3 Still, J. L., and Sperling, E., J. biol. Chem. *182* (1950) 585.
4 De Marco, C., and Crifo, C., Enzymologia *33* (1967) 325.
5 Dixon, M., and Kenworthy, P., Biochim. biophys. Acta *146* (1967) 54.
6 Yusko, S. C., and Neims, A. H., J. Neurochem. *21* (1973) 1037.
7 Shack, J., J. natl Cancer Inst. *3* (1943) 389.
8 Nagata, Y., Shimojo, T., and Akino, T., Comp. Biochem. Physiol. *91B* (1988) 503.
9 Nagata, Y., and Akino, T., Comp. Biochem. Physiol. *89B* (1988) 179.
10 Yamada, R., Nagasaki, H., Wakabayashi, Y., and Iwashima, A., Biochim. biophys. Acta *965* (1988) 202.
11 Yamada, R., Nagasaki, H., Wakabayashi, Y., and Iwashima, A., Biochim. biophys. Acta *990* (1989) 325.
12 Konno, R., and Yasumura, Y., Genetics *103* (1983) 227.
13 Konno, R., Isobe, K., Niwa, A., and Yasumura, Y., Biochim. biophys. Acta *967* (1988) 382.
14 Konno, R., and Yasumura, Y., Lab. Anim. Sci. *38* (1988) 292.
15 Konno, Y., Nagata, Y., Niwa, A., and Yasumura, Y., Biochem. J. *261* (1989) 285.
16 Aswad, D. W., Analyt. Biochem. *137* (1984) 405.
17 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. biol. Chem. *193* (1951) 265.
18 Nagata, Y., Konno, R., Yasumura, Y., and Akino, T., Biochem. J. *257* (1989) 291.
19 Negri, A., Massey, V., and Williams, Jr, J. biol. Chem. *262* (1987) 10026.
20 Hamilton, G. A., in: Advances in Enzymology, vol. 57, p. 85. Ed. A. Meister. Academic Press, New York and London 1985.

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Creatol (5-hydroxycreatinine), a new toxin candidate in uremic patients

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Summary. Both 5-hydroxy-1-methylhydantoin (**3**) and the hitherto unrecognized 5-hydroxycreatinine (2-amino-5-hydroxy-1-methyl-imidazol-4(5*H*)-one or creatol) (**6**) can be isolated from the urine of uremic patients, in whom these compounds probably arise as oxidative metabolites of creatinine (**1**). The enhanced production of the well-known uremic toxin, methylguanidine (**8**), from creatinine (**1**) in such patients, almost certainly occurs via the newly recognized metabolite (**6**).
Key words. Creatinine; 5-hydroxycreatinine; creatol; 5-hydroxy-1-methylhydantoin; uremic toxin; uremia.

During our investigation of novel metabolites produced in animals under abnormal conditions such as viral infection, we isolated two hydantoins, 1-methylhydantoin (**2**) and its 5-hydroxy derivative (**3**), which eventually proved to be bio-active as plant growth regulators[1]. Although neither hydantoin has been detected in normal animals, we recently showed that mammals do have the capacity to metabolize (**2**) into (**3**) and thence into methylurea (**5**)[2,3], as represented in figure 1. We therefore concluded that the production of the new metabolite (**3**) in infected animals probably depended on the formation[4,5] of its precursor (**2**) from creatinine (**1**) under the abnormal conditions pertaining. Hence, we expected to find evidence for the metabolite (**3**) in uremic patients, who were already known[4] to be capable of converting creatinine (**1**) into the precursor (**2**).

A greatly increased production of the uremic toxin[6,7] methylguanidine (**8**) has been observed in mammals with chronic renal failure, and creatinine (**1**) has been implicated as one of its precursors. The involvement of 'active oxygen' in the oxidative conversion of creatinine (**1**) into methylguanidine (**8**) has been demonstrated[8,9], as has the increased capacity of uremic mammals to produce such active species[10]. However, the mechanism by which the production of (**8**) is increased[6,11,12] in uremic mammals has remained an open question. Seeking to answer this question, we now postulate an alternative oxidative pathway, operative in uremic mammals, for the conversion of creatinine (**1**) to methylguanidine (**8**) via 5-hydroxycreatinine ('creatol': **6**) and perhaps 2-amino-1-methyl-1*H*-imidazole-4,5-dione (**9**) (fig. 1).

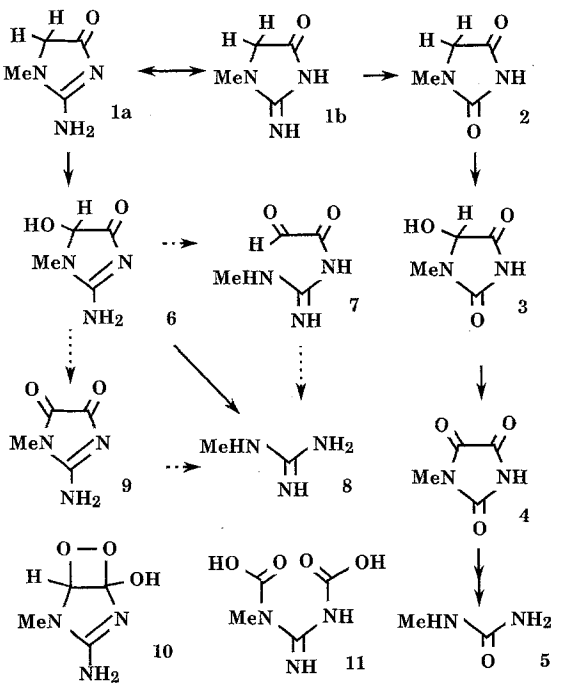

Figure 1. The metabolic pathway of creatinine (**1**) in uremia.

As evidence for the proposed dual oxidative metabolism of creatinine (**1**) in uremic patients, we sought and isolated the two key metabolites (**3**) and (**6**) in the urine of such patients. Urine samples from two uremic patients, whose blood creatinine levels were around 10 mg/dl, were collected during 24 h. T. F., female, suffering from diabetes and K. M., male, with chronic renal failure gave urine samples (27 and 35 ml, respectively); they had corresponding blood creatinine values of 11.0 and 11.5 mg/dl.

From both urines, (**3**) and (**6**) were isolated as follows. The urine obtained from one patient was divided equally into two portions. The first half was evaporated to dryness in vacuo. The residue was applied to a Biolex-70 ion exchange column (H^+ form). After washing with H_2O in order to remove salts, an initial eluate with 0.1 N-AcOH (before the creatinine fraction) was evaporated in vacuo to give a residue, from which the product (**6**) was isolated by reverse-phase HPLC using a Develosil ODS-5 μ column and eluting with 0.1 % aqueous TFA (trifluoroacetic acid). The isolated (**6**) gave a single peak in each of two HPLC analyses using Develosil ODS-5 μ and Asahi-pak GS-320H columns; a recovery value for (**6**) was obtained from the first HPLC analysis. From the other half of the urine, (**3**) was isolated as reported previously from animal urines[2,3]; the sample was diluted with a two-fold volume of methanol and evaporated to give a solid which was added to methanolic ethyl acetate (1:6). After removal of insoluble material by filtration, the filtrate was concentrated and purified by silica gel column chromatography (chloroform-methanol, 9:1) to give pure (**3**); a recovery value for (**3**) was obtained from HPLC analysis. Both patients had similar amounts of (**3**) and (**6**), ca 0.5 and ca 1.5 μg/ml, respectively.

Although each was isolated from a different fraction, the two creatinine metabolites, (**3**) and (**6**), had rather similar ^{1}H-NMR spectra in D_2O, consisting of a single methyne proton around 5 ppm and a single methyl signal around 3 ppm (fig. 1). The first metabolite was clearly 5-hydroxy-1-methylhydantoin (**3**) because its ^{1}H-NMR and EI-mass spectra were identical with those of an authentic specimen[1,3]. The second metabolite was assigned the structure, 2-amino-5-hydroxy-1-methylimidazol-4(5*H*)-one, i.e. 5-hydroxycreatinine (**6**: 'creatol'), from the spectral data (fig. 2); this was subsequently confirmed by direct comparison of the isolated and synthetic specimens: the hydrochloride, m.p. 190 °C, of 5-hydroxycreatinine (**6**) was prepared for analysis by the oxidation of a creatinine derivative (procedure not shown). Creatol (**6**) could not be detected in normal mammals even after i.v. or p.o. administration of creatinine (**1**), whereas the optically active (**3**) was isolated from mammals to which (**2**) had been administered[3].

Although Aoyagi et al. have proposed without evidence a hypothetical pathway for conversion of creatinine (**1**) into methylguanidine (**8**) via a type of dioxetane (**10**) and its ring-fission product (**11**)[8], our demonstration of the existence of creatol (**6**) in uremic patients now provides the key to a more reasonable explanation for the increased production of (**8**) in such patients. Further studies on the in vivo mechanism for the formation and/or degradation of creatol (**6**) (involving the role of 'active oxygen') are now in progress. To date, we have observed that creatol (**6**) is converted slowly into methylguanidine (**8**) in vitro by simply stirring in water ($t_{1/2}$ = 5d: at 37 °C), whereas the same conversion occurs much more quickly in Fenton's reagent which provides 'active oxygen'. This fact is consistent with our proposed in vivo mechanism for the formation of (**8**) via (**6**), but it is not possible yet to conclude whether (**8**) is formed from (**6**) via (**7**) or (**9**).

If it is true that uremia (creatininemia) induces the catabolic pathway of conversion of creatinine (**1**) into methylguanidine (**8**) via creatol (**6**), quantification of creatol (**6**) in urine and/or serum will become important in order to judge the possibility whether the value for cre-

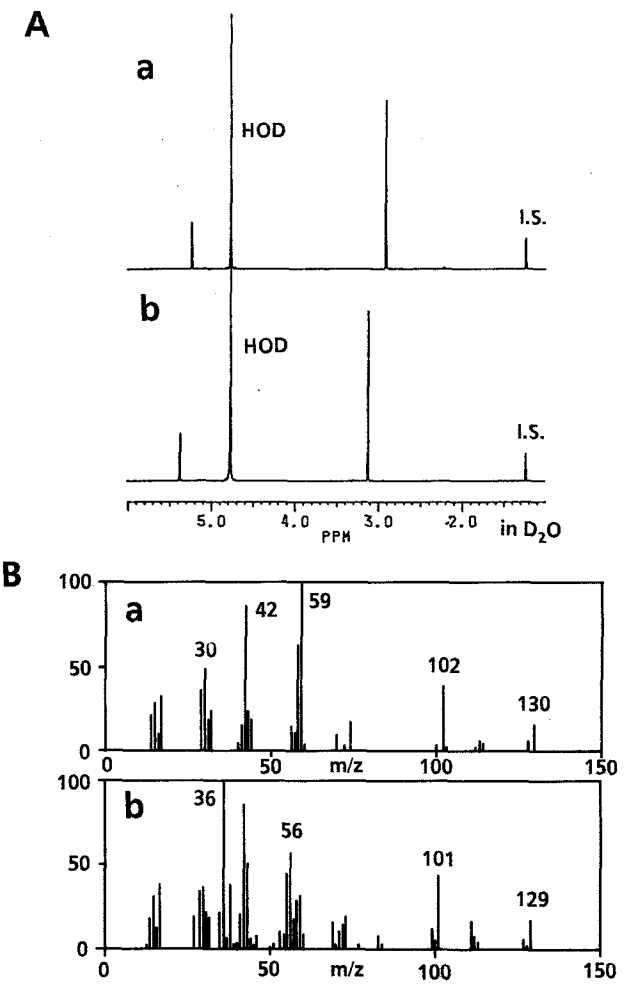

Figure 2. Creatol (**6**) and 5-hydroxy-1-methylhydantoin (**3**): metabolites isolated from uremic patients. *A* 400 MHz ^{1}H-NMR analysis. *B* EI Mass analysis. (a) 5-Hydroxy-1-methylhydantoin (**3**). (b) Creatol (**6**). ^{1}H-NMR conditions were as follows: Instrument: Bruker AM-400 spectrometer; internal standard: t-butanol (1.23 ppm); solvent: D_2O. EI Mass conditions were as follows: Instrument: Hitachi M-80B spectrometer; ionization voltage: 70 eV.

atol itself, and/or the ratio of (**6**) versus creatinine or another creatinine metabolite, provide a useful diagnostic tool for judging the severity of uremia in patients. Moreover, creatol (**6**) should now be considered as a candidate uremic toxin, mainly because it is a pro-toxin as the precursor of methylguanidine (**8**), which is already accepted as a toxin, but also because creatol (**6**) itself contains an intrinsically toxic guanidino-structure. Studies on the toxicity of creatol (**6**) using animals, including rats with chronical renal failure [7, 12], are now in progress. Each of the two catabolic pathways for creatinine (**1**) via hydroxylated heterocycles, (**3**) or (**6**), in creatininemia might be characteristic enough to be classified, respectively, as a detoxification route and a toxin-producing process.

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1 Ienaga, K., Nakamura, K., Goto, T., and Konishi, J., Tetrahedron Lett. *28* (1987) 4587.
2 Ienaga, K., Nakamura, K., Naka, F., and Goto, T., Biochim. biophys. Acta *967* (1988) 441.
3 Ienaga, K., Nakamura, K., Ishii, A., Taga, T., Miwa, Y., and Yoneda, F., J. chem. Soc., Perkin Trans. *I* (1989) 1153.
4 Szulmajster, J., Biochem. biophys. Acta *30* (1958) 154.
5 Jones, J. D., and Burnett, P. C., Kidney Int. *7* (1975) 294.
6 Giovannetti, S., Balestri, P. L., and Barsotti, G., Archs int. Med. *131* (1973) 709.
7 Yokozawa, T., Mo, Z. L., and Oura, H., Nephron *51* (1989) 388.
8 Aoyagi, K., Nagase, S., Narita, M., and Tojo, S., Kidney Int. *22* (1987) 229.
9 Nagase, S., Aoyagi, K., Narita, M., and Tojo, S., Nephron *40* (1985) 470.
10 Giardini, O., Taccone-Gallucci, M., Lubrano, R., Ricciardi-Tenore, G., Bandino, D., Silvi, I., Ruberto, U., and Casciani, C. U., Nephron *36* (1984) 235.
11 Giovannetti, S., Biagini, M., Balestri, P. L., Navalesi, R., Giagnoni, P., de Matleis, A., Ferro-Milone, P., and Perffetti, C., Clin. Sci. *36* (1969) 445.
12 Yokozawa, T., Zheng, P. D., Oura, H., and Koizumi, F., Nephron *44* (1986) 230.

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The effect of endotoxin on membrane fatty acid composition in BCG-sensitized mice

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Summary. The effects of endotoxin on mouse liver phospholipid fatty acid composition have been investigated. Administration of endotoxin from *Salmonella abortus equi* led to a decrease in the polyunsaturated fatty acid content of livers from mice sensitized with Bacille Calmette Guérin (BCG). The content of arachidonic acid fell significantly in both the phosphatidylcholine and phosphatidylinositol fractions whereas in the phosphatidylethanolamine fraction the linoleic acid content was significantly reduced. The polyunsaturated fatty acids were replaced by increased amounts of oleic acid and palmitic acid, leading to a reduction in the polyunsaturated to saturated fatty acid ratio.
Key words. Endotoxin; fatty acid; phospholipid; BCG.

Mice can be made highly sensitive to bacterial endotoxin by the intravenous injection fourteen days beforehand of attenuated live mycobacteria (BCG, Bacille Calmette Guérin) [1]. The mechanisms responsible for this enhanced susceptibility are not fully defined. Recently we have shown that mice infected with BCG exhibit changes in their phospholipid fatty acid composition and triacylglycerol content [2]. In particular, we found that BCG infection resulted in an increased polyunsaturated to saturated fatty acid ratio in the phospholipids of the liver, spleen and peritoneal macrophages. This change in membrane fatty acid composition might form the basis of the increased sensitivity to endotoxin.

BCG infection is associated with a state of profound macrophage hyperactivity [3]. Such 'primed' macrophages may be stimulated into a fully 'activated' state by endotoxin in which they produce increased amounts of tumour necrosis factor, prostaglandins, leukotrienes and superoxide anion [4, 5].

After administration of endotoxin, the polyenoic content might be expected to fall due to the conversion of these fatty acids to eicosanoid derivatives such as prostaglandins and leukotrienes [5, 6].

This paper details the changes in membrane fatty acid composition in livers from sensitized mice after injection with bacterial endotoxin.

Materials and methods

Mice: Adult TO strain mice (25 g) were given i.v. approximately 10^7 live organisms of BCG (Glaxo) suspended in 0.4 ml of phosphate buffered saline (PBS) to establish the