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THE EFFECT OF A PROTEIN-FREE HIGH-CARBOHYDRATE DIET ON THE METABOLISM OF DIMETHYLNITROSAMINE IN THE RAT

BY P. F. SWANN*
S. A. Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London W1P 5PR, U.K.

AND A. E. M. McLEAN
Division of Experimental Pathology, University College Hospital Medical School, University Street, London WC1E 6JJ, U.K.

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1. Rats fed on a protein-free high-carbohydrate diet for 7 days metabolized dimethylnitrosamine at only 55% the rate of rats fed on a commercial diet. 2. Dimethylnitrosamine was metabolized by liver slices from rats fed on the protein-free diet at less than half the rate attained by slices from rats fed on a commercial diet. But kidney slices from these rats metabolized dimethylnitrosamine at the same rate as kidney slices from rats on a commercial diet. 3. Methylation by dimethylnitrosamine (70mg/kg body wt.) of N-7 of guanine of the liver RNA and DNA of rats fed on a protein-free diet was only slightly higher than in rats fed on a normal diet given 27mg/kg body wt. In contrast, the methylation by dimethylnitrosamine of guanine in kidney nucleic acids of these rats was three times that in the rats fed on a normal diet. 4. In rats fed on a protein-free diet the incidence of kidney tumours produced by a single dose of dimethylnitrosamine is increased.

Rats fed on a protein-free diet for a short period became resistant to the lethal effects of dimethylnitrosamine. The median lethal dose is 45mg/kg body wt. in rats fed on a commercial pelleted diet (M.R.C. 41B) and 79mg/kg for rats fed on the protein-free diet (McLean & Witschi, 1966). During the feeding of a protein-free diet the activity of the enzyme system that metabolizes drugs, such as Pyramidon (aminopyrine), and foreign compounds, such as benzo[*a*]pyrene, falls to a low value in 4 days (McLean & McLean, 1966). In the present paper we report that a protein-free diet lowers the activity of the enzyme system metabolizing dimethylnitrosamine in the liver, but does not affect this enzyme system in the kidney. Because the rat is unable to excrete more than a small part of a dose of dimethylnitrosamine (Heath, 1962) the decrease in the amount metabolized in the liver leaves more to be metabolized in the kidney. A single dose of dimethylnitrosamine produces tumours of the kidney in all of the rats fed on this protein-free diet. This adds to the circumstantial evidence that the carcinogenic activity of dimethylnitrosamine is caused by some product of nitrosamine metabolism.

A preliminary report of this work has been published (Swann & McLean, 1968).

* Present address: Chemistry Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014, U.S.A.

MATERIALS AND METHODS

Animals. Porton–Wistar-derived albino rats from the Medical Research Council, Carshalton, Surrey, U.K., or Sprague–Dawley rats from the National Institutes of Health, Bethesda, Md., U.S.A., were maintained in cages with wire-grid bottoms and so denied access to sawdust and their own faeces as far as possible.

Diets. Rats were reared on an unlimited diet of M.R.C. 41B (Bruce & Parkes, 1956) or Wayne Lab-Blox (Allied Mills, Chicago, Ill., U.S.A.). The protein-free diet used was that described by McLean & McLean (1966). The bulk of this diet is carbohydrate (65% corn starch; 30% sucrose; 5% olive oil).

Rate of metabolism of dimethylnitrosamine by rats fed on diet 41B and a protein-free diet. The control rats for this experiment were fed on M.R.C. diet 41B up to and during the experiment. Twenty-five male rats (mean weight 180g) were given dimethylnitrosamine in 0.9% NaCl (50mg/kg body wt.) by intraperitoneal injection. The rats to be fed on the protein-free diet were reared on M.R.C. diet 41B until 8 days before the experiment. They were then transferred to the protein-free diet until and during the experiment. Twenty-five male rats, weighing an average of 170g at the start of the feeding period and an average of 140g on the day of injection, were each given dimethylnitrosamine in 0.9% NaCl (50mg/kg body wt.) by intraperitoneal injection.

Rats were anaesthetized in groups by intraperitoneal injection of sodium pentobarbitone (veterinary Nembutal; Abbott, Queenborough, Kent, U.K.) at various times after the injection and blood (3ml) was taken from the aorta. Dimethylnitrosamine concentration in the

blood samples was measured polarographically by using the variation of the method of Heath & Jarvis (1955) described by Heath (1962).

Rate of metabolism of dimethylnitrosamine by liver and kidney slices from rats on a normal diet and rats on a protein-free diet. Female rats weighing 170g, which had been reared on M.R.C. diet 41B, were fed on either M.R.C. diet 41B or a protein-free diet for 7 days before the experiment. At each occasion that the slice preparations were made, one rat fed on a normal diet and one rat fed on a protein-free diet were killed by a blow on the head. The livers and kidneys were placed in ice-cold Krebs–Ringer phosphate buffer (Umbreit, Burris & Stauffer, 1964) with the addition of glucose (2mg/ml). Slices (approx. 0.008in thick) from liver and kidney were cut, blotted and weighed. The slices (150–250mg total wt.) were equilibrated with O_2 in Warburg flasks each containing 2.8ml of the Krebs–Ringer buffer. Three separate samples were taken from each tissue. [^{14}C]Dimethylnitrosamine (40μg, 0.010μCi in 0.2ml of solution) was added from the side arm and the $^{14}CO_2$ produced taken up by NaOH (0.2ml; 10%, w/v) in the centre well. The slices were incubated for 2h. The NaOH was then removed from the well, the [^{14}C]carbonate precipitated as barium carbonate, and the radioactivity determined (Heath & Dutton, 1958; Swann, 1968).

Subsequently, Sprague–Dawley rats, maintained on Wayne Lab-Blox but otherwise treated exactly as described above, were used in an experiment analogous to that described above. Slices (about 500mg total wt.) cut from the livers of rats fed on the protein-free diet or Wayne Lab-Blox were incubated for 2h in flasks containing dimethylnitrosamine (150μg) in Krebs–Ringer phosphate buffer (5ml) with O_2 as the gaseous phase. The metabolism was stopped by addition of sulphosalicylic acid (5ml; 10%, w/v) and the amount of dimethylnitrosamine remaining determined by the method of Heath & Jarvis (1955).

Methylation in vivo *of guanine in liver and kidney RNA and DNA after a single dose of* [^{14}C]*dimethylnitrosamine was given to rats previously fed on a protein-free diet.* Seven male rats weighing 150–160g were fed on the protein-free diet for 7 days before the experiment. On the day of injection the mean weight of the rats was 125g. Each rat was given 70mg of dimethylnitrosamine/kg body wt. Water but no food was given to the rats between the injection of dimethylnitrosamine and their death. At 19h after the injection of dimethylnitrosamine the rats were anaesthetized with sodium pentobarbitone and blood was taken from the aorta. The dimethylnitrosamine concentration in the blood was measured by polarography (Heath, 1962). The liver and kidneys of the rats were removed and the amount of 7-methylguanine in RNA and DNA was measured (Swann & Magee, 1968).

RESULTS

The effect of feeding a protein-free diet on the rate of metabolism of dimethylnitrosamine (50mg/kg body wt.) was assessed by measuring the rate of disappearance of dimethylnitrosamine from the blood of the animals by using the method of Heath (1962). The rate of metabolism in the period in which the concentration fell to 20% of the initial value was calculated by linear regression analysis from the results shown in Fig. 1 (Snedecor, 1946). The results in terms of change of concentration were expressed in terms of mg metabolized/h per kg body wt. by extrapolating the regression line to find the concentration at zero time (68.7μg/ml for rats on normal diet, 67.0μg/ml for rats on a protein-free diet) and assuming that this corresponded to 50mg/kg body wt. The rate of metabolism in male rats fed on diet 41B was 4.9mg/h per kg and that for rats on the protein-free diet 2.7mg/h per kg, which may be compared with the results of Heath (1962), who found that female rats from the same colony fed on M.R.C. 41B diet metabolized 5.7mg/h per kg body wt.

The metabolism of dimethylnitrosamine takes place predominantly in the liver, and to a lesser extent in other tissues such as kidney and probably lung (Magee & Vandekar, 1958). To discover if the decrease in the rate of metabolism of dimethylnitrosamine in the living rat was the result of a change in the rate at which the liver metabolized dimethylnitrosamine, the rate of production of $^{14}CO_2$ from [^{14}C]dimethylnitrosamine by liver

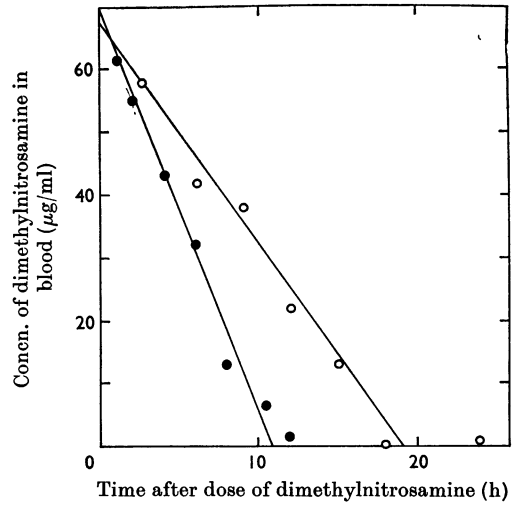

Fig. 1. Metabolism of dimethylnitrosamine in rats fed on normal and protein-free diets. The concentration of dimethylnitrosamine remaining in the blood of male rats fed on a normal (M.R.C. 41B) (●) or protein-free (○) diet at various times after a dose of dimethylnitrosamine (50mg/kg body wt.) was measured by polarography. The results at 1, 2, 8, 10½ and 12h for rats on a normal diet are each the mean of determinations on four rats; that for 4h on three rats; and that for 6h on two rats. Except for the result at 6h, which is the mean of determinations on four rats, each result for the rats on a protein-free diet is the mean of determinations on five rats.

slices *in vitro* was measured. The results (Table 1) show that liver slices from rats fed on a protein-free diet for 7 days before the experiment produced $^{14}CO_2$ from [^{14}C]dimethylnitrosamine at less than half the rate of slices from rats fed on M.R.C. diet 41B. By contrast, the slices of the kidneys of rats fed on a protein-free diet produced $^{14}CO_2$ from [^{14}C]dimethylnitrosamine at a rate not significantly different from that of kidney slices from rats fed on a commercial diet. Slices of liver from the rats fed on a protein-free diet respired much less than slices from the rats fed on M.R.C. diet 41B. This might suggest that the rate of metabolism of dimethylnitrosamine in these slices is not impaired but because of the change in respiratory rate the production of $^{14}CO_2$ does not accurately reflect the rate of metabolism. For this reason the experiment was repeated but this time the rate of disappearance of dimethylnitrosamine from the incubation medium and the slices was directly measured. Because of a change in laboratory, rats of a different breed, Sprague–Dawley instead of Porton–Wistar, were used, and Wayne Lab-Blox had to be used in place of M.R.C. diet 41B. Otherwise the experiment was essentially the same except that the disappearance of dimethylnitrosamine from the slices and medium during a 2h incubation with the slices was measured polarographically (Heath & Jarvis, 1955). In this experiment also liver slices from rats fed on a protein-free diet metabolized dimethylnitrosamine at only 55% of the rate obtained with slices from rats on a normal diet (Table 1).

This result would suggest that the effect of a protein-free diet on the rate of metabolism of dimethylnitrosamine in the living rat is the result of a change in the rate at which the liver can metabolize this compound, but that the rate at which the kidney metabolizes dimethylnitrosamine is unaltered. It is possible to make an estimate of the amount of metabolism of dimethylnitrosamine taking place in each tissue *in vivo* and to show that this difference between liver and kidney is not an artifact of the system *in vitro*. During the metabolism of dimethylnitrosamine a methylating agent is produced, which reacts with susceptible sites in the components of the organ in which the metabolism takes place. There is no evidence for migration of this active intermediate from the tissue in which it is produced to other tissues, and the amount of methylation of susceptible sites seems to parallel the rate of metabolism of the dimethylnitrosamine measured by other means. The amount of methylation at N-7 of guanine in rat liver and kidney nucleic acids produced by a dose of 70mg of dimethylnitrosamine/kg body wt. in rats that had been fed on a protein-free diet for 7 days previously was compared (Table 2) with the amount of methylation at N-7 of guanine produced in the nucleic acids of liver and kidneys of rats maintained on a normal diet (M.R.C. 41B) and given 27mg of dimethylnitrosamine/kg body wt. (Swann & Magee, 1968). The time-interval between the injection of the dimethylnitrosamine and the death of the animals is different in each case. Each group was allowed to live long enough to metabolize most, but not all, of the nitrosamine. Thus, the rats on the normal diet given 27mg of dimethylnitrosamine/kg body wt. were killed 5h after the dose. Those on the protein-free diet given 70mg of dimethylnitrosamine/kg body wt. were killed 19h after the dose. The mean proportion of dimethylnitrosamine not metabolized at this time was

Table 1. *Metabolism of dimethylnitrosamine by liver and kidney slices taken from rats fed on a normal or protein-free diet*

Slices were cut from the liver and kidneys of rats fed on either a normal or protein-free diet. For the measurement of respiration and [^{14}C]dimethylnitrosamine metabolism Porton–Wistar rats were used and in these experiments the normal diet was M.R.C. 41B. In the experiment in which dimethylnitrosamine metabolism was measured Sprague–Dawley rats were used and the normal diet was Wayne Lab-Blox. Experimental details and numbers of animals are given in the text. Results are given as means ±S.E.M. Estimates of statistical significance were by Student's '*t*' test (Snedecor, 1946): *$P = 0.001$; †$P = 0.1$; ‡$P = 0.025$.

	Normal diet		Protein-free diet	
	Liver	Kidney	Liver	Kidney
Respiration				
(μl of O_2/h per g wet wt.)	756± 39	2628±60	322± 35	2585±123
(% of normal)			(50%)*	(98%)
[^{14}C]Dimethylnitrosamine metabolism				
(d.p.m. of CO_2/h per g of tissue)	3575±265	550±55	1347±161	423± 50
(% of normal)			(38%)*	(77%)†
Dimethylnitrosamine metabolism				
(μg metabolized/2h per g of tissue)	106± 15		58± 5.0	
(% of normal)			(55%)‡	

Table 2. *Methylation of* N-7 *of guanine in the nucleic acids of the living rat fed on a normal diet (M.R.C. 41B) or a protein-free diet*

Results for the protein-free diet are from the pooled livers and pooled kidneys of seven rats killed 19h after the dose of 70mg of dimethylnitrosamine/kg body wt. Results for the normal diet, taken from Swann & Magee (1968), are for the pooled livers and pooled kidneys of six rats killed 5h after a dose of 27mg of dimethylnitrosamine/kg body wt. The methods for preparation of nucleic acids and the determination of 7-methylguanine are given in the Materials and Methods section.

	Proportion of guanine residues methylated (% of total guanine)	
	Normal diet (M.R.C. 41B)	Protein-free diet
Liver RNA	1.16	1.4
Liver DNA	0.87	1.19
Kidney RNA	0.16	0.48
Kidney DNA	0.106	0.36

estimated by polarography to be approx. 6% of the dose, by use of the method of Heath (1962). Despite the much higher dose given to the rats on the protein-free diet methylation at N-7 of guanine in rat liver DNA and RNA was approximately the same as in the normally fed animals. In contrast, methylation of kidney nucleic acids was more than three times as great in the rats fed on a protein-free diet as in those fed on a normal diet.

DISCUSSION

The work reported above shows that the feeding of a protein-free diet, even for the short period of 7 days, decreases the rate of dimethylnitrosamine metabolism by the living rat to half the rate achieved by rats fed on a commercial diet (M.R.C. 41B). Experiments with liver and kidney slices *in vitro* show that the rate of metabolism of dimethylnitrosamine by liver slices from the Porton–Wistar or Sprague–Dawley rats fed on a protein-free diet was half that of liver slices obtained from rats on a commercial diet (M.R.C. 41B or Wayne Lab-Blox). The rate of metabolism of dimethylnitrosamine by kidney slices from Porton–Wistar rats fed on a protein-free diet was not significantly different from that of kidney slices from rats fed on a normal diet (M.R.C. 41B). This difference between the effect on liver and kidney found *in vitro* is probably a true representation of the changes occurring in the living rat, for it corresponds to the changes in the methylation of guanine found in these tissues. Although the dose of dimethylnitrosamine given to the rat fed on a protein-free diet is more than twice that given to the rat on the normal diet, the amount of methylation of rat liver DNA and RNA is scarcely increased. In contrast, the methylation of the guanine in the kidney nucleic acids of the rats given a protein-free diet before administration of 70mg of dimethylnitrosamine/kg is more than three times as great as the methylation achieved in the rats fed on a commercial diet and given 27mg of dimethylnitrosamine/kg.

The results of Heath (1962) show that the rate of dimethylnitrosamine metabolism is not dependent on the concentration of dimethylnitrosamine in the range of concentrations used in this experiment. Therefore, as the kidney of the rat fed on a protein-free diet is exposed to dimethylnitrosamine for nearly four times as long (19h instead of 5h) and metabolizes it at 77% the rate of the kidney of the rat on a commercial diet, it would be expected that if the result *in vitro* is correct the amount of methylation found in the kidney nucleic acids of the rat fed a protein-free diet should be $0.77 \times 19/5 = 2.93$ times as great as the amount of methylation of kidney nucleic acids found in the rat fed on a normal diet, i.e. the percentage methylation at N-7 of guanine in kidney DNA in the rat fed on a protein-free diet might be 0.31 and that in RNA 0.47. In the experiment *in vivo* the results were DNA 0.36, RNA 0.48. The experiments *in vitro* showed that liver slices from rats fed on a protein-free diet metabolized dimethylnitrosamine at only 38% of the rate in liver slices from rats fed on a normal diet. If this were a true representation of the change in the activity of the liver in the living rat, one would expect the methylation of guanine by dimethylnitrosamine in the liver nucleic acids of the rat fed on a protein-deficient diet to be $0.38 \times 19/5 = 1.44$ times as great as in the liver of the rat fed a normal diet, i.e. the percentage methylation at N-7 of guanine in liver RNA would be 1.67 and of DNA 1.25. The amount found in the experiment *in vivo* (RNA 1.4, DNA 1.19) was slightly less than predicted. The discrepancy is greater for the RNA than for the DNA, which would be compatible with the results of Craddock & Magee (1963) that 7-methylguanine is lost more rapidly from RNA than

DNA. Craddock & Magee (1963) also showed that the loss of 7-methylguanine from the kidney was proportionately very much less than from the liver, which would account for the better correspondence between the predicted and actual results for the methylation of N-7 of guanine in the kidney than in the liver.

This short period of feeding on a protein-free diet increases the median lethal dose 1.7-fold and probably decreases the damage to the liver (McLean & Verschuuren, 1969). The toxicity of dimethylnitrosamine is the result of metabolism of the compound (Heath, 1962), but dimethylformamide, a competitive inhibitor of dimethylnitrosamine metabolism, although considerably more effective than the protein-free diet in decreasing the rate of metabolism of dimethylnitrosamine, had less effect in decreasing the toxicity of dimethylnitrosamine (Heath, 1962). The explanation for this is probably that the protein-free diet does not affect the metabolism of dimethylnitrosamine in all organs to an equal degree: the metabolism in the liver, which is the organ most badly damaged normally, is selectively inhibited. The metabolism in the kidneys, which normally show no overt damage, is not affected and they metabolize a greater proportion of the dose. A single dose of dimethylnitrosamine (60mg/kg body wt.) produces kidney tumours in every rat on a protein-free diet (Swann & McLean, 1968; Hard & Butler, 1970*a*; McLean & Magee, 1970) but in only 35% of rats on a commercial M.R.C. 41B diet (Hard & Butler, 1970*a*). Studies of the relationship between chemical structure and carcinogenic activity have suggested that the carcinogenic activity of nitroso compounds is the result of the production of an active intermediate in their breakdown (Druckrey, Preussmann, Ivancovic & Schmähl, 1967). The results reported here are compatible with that view and it is probable that the increased incidence of tumours in the rats on a protein-free diet is the result of the relative increase of the amount of dimethylnitrosamine metabolized in the kidney.

The change in the activity of the enzyme system metabolizing dimethylnitrosamine in the livers of rats fed a protein-free diet is probably the result of lack of protein, rather than the high content of carbohydrate in the diet. The effect of a protein-free diet on the toxicity of carbon tetrachloride (McLean & McLean, 1966), which seems analogous to the effect on dimethylnitrosamine toxicity, is reversed by addition of only 10% casein to the diet, and thus protein would appear to play a more positive role than carbohydrate. Venkatesan, Arcos & Argus (1970) showed that the metabolism of dimethylnitrosamine *in vitro* by rat liver microsomal fraction is inhibited by feeding only glucose for 24h, but is increased by feeding cellulose for 24h or by 24h starvation. Feeding pure casein for 24h produced an even greater stimulation of activity.

In the experiments reported in the present paper the high content of carbohydrate in the diet suppresses the breakdown and utilization of protein from muscle, but starving the rat, either by feeding cellulose or by withholding food altogether, would lead to a mobilization of the animal's own protein and causes a high protein influx to the liver. Venkatesan *et al.* (1970) believe that their results show a direct inhibition of dimethylnitrosamine metabolism by carbohydrate, but the stimulation by starvation and the greater stimulation by a pure casein diet show that the inhibition of dimethylnitrosamine metabolism by a pure glucose diet could be caused by lack of protein rather than presence of carbohydrate.

The experiments reported in the present paper show the intimate relationship between the diet and the carcinogenic and toxic effects of dimethylnitrosamine. In this, they add to the already considerable literature on the effect of diet and enzyme inducers on the carcinogenic activity of chemicals. In addition, this experiment provides a means for the production of kidney tumours, resembling the Wilms tumours found in children, selectively in all the animals treated with a single dose of carcinogen. This procedure has been used in a microscopic study of the development of kidney tumours (Hard & Butler, 1970*a*, *b*) and promises to be a powerful tool in experimental cancer research.

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REFERENCES

Bruce, H. M. & Parkes, A. S. (1956). *J. Anim. Techns Ass.* **7**, 54.

Craddock, V. M. & Magee, P. N. (1963). *Biochem. J.* **89**, 32.

Druckrey, H., Preussmann, R., Ivancovic, S. & Schmähl, D. (1967). *Z. Krebsforsch.* **69**, 103.

Hard, G. C. & Butler, W. H. (1970*a*). *Cancer Res.* **30**, 2796.

Hard, G. C. & Butler, W. H. (1970*b*). *Cancer Res.* **30**, 2806.

Heath, D. F. (1962). *Biochem. J.* **85**, 72.

Heath, D. F. & Dutton, A. (1958). *Biochem. J.* **70**, 619.

Heath, D. F. & Jarvis, J. A. E. (1955). *Analyst, Lond.*, **80**, 613.

McLean, A. E. M. & Magee, P. N. (1970). *Br. J. exp. Path.* **51**, 587.

McLean, A. E. M. & McLean, E. K. (1966). *Biochem. J.* **100**, 564.

McLean, A. E. M. & Verschuuren, H. C. (1969). *Br. J. exp. Path.* **50**, 22.
McLean, A. E. M. & Witschi, P. (1966). *Biochem. J.* **100**, 11P.
Magee, P. N. & Vandekar, M. (1958). *Biochem. J.* **70**, 600.
Snedecor, C. W. (1946). *Statistical Methods.* Ames: Iowa State Press.
Swann, P. F. (1968). *Biochem. J.* **110**, 49.
Swann, P. F. & McLean, A. E. M. (1968). *Biochem. J.* **107**, 14P.
Swann, P. F. & Magee, P. N. (1968). *Biochem. J.* **110**, 39.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1964). *Manometric Techniques*, p. 149. Minneapolis: Burgess Publishing Co.
Venkatesan, N., Arcos, J. C. & Argus, M. F. (1970). *Cancer Res.* **30**, 2563.