TCDD. 2 additional chlorinated compounds (**IVa** and **b**), a major and a minor, were identified as trichloro-dimethoxydibenzo-p-dioxins. The exact position of the substituents in these 2 metabolites remains unknown. Hydroxylation could take place at position 1, by direct chlorine replacement or via NIH-shift. In another metabolic study on various chlorinated dibenzo-p-dioxins different hydroxylated compounds were found but it was not possible to decide whether hydroxylation at position 1 does occur.[11] The mass-spectrum of compound **V** did not show the characteristic fragments of laterally methoxylated dioxins (M^+-15, M^+-43). Exact mass-measurements using high-resolution MS (Varian MAT 212 instrument, resolution 4000) indicated a composition of $C_{14}H_{10}O_3Cl_4$ (experimental mass 365.9352, exact mass 365.9385, courtesy J. Schmid, Givaudan Research Company Ltd, Dübendorf, Switzerland), suggesting a tetrachloro-dimethoxydiphenylether. The fragment M^+-50 (CH_3Cl) has been found with other chlorinated 2-methoxydiphenyl ethers[12]. The formation of this compound would have to involve cleavage of one of the ethereal bridges in the dioxin molecule, perhaps after epoxide formation at the angular carbon atom. Compound **VI** was identified as 1,2-dichloro-4,5-dimethoxybenzene based on its mass-spectrum and its co-elution with a synthetic reference sample (courtesy C. Rappe, University of Umea, Umea, Sweden). This metabolite must have been formed in a reaction involving cleavage of both ethereal bridges in the dioxin system.

The proposed metabolic breakdown scheme for TCDD is shown in figure 3. The biotransformation of TCDD in the dog appears to be a detoxification process, since it could be shown that the acute toxicity of the metabolites (using a crude extract from the bile of TCDD-treated dogs, which contained a mixture of all the compounds mentioned) in the guinea-pig is at least 100 times lower than that of the parent compound.[13] To our knowledge this is the 1st report on the identification of mammalian metabolites of TCDD and it demonstrates that this compound, in some organisms, is not as inert to metabolic attack as has hitherto been believed.

1 Reprint requests should be addressed to: Ch. Sch., Institute of Toxicology, Federal Institute of Technology and University of Zurich, Schorenstr. 16, CH-8603 Schwerzenbach (Switzerland).

2 H. Poiger and Ch. Schlatter, Nature *281*, 706 (1979).
3 J.R. Olson, T.A. Gasiewicz and R.A. Neal, Toxic. appl. Pharmac. *56*, 78 (1980).
4 J.C. Ramsey, J.G. Hefner, R.J. Karbowsky, W.H. Braun and P.J. Gehring, Toxic. appl. Pharmac. *48*, A126 (1979).
5 F. Matsumura and H.J. Benezet, Envir. Health Perspec. *5*, 253 (1973).
6 B.A. Schwetz, J.M. Norris, G.K. Sparschu, V.K. Rowe, P.J. Gehring, J.L. Emerson and C.G. Gerbig, Envir. Health Perspec. *5*, 87 (1973).
7 J.R. Olson, M.A. Holscher and R.A. Neal, Toxic. appl. Pharmac. *55*, 67 (1980).
8 H. Poiger, H. Weber and Ch. Schlatter, Pergamon Ser. Envir. Sci. *5*, 317 (1982).
9 M.Th.M. Tulp and O. Hutzinger, Biomed. Mass Spectrom. *5*, 224 (1978).
10 J.W. Daly, D.M. Jerina and B. Witkop, Experientia *28*, 1129 (1972).
11 M.Th.M. Tulp and O. Hutzinger, Chemosphere *7*, 761 (1978).
12 C.A. Nilsson, Doctorial Dissertation, University of Umea, Umea, Sweden (1977).
13 H. Weber, H. Poiger and Ch. Schlatter, submitted to Toxic. Lett. (1981).

Urinary catecholamine metabolites (vanylmandelic and homovanillic acids) in the rat after subchronic treatment with sodium nitrate or nitrite

S. Laurens, P. Fritsch and G. de Saint Blanquat

Groupe de Recherches sur la Toxicologie des Aliments et des Boissons, INSERM U-87, Université Paul Sabatier, 2 rue François Magendie, F-31400 Toulouse (France), 24 November 1980

Summary. Sodium nitrate and nitrite in rat food (5 and 0.5% respectively) result in a decrease of both food intake and growth. These agents induce a specific increase in the urinary excretion of 2 catecholamine metabolites: vanylmandelic and homovanillic acids.

According to the studies of Goodall et al.[2-4] vanilmandelic acid (VMA), arising principally from the peripheral organs, is the main catabolite of adrenaline and noradrenaline after methylation and oxidative deamination. Homovanillic acid (HVA), generated through similar degradation pathways, is the main catabolite of circulating dopamine[5]. Both these acids are eliminated in their free form in urine and correspond to the most important forms of excretion for 3-O-methylated amines like metadrenaline, normetadrenaline and 3-O-methyldopamine. So, the measurement of these 2 urinary catabolites of catecholamines can provide additional information in this field[6].

The metabolism of catecholamines, and also their role in certain physiological functions (e.g. blood pressure regulation) have been the subject of many investigations. In the present report we consider these basic physiological data with respect to nutrition and food toxicology where nitrates and nitrites are involved. These compounds pose an important problem because of their high concentrations in foodstuffs[7,8], which is due to various factors: accumulation by plants, high levels in drinking water, use as preservatives in meat products. Their physiopathological effects have often been described[9-13] and it is known for example that nitrates and nitrites affect the vasomotricity of the blood pressure[14,15] and the blood supply to various organs[16-18]. Furthermore, in an epidemiological study, Malberg et al.[19] found a relationship between the frequency of hypertension in a population in the state of Colorado (USA) and the high level of nitrate in the drinking water (up to 125 ppm). Egashira et al.[20] reported that nitrates and nitrites disturbed monoamine oxidase activity in vitro in various tissues in the rat. We have investigated the *in vivo* impact of these compounds on cardiovascular activity by investigating their possible effects on the metabolism of catecholamines. It was with this aim that we studied the urinary excretion of the two major metabolites of dopamine and noradrenaline after subchronic administration of sodium nitrate or nitrite.

Materials and methods. 1. Experimental material: 49 Sprague-Dawley rats weighing 75 g at the beginning of experiment were maintained at 23 ± 1 °C in a thermostated

and ventilated animal house lit at regular intervals (12 h light, 12 h dark). They were placed in individual metabolic cages for 21 days. Seven identical groups were set up: the control group had free access to solid chow (U.A.R. Villemoisson-sur-Orge, France); one group of animals was fed a diet containing 68.55 g Na NO_3/kg (5% NO_3^- ion) and another received 7.5 g $NaNO_2$/kg chow (0.5% NO_2^- ion); 2 corresponding pair-fed groups were set up i.e. they received a quantity of chow which was determined each day by the average intake of the rats treated with either nitrate or with nitrite; 2 groups of sodium-equivalent animals i.e. rats which had access to chow containing an amount of NaCl equivalent, in terms of Na^+ concentration, to the nitrate diet (47.18 g NaCl/kg) or the nitrite diet (6.36 g NaCl/kg). Water was given ad libitum.

The animals were weighed every day and the intake of water and chow as well as the diuresis was measured. At the end of the experiment the levels of haemoglobin and methaemoglobin were assayed in the blood - these parameters giving an indication of the effect of nitrates and nitrites. During the last 24 h, the urine was collected in 6 M HCl in order to avoid catecholamine degradation. It was stored for not more than 4 weeks at −30 °C.

2. Assay techniques: The level of haemoglobin was determined heparinized blood according to Van Kampen and Zijlstra's method[21] and methaemoglobin using the technique of Hagesh et al.[22]. For the urine, measurements were made of the pH, the level of protein using the biuret method, glucose by glucose oxidase, sodium and potassium by photometry and creatinine by colorimetry at 520 nm in the presence of picric acid and NaOH.

VMA was assayed using the technique of HYCEL (Houston, Texas, USA): the urine is treated with activated magnesium silicate to eliminate most of the chromogens. This step is followed by extraction with ethyl acetate then with 2.44% sodium carbonate. The VMA recovered is mixed with a solution of diazo-p-nitroaniline and a diazo-VMA derivative obtained. After washing several times with NaOH solution, ethanolamine in methanol is added to stabilize the color which is measured at 530 nm.

The HVA was assayed with Sato's technique[23]: the urine is brought to pH 4.5 with acetate buffer then passed through a chloride-form Dowex 50×4 100 to 200 mesh ion exchange resin. The HVA is eluted out with a NaCl solution and the eluate is extracted with a chloroform/toluene (1:4 v/v) mixture then with a pH 8.5 tris buffer. After elimination of the organic phase, the aqueous phase is centrifuged and the last traces of organic solvent removed.

Part of the aqueous phase is used for the fluorescence reaction in strongly basic medium and in the presence of potassium ferricyanide and cysteine. The assay is carried out at 320 nm (excitation) and 420 nm (fluorescence).

Results. It can be seen in the table that the reduced growth of the treated and the pair-fed animals is linked to a decrease in food intake in as much as the protein efficiency coefficient remained unchanged and the presence of sodium in the diet did not cause any weight change. Diuresis was greatly increased by both nitrate and sodium chloride at high concentration in the diet; this increase was generally linked to water intake. It can also be noted that the level of creatinine in the urine and the ratio of urinary Na: ingested Na did not vary. The results for pH, protein and urinary glucose were unaltered and are not reported in the table. As for the blood haemoglobin and methaemoglobin levels, we found the methaemoglobinaemia characteristic of nitrate and nitrite treatment.

The catecholamine measurements show that under our working conditions, nitrate and nitrite increase the excretion of VMA (fig. 1) and HVA (fig. 2) whereas the pair-fed and sodium-equivalent animals show excretion levels

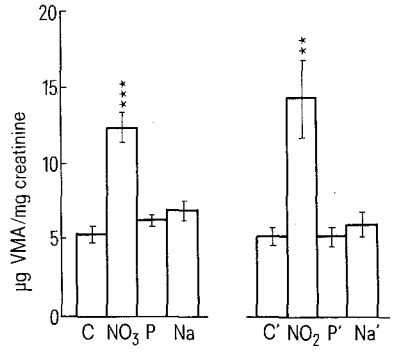

Figure 1. Levels of vanilmandelic acid in the urine of rats undergoing various treatments: C and C': Controls; NO_3 and NO_2: animals receiving sodium nitrate or nitrite; P and P': pair-fed animals; Na and Na': animals receiving an equivalent ammount of sodium (NaCl) to those treated with sodium nitrate or nitrite.

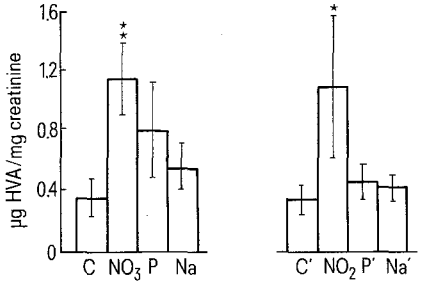

Figure 2. Levels of homovanillic acid in the urine of rats undergoing various treatments (legends as fig. 1).

Nutritional and urinary parameters in the rat after 21 days of treatment with sodium nitrate or nitrite (including a control group, treated groups, pair fed groups and sodium-equivalent groups)

	P (g/21 days)	PER[a]	Food intake (g/day)	Water intake (ml/day)	Diuresis (ml/day)	Urinary creatinine (mg/kg/day)	Na in the urine / Ingested Na
Control	135.7±7.91	11.3±0.26	23.7±0.66	30.0±1.30	15.5±1.10	41.5±1.93	0.54±0.025
NO_3	108.4±5.93*	10.1±0.37	19.9±1.24*	66.5±2.22***	55.2±2.60***	44.2±2.29	0.62±0.045
PF	103.1±6.02*	9.7±0.49	19.3±0.59	28.0±1.91	12.5±1.92	42.6±3.48	0.52±0.031
equivalent Na	131.2±7.56	10.9±0.54	20.4±0.92*	62.1±2.89***	45.4±2.80	43.9±1.54	0.64±0.026
NO_2	91.5±6.67***	11.1±0.51	14.1±0.92***	32.5±2.31	16.8±2.23	34.5±3.58	0.63±0.020
PF	71.9±1.61***	9.3±0.17***	13.6±0.15***	24.0±1.50	11.4±1.63	38.6±4.84	0.58±0.032
equivalent Na	138.2±8.43	12.3±0.62	19.3±0.92*	29.6±3.33	20.1±1.43*	41.9±2.61	0.68±0.021

[a]PER: Protein efficiency ratio: weight gain over 21 days/protein nitrogen intake over 21 days. *$p<0.05$; **$p<0.01$; ***$p<0.001$ (t-test with respect to controls). [b]t-test with respect to NO_2 group ($p<0.05$); [c]t-test with respect to NO_2 group ($p<0.001$).

which are more or less identical with those of the controls. As an example VMA (expressed as µg per mg creatinine) goes from 5.41 ± 0.52 in the controls to 12.42 ± 1.10 in the nitrate-treated group and to 14.31 ± 2.58 in the nitrite-treated group. Similarly, the level of HVA expressed in the same way goes from 0.38 ± 0.12 in the controls to 1.15 ± 0.22 and 1.08 ± 0.46 in the nitrate and nitrite-treated groups respectively.

Discussion. Weight variations observed in animals receiving sodium nitrate or nitrite have already been reported[9,24]; the technique used in the present investigation also shows that the presence of these substances in the diet causes a drop in food intake. Pankow et al.[25] and Csallany and Ayaz[26] suggested that the rat modulates its intake of nitrate/nitrite according to its capacity to resist methaemoglobinaemia through adaptation of the corresponding enzyme systems. The results which we obtained with the pair-fed and sodium-equivalent groups indicate that it is the decrease in food intake which is the cause of the reduced growth of the treated animals.

We also observed the well-known diuretic effect of nitrate with concomittant high liquid intake linked to Na^+ ion urinary excretion. Sodium nitrate and chloride are both osmotic diuretics and recent studies have shown that nitrate has no specific effects on filtration in the glomerule or reabsorption in the tubules[27,28]. In our experiment this is confirmed by the fact that a certain number of urinary parameters remain unaffected by nitrate treatment.

The catecholamine study shows that nitrates and nitrites clearly increase the urinary excretion of VMA and HVA. This is probably indicative of a modification of the metabolism of the amines in question. The modification could be related to various vasomotor effects. The variations observed suggest an increase in the metabolism of the amines - this would allow a relationship to be established with known experimental pharmacological effects such as rise in blood pressure in the dog[14] and the rat[15] or increase in the blood supply to various organs in the dog[29] the rabbit[16,17] and the rat[18]. These vasomotor effects already observed under the influence of nitrate or nitrite have sometimes been explained by an action on the muscle fiber[30,31] and also by displacement of the equilibrium of the fluid compartments of the organism. In any case these phenomena can be influenced to a greater or lesser extent by catecholamines. Our results therefore complement these previous observations and it is probable that the action of nitrates/nitrites on catecholamine metabolism (through a mechanism as yet to be elucidated), is one of the key points enabling an explanation to be given of the very numerous effects of these compounds in the various physiological functions mentioned above.

1 This work was supported by D.G.R.S.T. grant No. 80.G. 0908 (Nutrition humaine - Toxicologie alimentaire).
2 Mc C. Goodall and H. Alton, Biochem. Pharmac. *14*, 1595 (1965).
3 Mc C. Goodall and H. Alton, Biochem. Pharmac. *17*, 905 (1968).
4 Mc C. Goodall and H. Alton, Biochem. Pharmac. *18*, 295 (1969).
5 Mc C. Goodall and H. Alton, Biochem. Pharmac. *21*, 2401 (1972).
6 J.M. Cottet-Emard, L. Peyrin and J. Bonnod, J. neural Transm. *48*, 189 (1980).
7 J.W. White, J. agric. Fd Chem. *23*, 886 (1975).
8 M. Jagerstad, A. Norden and R. Nilsson, Ambio *6*, 276 (1977).
9 WHO Food Additives Series No. 10, 86 (1978).
10 J. Gehrmann-Fink und H.J. Kerber, Dt. tieräztl. Wschr. *85*, 50 (1978).
11 A.E. Wasserman, Proc. a. Rec. Meat Conf., University of Connecticut 1978; abstr. p. 121.
12 W. Lijinsky, Adv. mod. Toxic. *1*, 149 (1979).
13 G. de Saint Blanquat, Annls Nutr. Aliment. *34*, 827 (1980).
14 A.A. Rubin, L. Zitowitz and L. Hausler, J. Pharmac. exp. Ther. *140*, 46 (1963).
15 M.J. Fregly, Exp. biol. Med. *115*, 386 (1964).
16 M. Mazur, P. Polakowski and J. Staszweski, Polskie Archwm Med. wewn. *54*, 27 (1975).
17 E.E. Galal, A. Kandil, M.A. Latif and T. Khedr, J. Drug Res. Egypt. *7*, 145 (1975).
18 C. Cazottes, G. de Saint Blanquat and P. Fritsch, Naunyn-Schmiedebergs Arch. Pharmak. *309*, 69 (1979).
19 J.W. Malberg, E.P. Savage and J. Osteryong, Envir. Pollut. *15*, 155 (1978).
20 T. Egashira, R. Koide, M. Ikeno, Y. Awaya, H. Ayokadi and K. Takahara, Showa Igakkai Zasshi *36*, 419 (1976).
21 E.J. Van Kampen und W.G. Zijlstra, Clin. chim. Acta *6*, 538 (1961).
22 E. Hegesh, N. Gruener, S. Cohen, R. Bochkovsky and H.I. Shuval, Clin. chim. Acta *30*, 679 (1970).
23 T.L. Sato, J. Lab. clin. Med. *66*, 517 (1965).
24 P. Fritsch, M.T. Canal, G. de Saint Blanquat and E. Hollande, Annls Nutr. Aliment. *34*, 1097 (1980).
25 D. Pankow, W. Ponsold, D. Liedke, G. Gessner and A. Pfordte, Acta biol. med. germ. *34*, 1205 (1975).
26 A.S. Csallany and K.L. Ayaz, Toxic. Lett. *2*, 141 (1978).
27 M.W. Weiner, Proc. Soc. exp. Biol. Med. *158*, 370 (1978).
28 J.M. Goldinger, B.D. Erasmus, Y.K. Song, F.J. Koschier and S.K. Hong, Biochem. biophys. Acta *598*, 357 (1980).
29 I. Greene and E.P. Hiatt, Am. J. Physiol. *176*, 463 (1954).
30 K.H. Jhamandas and C.W. Zash, Can. J. Physiol. Pharmac. *45*, 675 (1967).
31 I. Dynarowicz, J. Wilczer and T. Garbulinski, Pol. J. Pharmac. Pharm. *27*, 521 (1975).

Host macrophages are involved in systemic adoptive immunity against tumors[1]

H. Van Loveren, Margriet Snoek and W. Den Otter

Department of Pathology, Rijksuniversiteit Utrecht, Pasteurstraat 2, NL-3511 HX Utrecht (The Netherlands), 29 July 1981

Summary. The positive systemic therapeutic results obtained with adoptive transfer of immune spleen cells could not be reproduced in macrophage depleted mice. Thus, host macrophages are involved in systemic adoptive immunity against tumors.

Immune spleen cells injected into tumor-bearing mice may have a systemic antitumor effect[2]. There are also many data suggesting that host macrophages exert an antitumor effect[3]. These data are consistent with the hypothesis that the systemic antitumor effect of injected immune spleen cells is due to co-operation of the injected lymphocytes with host macrophages. In this study this hypothesis was tested by elimination of macrophages from the tumor-bearing mice before the transfer of immune lymphocytes. The macrophages were eliminated with silica. I.p. silica treatment