Influence of Germ-Free Status on the Excretion of Simple Phenols of Possible Significance in Tumour Promotion

Local application of phenol, *p*-cresol and 2-ethylphenol promotes the development of skin tumours in mice after a single initiating application of dimethylbenzanthracene[1]. The former 2 compounds and the *para* isomer of ethylphenol are the major urinary simple phenols when natural plant-containing diets are fed to rats[2], and it has been shown that the intestinal microflora plays a major role in the formation of these substances[3]. It was therefore deemed of interest to study the excretion of simple phenols in germ-free and conventional rats.

Germ-free rats of the CDF-strain were raised and reared as described elsewhere[4]. Control animals were of the same strain and were from the 4th generation after conventionalization. 4 rats aged 110–130 days and weighing 150–200 g were used in each group. 10 days before and during the study a commercial pellet diet (Felleskjøpet, Oslo) described previously[2] was fed. The diet was autoclaved at 120 °C for 20 min. The animals were allowed free access to food and water. The rats were placed in metabolic cages and the individual 48-h urines were obtained by the use of separators which disposed of the faeces. Control urines from the conventional rats were collected in containers placed in solid carbon dioxide whereas the urines from the germ-free rats were collected at room temperature and removed from the isolators every 24 h. The urines were stored at − 20 °C and thawed, filtered and diluted to 20 ml immediately prior to analysis. Samples of 10 ml of the diluted urines were hydrolyzed with β-glucuronidase (type H2 containing sulphatase, Sigma Chemical Co.), extracted and analyzed by gas chromatography as described previously[2,5].

Except for traces of phenol (< 0.005 mg/24 h), simple phenols were not detected in the hydrolyzed urine of the germ-free rats. The excretion of the major urinary phenols in the conventional rats was as follows (mg/24 h, mean with range in parentheses): phenol 0.09 (0.06–0.16), *p*-cresol 0.28 (0.08–0.73), and 4-ethylphenol 0.38 (0.31–0.47). Smaller amounts of catechol, 4-methylcatechol and resorcinol and traces of guaiacol were also identified in these urines. The almost complete absence of urinary simple phenols is a previously unreported biochemical feature of germ-free animals. This finding demonstrates that with the exception of phenol which appears to arise to a very small extent in the tissues the normal microflora is essential for the formation of these compounds.

The process of continuous absorption of small amounts of simple phenols produced in the intestinal lumen is quite different from the experimental conditions used in the study of tumour promotion. Nevertheless, the fact that tumour-promoting activity is a property of some volatile simple phenols[1,6] should not be disregarded although the significance of the normal production of these compounds has not been investigated.

TANNENBAUM and SILVERSTONE[7] found that the feeding of a low protein diet reduced the incidence of spontaneous hepatomas in C3H mice and this suggests that protein or protein metabolites may be involved in the formation of these tumours. The urinary excretion of phenol and *p*-cresol in rats also decreases when the dietary protein intake is reduced[8]. Recently, GRANT and ROE[9] reported that germfree C3H mice are more resistant to hepatoma induction by dimethylbenzanthracene than mice of the same strain removed from the isolators and kept under 'minimal disease' conditions. They discussed whether immunological factors, virus etiology or microbial destruction of a protecting agent may explain this observation. In view of the information now available this difference in susceptibility might also be related to tumour promotion by some simple phenols produced through the action of the intestinal microflora on precursors normally present in the diet.

Zusammenfassung. In keimfrei aufgezogenen Mäusen werden fast keine einfachen Phenole mit dem Urin ausgeschieden, was auf den Mangel an Darmbakterien zurückzuführen ist.

O. M. BAKKE[10] and T. MIDTVEDT[11]

Department of Pharmacology, University of Bergen, Bergen, and Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt, Rikshospitalet, Oslo (Norway), 4 December 1969.

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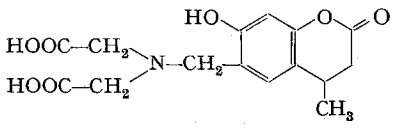
[10] Department of Pharmacology, University of Bergen, Bergen (Norway).
[11] Kaptein W. Wilhelmsen og frues bakteriologiske institutt, Rikshospitalet, Oslo (Norway).

Calcein Blue as a Fluorescent Label in Bone

Some fluorescent compounds which are fixed in newly formed calcified tissues may be used to label bone deposition. Such labels help to determine the time sequence of bone growth, repair and adaptation to functional demand. The use of alizarin red S[1,2], tetracyclines[3], porphyrines[4] and the fluoresceins Calcein[5] and DCAF[5,6] has been reported. These labels may be given singly or sequentially, in the latter case to enhance the effectiveness of the method in monitoring modelling[7] and remodelling. A double label is obtained by combining 2 different tetracyclines[8] or by combining a single tetracycline with alizarin red S[9] or haematoporphyrine[4]. To provide a trichrome label, alizarine red S[6] or haematoporphyrine[10] may be used with DCAF and tetracycline. Four different tetracyclines have also been administered[11]. There are disadvantages in the use of some of these substances. Alizarin red S has been claimed to inhibit further bone growth[9] and it is hardly detectable in the presence of

fuchsin stain. The use of haematoporphyrine is also limited, primarily because the high doses (300 mg/kg) necessary to obtain distinct labelling[12] may result in lethal intestinal complications. Furthermore, it has been observed that haematoporphyrine as well as alizarin red S is deposited at a somewhat different site from that of tetracycline and DCAF[13]. The advantages of multiple labelling and the above-mentioned difficulties prompted a search for other compounds for possible use as fluorescent labels.

Calcein-blue



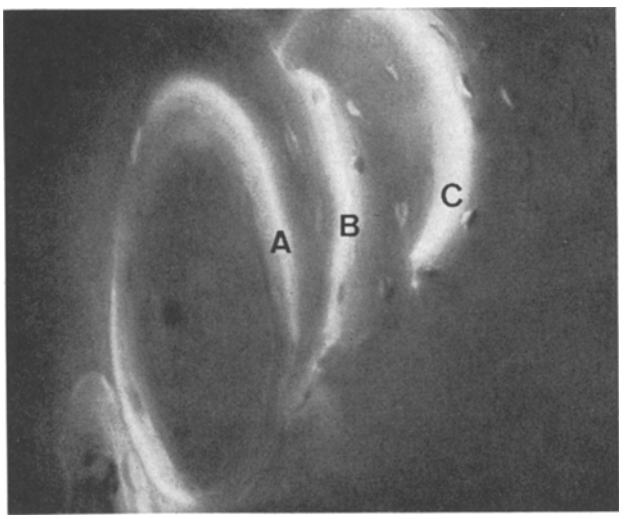
Calcein-blue[14], an indicator for the complexometric determination of Ca, Sr and Ba was tested. It was dissolved as a 3% solution in 2% $NaHCO_3$ and injected in sheep (i.v.), rabbits (i.v.) and rats (i.p.) at a dose of 30 mg/kg. The experimental animals were sacrificed from 24 h to 5 weeks after injection and the long bones fixed in alcohol. Sections 50 μ thick were prepared and examined microscopically under UV-illumination.

Calcein-blue was found to produce a blue fluorescence in bone upon UV-excitation[15]. The blue fluorescence appears at the same site as tetracycline and the fluoresceins. At a dose of 30 mg/kg no toxic effect was detected in the experimental animals and bone deposition was found to be unaffected. The fluorescence of Calcein-blue fades somewhat more quickly than the fluorescence of other labels, but sufficient time is provided to prepare photomicrographs. The contrasting fluorescence of Calcein-blue encourages its combination with a tetracycline[16] and a fluorescein[5,6] to obtain a trichrome UV-fluorescent labelling compatible with fuchsin counterstain[17].

Zusammenfassung. Calceinblau ergibt, in Knochen eingebaut, unter UV-Bestrahlung eine klar erkennbare blaue Fluoreszenz. Calceinblau eignet sich vor allem für Mehrfarbmarkierung des Knochens in Kombination mit Tetracyclin und Calcein oder DCAF. Solche Mehrfarbmarkierungen erleichtern das Studium des Knochenumbaus.

B. A. RAHN and S. M. PERREN

Laboratory for Experimental Surgery,
Swiss Research Institute,
CH-7270 Davos (Switzerland), 3 December 1969.



A contrasting, trichrome fluorescent label in a rabbit osteon (A, blue; B, green; C, yellow) is provided by combining Calcein-blue with the fluorescein DCAF and the tetracycline Achromycine®. The labels were injected at time intervals of 10 days.

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[14] Source: Fluka AG, CH-9470 Buchs.
[15] To observe the blue fluorescence a yellow barrier filter must be avoided.
[16] Achromycine® was kindly submitted – through the intermediary of their distributors Opopharma AG, Zürich – by Lederle Laboratories.
[17] We should thank Miss W. HUNTER for her skilful technical assistance.

Carcinogenic Action of Dimethylnitrosamine in Trout not Related to Methylation of Nucleic Acids and Protein in vivo

The carcinogenic action of dimethylnitrosamine has been correlated with the in vivo methylation of nucleic acids and proteins in the target organs[1]. Dimethylnitrosamine is not only a strong, mainly hepatocarcinogen in mammalia[2–5] but is also highly active in rainbow trout[6]. It is generally agreed that methylation is not due to the direct reaction of the unchanged molecule but to an intermediate formed by enzymatic oxidation[1,7,8], even if the formation of diazomethane in vivo is excluded[8].

Comparative studies by GAUDETTE et al.[9] have revealed that fish and certain type of amphibia lack the ability of oxidative drug metabolism, and we have now investigated whether 7-methylguanine is formed in the RNA of pigeon, frog and rainbow trout liver, species of different evolutionary stages, after application of ^{14}C-dimethylnitrosamine.

^{14}C-dimethylamine-hydrochloride was purchased from NEN, Chicago, and converted to the corresponding nitro-