ₂ 0.0
	390	29.0	Liver slices	515	Air 22.3
Kidney slices	205	57.0		521	Air 23.0
	256	57.0	Liver slices	420	N ₂ 29.0
Brain minced	317	57.0		474	N ₂ 27.0
Brain slices	360	58.2	Boiled slices	430	O ₂ 28.2
Brain minced	370	57.0			29.0
Blood	0.5 ml.	58.2			
	0.5 ml.	60.2	No tissue added	—	O ₂ 29.3
	0.5 ml.	57.8			
Control	Boiled liver slices (300 mg.)	55.6			
	No tissue added	54.8			
		59.80			

Table 2. *Effect of variation of amount of added dimethylnitrosamine on recovery after incubation with rat-liver slices*

Liver slices were incubated with dimethylnitrosamine for 75 min. at 37° in an atmosphere of O₂.

Wt. of liver slices (mg.)	Dimethyl-nitrosamine added (μg.)	Dimethyl-nitrosamine recovered (μg.)	Difference	
			Wt. (μg.)	Percentage
449	30.0	0	30.0	100
432	60.0	24.2	35.8	60
461	150.0	119.6	30.4	20
433	300.0	262.0	38.0	13

Fig. 2. Respiration of rat-liver slices in the presence of dimethylnitrosamine. A, Liver slices (115–141 mg.) were incubated in Krebs–Ringer phosphate containing glucose (0.01M): oxygen uptake was measured manometrically. B, Similar system to A, but with addition of dimethylnitrosamine to 13.5 mM final concentration.

Table 3. *Recoveries of dimethylnitrosamine after incubation with homogenates of various rabbit tissues in oxygen or nitrogen*

Homogenates of the different tissues were incubated with 34 μg. of dimethylnitrosamine under the standard conditions in atmospheres of N₂ or O₂.

Tissue	Dimethyl-nitrosamine recovered in N ₂ (μg.)	Dimethyl-nitrosamine recovered in O ₂ (μg.)	Difference (%)
Liver	31.0	19.5	37
Kidney	29.5	29.0	1.7
Spleen	30.0	30.0	0
Heart	30.0	30.0	0
Lung	31.0	31.0	0

Experiments with broken-cell preparations of rabbit tissue

In preliminary experiments, breakdown of dimethylnitrosamine in the presence of rat-liver homogenates was found to be capricious and slow,

but preparations of rabbit liver were found to be more active. Even with rabbit liver, however, the activity was very variable and, from time to time, completely inactive preparations were obtained.

As with rat-liver slices, breakdown of dimethylnitrosamine by rabbit-liver homogenates was found to require O₂. A substantial loss of dimethylnitrosamine occurred aerobically, but almost all of the added compound was recovered after incubation in N₂. Moreover, the recoveries from the anaerobic system were the same as that after incubation with the standard medium alone in O₂.

Dimethylnitrosamine was incubated with homogenates prepared from rabbit liver, kidney, spleen, heart or lung under the standard conditions, the recoveries being shown in Table 3. Apart from the very small loss in the presence of kidney homogenate, which is probably within the limits of error, the liver was the only tissue which caused breakdown of dimethylnitrosamine. This experiment also provides a good check on the experimental technique, since there is good agreement between the recoveries after incubation in O₂ or N₂ with all the inactive tissues, and with the liver homogenate incubated in N₂.

The results shown in Table 3 suggested that flasks containing the complete system under study and incubated in N₂ could be used as controls for the same systems incubated simultaneously in O₂, and this method was used in all subsequent experiments. Loss of dimethylnitrosamine was measured as the difference in the recoveries from the anaerobic and the aerobic systems, usually expressed as a percentage of the anaerobic recovery.

Fractionation of the homogenate. Rabbit-liver homogenate was centrifuged at 8500 g for 10 min. to sediment the nuclear and mitochondrial fractions. The supernatant fluid, containing the microsome and cell-sap fractions, was decanted, and the sediment was resuspended in 0.2M-phosphate buffer containing 0.1% of nicotinamide so that the volume was equal to that of the original homogenate. The activity of these two fractions was compared with that of the whole homogenate, a portion of which was kept chilled in ice during the centrifuging. All the activity of the whole homogenate was recovered in the microsome + cell-sap

fraction (Table 4, Expt. A) and none remained in the nuclear+mitochondrial fraction. In similar experiments adequate amounts of TPN and DPN (cf. Fig. 3) were added to nuclear+mitochondrial fractions incubated with dimethylnitrosamine, but no breakdown was obtained.

In another experiment, after preliminary centrifuging as above, the sediment was discarded and the supernatant fluid was centrifuged at 18 000 g for 1 hr. in order to effect a further separation into the microsome and cell-sap fractions. The activity of each separate fraction was then compared with that of the two fractions recombined. Negligible breakdown of dimethylnitrosamine was found with either fraction separately, but there was substantial

activity when the two fractions were recombined (Table 4, Expt. B). Several attempts were made to restore the activity of the microsome fraction alone by the addition of TPNH. The additions were made at 15 min. intervals during the incubation period (cf. Axelrod, 1955), but no activity was obtained.

In most of the following experiments the microsome + cell-sap fraction only was used.

Effect of dialysis and requirement for cofactors. In preliminary experiments much variation was found in the ability of preparations to retain their activity on standing at 2°. Occasionally activity was retained overnight, but sometimes it was lost after only a few hours. The time of dialysis therefore could be determined only by trial and error, in the hope that undialysed preparations, kept in the cold, would retain their activity. A microsome + cell-sap fraction was tested immediately after preparation under the standard conditions and found to break down 73 % of the added dimethylnitrosamine. A sample of this preparation was dialysed for 3 hr. against a large volume of 0.02M-phosphate buffer, pH 7.4, and another sample was kept chilled on ice during the period of dialysis. The dialysed sample was completely inactive, whereas the chilled undialysed sample retained full activity. This finding suggested a requirement for dialysable cofactors, and the effect of adding DPN and TPN to dialysed preparations was tried, with the results shown in Table 5. Activity was completely removed by dialysis and a substantial degree of recovery was obtained by the addition of TPN or DPN. There was no extra effect when the two cofactors were added together.

The effect of varying amounts of added coenzyme is shown in Fig. 3. TPN was always found to be rather more effective than DPN. The addition of ATP to dialysed preparations, in amounts as high as 10 mg./flask, did not cause any significant return of activity. The addition of DPN and TPN to undialysed homogenates prepared in the presence of nicotinamide caused only slight increase in activity, suggesting that there was adequate endogenous pyridine nucleotide.

Effect of variation of the standard conditions. Volumes of a microsome + cell-sap fraction varying from 0.5 to 4 ml. were incubated for 2 hr. under the standard conditions with 40 µg. of dimethylnitrosamine. The activity at each concentration is shown in Fig. 4. There was a linear relationship between loss of dimethylnitrosamine and quantity of liver preparation.

The amount of dimethylnitrosamine broken down by a fixed quantity of microsome + cell-sap preparation increased in an approximately linear relationship with time during the first 2 hr. of incubation (Fig. 1B), but after longer periods there was little further activity.

Table 4. Breakdown of dimethylnitrosamine by subcellular fractions of rabbit liver

Expt. A. Rabbit-liver homogenate was centrifuged at 8500 g for 10 min. to sediment the nuclear + mitochondrial fraction, which was resuspended in 0.2M-phosphate buffer, pH 7.4, containing 0.1 % (w/v) of nicotinamide. The two fractions were then incubated with 40 µg. of dimethylnitrosamine under the standard conditions. Expt. B. Liver homogenate from another rabbit was centrifuged as in Expt. A and the nuclear and mitochondrial fraction discarded. The supernatant was centrifuged at 18 000 g for 2 hr. to sediment the microsome fraction, which was resuspended in buffer and both fractions were incubated with dimethylnitrosamine as before.

Expt.	Preparation	Breakdown of added dimethylnitrosamine (%)
A	Whole homogenate	20
	Nuclear and mitochondrial fraction	0
	Microsome and supernatant fraction	20
B	Microsome fraction	2
	Supernatant fraction	5
	Recombined microsome and supernatant fraction	40

Table 5. Cofactor requirements for breakdown of dimethylnitrosamine by the microsome and supernatant fraction of rabbit-liver homogenate

The microsome + supernatant fraction was dialysed for 3.5 hr., and its activity compared with that of an undialysed sample of the same fraction under the standard condition of incubation. The effect of TPN and DPN was tested by adding 1 mg. of the appropriate nucleotide per flask.

Preparation	Breakdown of added dimethylnitrosamine (%)
Undialysed	71
Dialysed	0
Dialysed + TPN	47
Dialysed + DPN	37

Effect of inhibitors. The effects of various inhibitors on the breakdown of dimethylnitrosamine by microsome + cell-sap preparations incubated under the standard condition are shown in Table 6.

DISCUSSION

From the evidence presented in this paper it appears that there is an enzyme in liver which catalyses the chemical breakdown of dimethylnitrosamine. With the possible exception of kidney, the liver appears to be the only tissue among those tested which is capable of metabolizing dimethylnitrosamine. This conclusion is supported by evidence from hepatectomized rats (Magee, 1956). It is not clear why broken-cell preparations of rat liver should have lower activity than rabbit preparations. However, similar findings have been reported by La Du *et al.* (1955) in their work on the metabolism of aminopyrine. The concentration of metabolic activity in the microsome + cell-sap fraction of the liver and the requirement for pyridine nucleotides suggest that enzyme systems similar to those described by Brodie *et al.* (1955) and by Booth & Boyland (1957) may be involved. The failure to obtain activity by the addition of

TPNH to microsomes alone does not necessarily indicate a fundamental difference as it may be due to instability of the enzyme system which metabolizes dimethylnitrosamine. Some of the liver enzymes which metabolize foreign compounds are very unstable (B. B. Brodie, personal communication).

The finding that DPN as well as TPN could restore activity after dialysis is contrary to the experience of Brodie *et al.* (1955). Other workers, however, have shown requirements for DPN in similar systems. Davison (1955) and O'Brien (1957) showed that the enzymic conversion of the insecticides schradan and parathion from weak into strong inhibitors of cholinesterase required

Fig. 3. Requirement of pyridine nucleotides for breakdown of dimethylnitrosamine. Microsome + cell-sap preparations of rabbit liver were dialysed until free from activity, and then incubated with dimethylnitrosamine under the standard conditions. A, Effect of added TPN; B, effect of added DPN.

Fig. 4. Breakdown of dimethylnitrosamine by different amounts of rabbit-liver preparation. Different volumes of rabbit-liver microsome + cell-sap fraction were incubated with 40 μg. of dimethylnitrosamine under the standard conditions.

Table 6. Effect of inhibitors on the breakdown of dimethylnitrosamine by broken-cell preparations

Microsome + cell-sap preparations of rabbit liver were incubated with dimethylnitrosamine under the standard conditions with inhibitors added in final concentrations as indicated.

Inhibitor	Concn. of inhibitor (mM)	Inhibition of breakdown of dimethylnitrosamine (%)
Iodoacetate	1.0	44
Mercuric chloride	1.0	100
p-Chloromercuribenzoate	0.1	13
p-Chloromercuribenzoate	1.0	74
Azide	0.1	12
Azide	1.0	90
Cyanide	0.1	0
Sodium fluoride	10.0	0
SKF 525 A	0.2	60
SKF 525 A	0.4	85

DPN. Mueller & Miller (1953) have described an enzyme system in rat liver which demethylates 4-dimethylaminoazobenzene and related dyes, and this requires both DPN and TPN. Hultin (1957) has shown that not only metabolism of the azo dyes but also the formation of the dye-liver-protein complex (Miller & Miller, 1947) can occur *in vitro*. In both these reactions DPNH can to some extent replace TPNH.

The inhibition of metabolism of dimethylnitrosamine by SKF 525 A is consistent with the findings of Brodie *et al.* (1955) in most of the metabolic conversions which they studied. The general pattern of inhibition (Table 6) resembles that reported for several other systems (Davison, 1955; Cooper & Brodie, 1955) and confirms the similarity of the enzyme system which breaks down dimethylnitrosamine to those involved in the metabolism of other foreign compounds. Little is known of the metabolic products of dimethylnitrosamine. Extensive demethylation must occur *in vivo* (Dutton & Heath, 1956) and some formaldehyde is produced on incubation of the compound *in vitro* with rat-liver slices (unpublished results). The nitroso group must also be altered since this is the group which is detected polarographically.

The main purpose of this work was to gain information on how dimethylnitrosamine injures the liver, and some of the findings may be relevant. It seems possible that the concentration of dimethylnitrosamine metabolism in the microsome + cell-sap fraction of the liver may be related to the early inhibition of incorporation of labelled amino acids into liver proteins *in vivo* which is caused by dimethylnitrosamine (Magee, 1957, 1958), since the microsome fraction is the most active in hepatic-protein synthesis (Hultin, 1950; Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950). Furthermore, the same combination of microsomes + cell sap has been shown to be required for the incorporation of labelled amino acids into protein *in vitro* (Keller & Zamecnik, 1956). This will be discussed in the following paper.

SUMMARY

1. The metabolism of dimethylnitrosamine *in vitro* has been studied in preparations of rat and rabbit tissues.

2. Slices and broken-cell preparations of liver destroyed dimethylnitrosamine, but the other tissues tested were inactive. The reaction required oxygen.

3. The ability to destroy dimethylnitrosamine was found only in the microsome + cell-sap fraction of liver-tissue suspensions. Neither the microsomes nor cell sap was appreciably active alone, but activity was restored on recombination.

4. Activity was removed from tissue suspensions by dialysis. It could be partially restored by the addition of tri- or di-phosphopyridine nucleotide; the former was the more effective.

5. The possible bearing of these results on the hepatotoxic action of dimethylnitrosamine is discussed.

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