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NITROSAMINE METABOLISM IN KWASHIORKOR RATS

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Abstract—The *in vitro* metabolism of nitrosodimethylamine (NDMA) was studied in liver tissue obtained from male weanling kwashiorkor wistar rats. The elimination of this compound and that of nitrosomorpholine (NMOR) from the blood, after a single intravenous dose, was also investigated. *N*-demethylase activity in liver microsomes of the test animals was not significantly different from that of the controls although the activity of this enzyme per gram wet liver tissue was considerably reduced in the model animals. On the other hand, the glutathione (GSH) content in liver cytosol of the kwashiorkor animals was much higher than that of the controls. The elimination of NDMA and NMOR from the blood of the experimental animals over 8 hr following i.v. administration of the carcinogens, showed that the clearance rate of each nitrosamine was significantly lower in the kwashiorkor rats.

In the tropics of Africa, Asia and Latin America, there is a high incidence of PEM† [1–3]. PEM is a spectrum of diseases arising from a deficiency of dietary protein especially in childhood [4, 5]. Kwashiorkor, one form of PEM, results from both the quantitative and qualitative deficiency of dietary protein with adequate caloric intake. This disease in experimental animals presents an ideal and interesting model with which to study the role of nutritional status in the toxicity and carcinogenicity of environmental chemicals. In particular it may test the hypothesis [6], that interactions between diet and chemical carcinogens might be partly responsible for the geographic and socio-economic variations in tumor incidences in human populations. Chronic wasting in experimental animals such as occurs in PEM with its attendant structural changes in the liver and its clinical manifestations, [4, 5, 7–10] may complicate the whole pattern of drug disposition.

The effects of acute low protein diets on the metabolism of NDMA its toxicity and carcinogenicity have been investigated in the past. Nitrosamines are a group of highly carcinogenic compounds which have to be metabolically activated to an alkylating species which is considered to cause the carcinogenic transformations [11–14]. McLean and Verschuuren [15] found the acute toxicity of NDMA to be reduced by half in animals fed a low protein diet for 7 days. The metabolism of NDMA in liver slices of such pretreated rats was reduced to 38% [16] or even 50% [17] of that in livers of adequately fed rats. No effect of this diet was observed on the metabolism of NDMA in the kidney [16]. The difference in the inhibition of the metabolism of NDMA in the two organs was also reflected in the alkylation of guanine in DNA by NDMA. As suggested by these results a change in organotropism of a single dose of NDMA from liver to kidney, in rats fed a protein free diet for a short time, was observed [16, 18–20]. Chronic administration of NDMA to animals fed a low protein diet during the whole treatment however, resulted in an increase in necrotic transformations and a lowering of regenerative activity in the liver [21].

The experimental models used in these studies are probably not a true reflection of the PEM observed in man because adult rats were fed low protein diets. The typical kwashiorkor syndromes appear in children chronically malnourished from an early age [4, 5]. To have a more adequate model for the human situation we induced kwashiorkor in rats fed a low protein high carbohydrate diet from weaning and studied the metabolism of NDMA and NMOR.

MATERIALS AND METHODS

Chemicals

NDMA and NMOR were synthesised by nitrosation with $NaNO_2$/HCl from the corresponding amines. Both nitrosamines were 99% pure (according to gas chromatography and U.V.-spectrometry). All solvents used were of analytical grade and supplied by Merck (Darmstadt, F.R.G.). NADP, glucose-6-phosphate and glusose-6-phosphate dehydrogenase were purchased from Boehringer (Mannheim, F.R.G.). All other chemicals used were available commercially and of reagent grade.

Induction of kwashiorkor in rats

Male weanling wistar rats (weighing between 30 and 50 g) were fed *ad libitum* for 35 days on the kwashiorkorigenic diet [22]. The diet was prepared by Altromin International (Lage, F.R.G.) and contained 3.5% protein (dietary casein) 81.5% carbohydrate, 8% fat oil, 4% mineral mixture and 3% all

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† Abbreviations: NDMA, *N*-nitrosodimethylamine; NDEA, *N*-nitrosodiethylamine; NMOR, *N*-nitrosomorpholine; PEM, protein energy malnutrition; GSH, reduced glutathione.

vitamin supplementation. The control rats were placed on a Standard Altromin Stock diet containing 19–20% protein.

The outset of the kwashiorkor syndrome was certified by the following parameters which were observed in the model animals as compared to the controls: (a) a drastic reduction in body weight gains (b) flaking-off of hair (c) reduced packed-cell volume (d) a drastic reduction of white blood cells (e) lowered rectal temperatures (f) a dramatic recovery of the affected animals within a few days of being placed on the control diet.

Microsomal preparation

Liver was perfused *in situ* with ice cold 0.15 M KCl, homogenised in 4 vol 0.06 M phosphate buffer pH 7.4, 0.15 M KCl with a Teflon–glass homogeniser. The supernatant of the 9000 × *g* centrifugation was recentrifuged at 105,000 × *g* for 60 min. This supernatant was designated cytosol: the pellet was washed in buffer, recentrifuged and taken up in 2 vol buffer per gram liver. Protein was determined according to Lowry [23].

Incubation assay

The complete microsomal incubation medium had a total vol of 6 ml and contained NADP (0.5 mM) glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (5 U), semicarbazide hydrochloride (10 mM), $MgCl_2$ (20 mM), 0.06 M phosphate buffer, 0.15 M KCl and 4 mg microsomal protein. The concn of NDMA in the incubation mixture varied between 0.1–5 mM.

All incubations were carried out in sealed 25 ml Erlenmeyer flask for 30 min at 37° in a water bath shaker. The reactions were terminated by the addition of 2 ml of 5% trichloroacetic acid, the protein precipitated and the formaldehyde concentration measured according to Nash [24]. The cytochrome P_{450} and b_5 concentrations were determined from the reduced CO difference spectrum by the method of Omura and Sato [25] in a Shimadzu UV 300 spectrophotometer. An extinction coefficient of 91/mM × cm between 450 and 490 nm was used. Reduced glutathione in cytosol was determined according to Ellmann [26] in the clear supernatant after protein precipitation in 5% trichloroacetic acid. Glutathione S-transferase in the cytosol was determined according to the method of Habig [27] with 1 mM 1-chloro-2,4-dinitrobenzene as substrate.

Determination of nitrosamines in blood

According to [28] each plasma sample was diluted to 20 ml with 0.9% saline, and extracted with dichloromethane on an Extrelut colomn (Merck, Darmstadt, F.R.G.). Quantification was achieved by gas-chromatographic analysis using chemoluminiscence detector (TEA 502, Thermo Electron, Waltham,U.S.A.).

Treatment of animals

Two groups of 5 animals each were given 4.0 mg/kg NDMA or 6.3 mg/kg (equimolar dose) NMOR in 0.9% saline intravenously. 0.1 ml blood was drawn from the plexus orbitalis with heparinised pipettes at incremental times between 0.5 and 8 hr and mixed immediately with 0.5 ml of 0.1 n NaOH to prevent *in situ* nitrosation. The samples were centrifuged for 5 min at 2000 rpm (MSE super minor centrifuge). The NDMA and NMOR content of each sample was determined in the supernatant as described above.

RESULTS

All data on the biological status of the animals are summarized in Table 1. The kwashiorkorigenic diet led to a drastic decrease in both body and liver weight, but the ratio of liver weight to body weight was comparable to the ratio in the control group. A

Table 1. Biological status of kwashiorkor rats in comparison to controls

	Kwashiorkor rats	Normal rats
Body weight (g)*	58 (40–70; 10)	265 (230–290; 10)
Liver weight (g)*	2.8 (1.7–4.1; 10)	9.6 (8.8–11.6; 10)
Ratio liver to body (g/g)	0.048	0.036
Protein content of:		
plasma (mg/ml)*	30.0 (24.7–37.4; 10)	52.1 (46.4–56.1; 10)
liver microsomes (mg/g liver)†	10.8 ± 0.3	19.3 ± 0.3
cytosol (mg/g liver)†	41.6 ± 1.3	68.0 ± 0.7
Cytochrome content of liver microsomes (nmole/mg protein):		
P_{450}‡	0.339	0.425
b_5‡	0.232	0.262
GSH content of liver cytosol:		
in (nmole/mg protein)†	41.6 ± 0.8	22.2 ± 0.4
in (μmole/g liver)†	1.73 ± 0.03	1.50 ± 0.03
GSH-S-transferase activity: in liver cytosol		
(nmole/mg protein·min)*	775 (758–844; 5)	1309 (1154–1464; 7)

* Median value (lower limit–upper limit; number of animals).
† Arithmetic mean of at least 4 determinations ± S.D.
‡ Values of pooled livers of 5 kwashiorkor or 7 control animals.
According to [30] the interindividual variation in one treatment group is less than 10%.

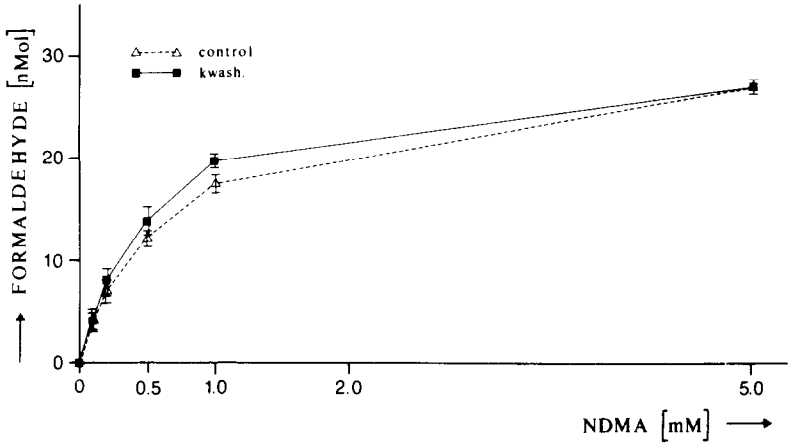

Fig. 1. NDMA-demethylase activity in rat liver microsomes. The ordinate indicates nmole formaldehyde produced per mg protein during 30 min. All values are arithmetic means with S.D. of 5 measurements.

lower protein content in plasma and liver was observed due to PEM and the livers of the malnourished rats had and extremely high fat content.* Two of the tested enzymatic values were decreased significantly, namely P_{450} and GSH-S-transferase. GSH concentration was remarkably higher in the treated animals both on mg cytosolic protein and on a gram liver basis.

NDMA-demethylase activity, however, only showed a difference (Fig. 2) between treated and control group if the values were related on a g liver basis. If the enzyme activity was based on mg microsomal protein no change in formaldehyde production between the two groups was seen (Fig. 1).

To study the metabolic efficiency for nitrosamine oxidation in kwashiorkor rats, we compared the blood level of NDMA and NMOR between 0.5 and 8 hr after the application of each compound in both animal groups. The results are shown in Fig. 3 and Fig. 4. For all four groups nearly linear kinetics were found in the measured time range. The concentration of NDMA at all time points was about 4-fold higher in the blood of kwashiorkor rats than in that of control animals, and the concentration of NMOR about 2-fold. This is consistent with the finding of Swann and McLean [16], who found a 2-fold decrease in NDMA metabolism in rats fed a protein free diet for 8 days prior to i.p. injection with 50 mg/kg NDMA.

The molar amounts of nitrosamines were higher for NMOR than those for NDMA in both kwashiorkor and control rats. This demonstrates a higher metabolic rate of the less lipophilic NDMA. No difference in NDMA content in blood was measurable between kwashiorkor and control rats 8 hr after application, whereas the concentration of NMOR in both groups could be extrapolated to coincide after 12 hr.

* Visible as a fatty layer during microsome preparation.

DISCUSSION

The results of the *in vitro* experiments (Fig. 1) have shown that NDMA was demethylated by liver microsomes at a similar rate in the kwashiorkor rats

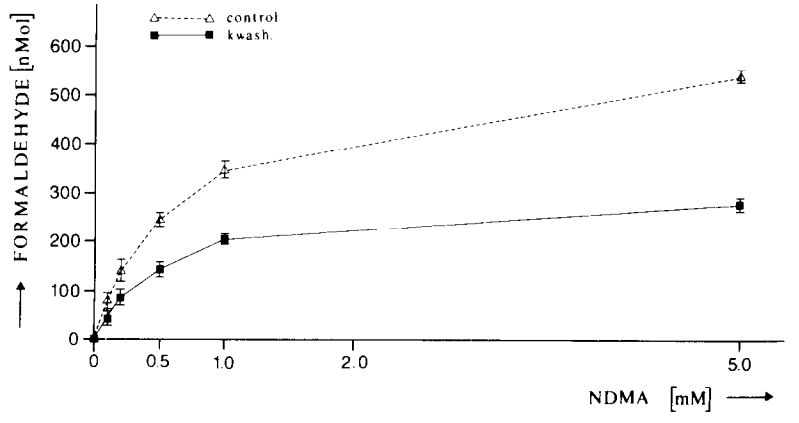

Fig. 2. NDMA-demethylase activity in rat liver microsomes. The ordinate indicates nmole formaldehyde produced per g liver in 30 min. All values are arithmetic means with S.D. of 5 measurements.

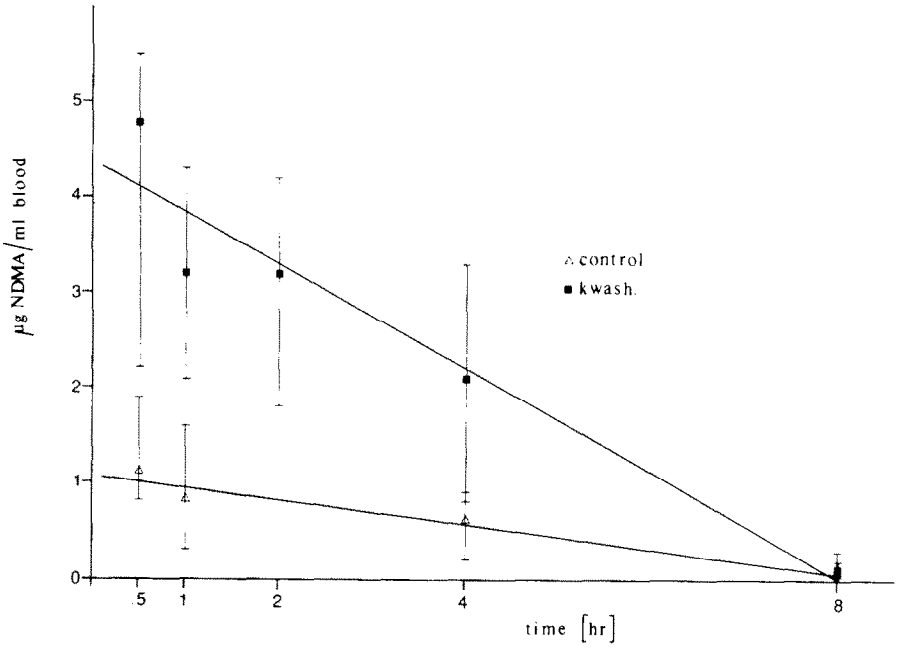

Fig. 3. Elimination of NDMA from the blood after i.v. application of 4.0 mg/kg. The points represent median values with their range; the lines were calculated by linear regression.

as in the normal animals. NDMA-demethylase activity in the liver of the kwashiorkor animals showed a highly significant difference to that of the normal rats when the values were calculated per gram liver weight (compare Fig. 1 and Fig. 2). This difference is due to a much lower protein content in the liver of kwashiorkor rats compared with the controls (Table 1). Indeed fatty liver is one of the reported features in kwashiorkor animals (7–10), and was also observed in our preparations.

Qualitatively (K_m, V_{max}) no change in the *N*-demethylase due to low protein diet is anticipated, but the quantity of enzyme is reduced because of lower protein synthesis (Table 1). This interpretation

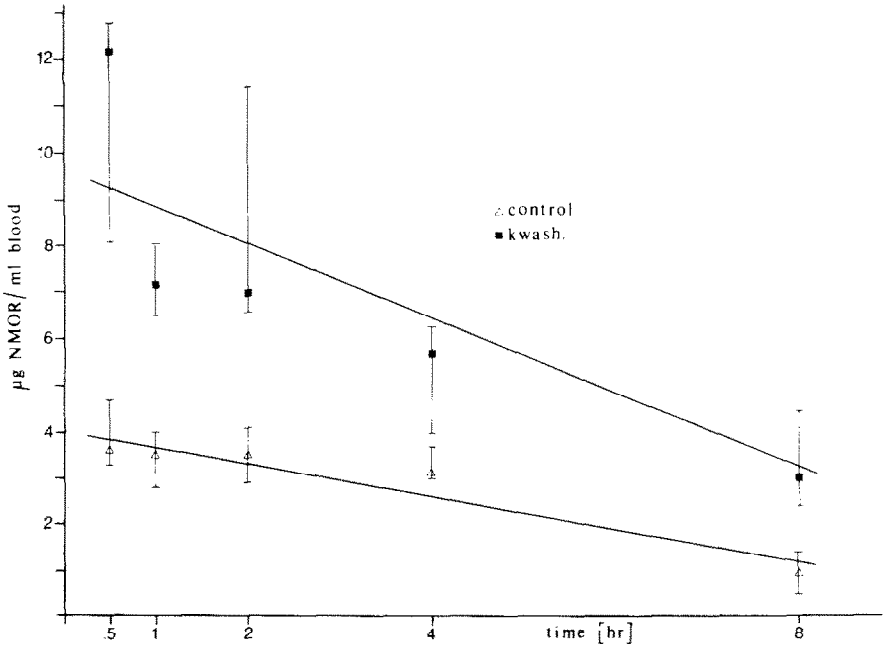

Fig. 4. Elimination of NMOR from the blood after i.v. application of 6.3 mg/kg. The points represent median values with their range; the lines were calculated by linear regression.

is supported by the observation of Standstead [29] that liver function remains unaltered despite structural changes in the liver occurring in PEM.

The higher content of GSH in the livers of kwashiorkor rats (Table 1) compared to the normal content reported here differs from previous reports [30–32] where lower GSH-levels were observed after protein-free or protein-deficient diets. This could be caused by the acute deprivation to which those animals were subjected. The kwashiorkor animals in our experiments underwent a chronic shortage of protein from their weaning and were at a stage where no further deterioration or improvement in condition was observable. The higher content of GSH might also indicate a condition of possible revitalisation of the liver parenchyma, which is known to be structurally affected in kwashiorkor [8], to enable the liver to maintain normal functions.

Zinc deficiency which has been monitored in plasma of kwashiorkor patients [29] and is known to increase GSH-content of liver [33] may also contribute to the elevated GSH concentration in the kwashiorkor rats. The elevated GSH concentration in the liver of kwashiorkor animals may reflect a higher ability of these animals to detoxify foreign compounds. If nitrosamines are detoxified by GSH, this could explain the dramatic increase in LD_{50} for NDMA observed by Swann *et al.* [15] in rats fed a low protein diet.

The metabolism of NDMA and NMOR should be directly correlated with the amount and activity of the metabolizing enzymes. If we assume that the oxidative metabolism of *N*-nitrosamines occurs at the same rate in kwashiorkor as in control rats (see Fig. 1) then the differences found (Figs. 3 and 4) can be explained by the different amount of protein per g liver (Table 1). Since the given dose is related to the body weight and consequently to the liver weight, the overall degradation depends on the activity of the nitrosamine metabolising enzymes per g liver; NDMA demethylase is about 2-fold higher in the control group (see Fig. 2).

As was demonstrated the metabolism of NDMA and NMOR is reduced in kwashiorkor rats. The elimination rate from the blood as well as the oxydative dealkylation in liver microsomes is lower. If rats were treated with disulfiram similar effects were observed: dealkylation of NDMA was inhibited [34] and elimination of NDMA and NDEA from the blood was lowered [35]. Furthermore the $^{14}CO_2$ exhalation of ^{14}C labelled NDMA, NDEA and *N*-nitrosopyrrolidine was inhibited [36–38]. As a consequence of the reduced metabolism the acute toxicity was drastically diminished [39]. Therefore we suggest a reduced toxicity of NDMA and NMOR in kwashiorkor rats, as it is described for NDMA after a protein deficient diet [15].

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