The Origin of Hexahydrohippurate (Cyclohexanoylglycine) in the Urine of Herbivores

M. T. BALBA and W. CHARLES EVANS

Department of Biochemistry and Soil Science, University College of North Wales, Bangor, Gwynedd LL57 2UW, Wales, U.K.

Hexahydrohippuric acid was first described as a metabolite in the urine of cattle at pasture by Suemitsu et al. (1971). It was present to the extent of 50 mg/litre; the biochemical origin of this component remained unknown.

Schogt & Haverkamp Begemann (1965) had identified 11-cyclohexylnundecanoic acid in butter and confirmed it by synthesis. Hansen & Gerson (1967) isolated it from the depot fat of ruminants and bovine rumen bacteria (Hansen, 1967). Subsequently, De Rosa et al. (1972) proved that the alicyclic ring of \( \omega \)-cyclohexyl fatty acids present in the cell lipids of an acidophilic thermophilic bacillus (\textit{Bacillus acidocaldarius}) was derived from shikimate. In view of our demonstration (see the following paper [Balba & Evans 1977]) that cyclohexanecarboxylate is an intermediate in the methanogenic fermentation of benzoate by an adapted consortium of rumen bacteria, there remained the possibility that, if this occurs in the intact animal, some might be absorbed and conjugated with glycine in the liver or kidney. These alternative hypotheses as to the origin of hexahydrohippurate in the urine have now been tested.

A Welsh Mountain wether (castrated ram) (35 kg, 3 years old), fistulated to the rumen, was placed in a metabolism crate fitted with a continuous urine-collection attachment and fed on a normal daily diet of hay (400 g) and sheep pellets (250 g, BOCM Silcock); water was offered ad libitum. The tight seal of the rumen cannula had a polythene tube passing through it directly into the rumen; externally this polythene tubing was attached to a peristaltic pump for the infusion of a measured volume of fluid (250 ml) over a predetermined time-span (48 h). An initial urinary collection over this period was made by using water as the drip-infusion fluid. In separate experiments, non-radioactively labelled substrates followed by the same compounds labelled with \( ^{14} \text{C} \) were administered in the above volume of water, and the individual urine samples collected, as follows.

The substrates used were: (1) \([\text{ring-U-}^{14}\text{C}]\text{benzoate}\) (The Radiochemical Centre, Amersham, Bucks., U.K.), 0.2 \( \mu \text{Ci/ml} \) (47719 c.p.m./ml; Philips liquid-scintillation counter); \([\text{ring-U-}^{14}\text{C}]\text{phenylalanine}\) (The Radiochemical Centre), 0.1 \( \mu \text{Ci/ml} \) (62226 c.p.m./ml); \([\text{U-}^{14}\text{C}]\text{shikimic acid}\) (New England Nuclear Corp., Boston, MA, U.S.A.), 0.1 \( \mu \text{Ci/ml} \) (59334 c.p.m./ml).

Urine samples were acidified to pH 3 and extracted with ethyl acetate; acidic components were taken into \( \text{NaHCO}_3 \) solution from this solvent, and, after acidification, the diethyl ether-soluble acids (e.g. benzoic acid and cyclohexanecarboxylic acid) separated from those that remained in the aqueous phase (e.g. hippuric acid and hexahydrohippuric acid). This fractionation was suitable for the identification of some of the components by t.l.c. and g.l.c., but the conjugated acids were not easily separable by these techniques.

The metabolites (from the urine) in the ethyl acetate extract were sharply separated by gel filtration by using Sephadex G-10 (Pharmacia, Uppsala, Sweden) (cf. Sinha & Gabrieli, 1968); the extract (10 mg), dissolved in sodium phosphate buffer (1 ml, 0.1 M, pH 7), was placed on a column (91 cm x 1.4 cm) of Sephadex G-10 (bed vol. 130 ml), developed with a similar buffer with a constant-flow-rate peristaltic pump and fractions (5 ml/tube) were collected (20 ml/h) (Combi Cold Cabinet at 4°C and LKB automatic fraction collector). The \( A_{260} \) of the effluent was automatically recorded to locate the
Fig. 1. Gel-filtration pattern of four authentic acids of interest as metabolites in urine

The fractions are: (1) $^{14}$C-cyclohexanecarboxylate; (2) $^{14}$C-hexahydrohippurate; (3) hippurate; (4) benzoate.
The Johns Hopkins Press, Baltimore

The Methanogenic Fermentation of Aromatic Substrates

M. T. BALBA and W. CHARLES EVANS

Department of Biochemistry and Soil Science, University College of North Wales, Bangor, Gwynedd LL57 2UW, Wales, U.K.

Tarvin & Buswell (1934), through their quantitative approach to the fermentation of biological materials by sewage sludge, showed that benzoate, phenylacetate, phenylpropionate and cinnamate were completely utilized by micro-organisms under strictly anaerobic conditions. Clarke & Fina (1952), Barker (1956), Fina & Fiskin (1960), Roberts (1962), Nottingham & Hungate (1969) and Ferry (1974) studied this phenomenon; the knowledge gained can be summarized as follows.

(i) A 'consortium' from rumen liquor or the black sewage-digester sludge can be established, which ferments benzoate anaerobically according to the equation:

\[ 4C_6H_5CO_2H + 18H_2O \rightarrow 15CH_4 + 13CO_2 \]

(ii) [carboxyl-\(^{14}\)C]Benzoate behaved like exogenous \(^{14}\)CO\(_2\) in that it was not primarily reduced to methane (less than 25\%). [ring-\(^{14}\)C\(_1\)]Benzoate appeared mainly as \(^{14}\)CH\(_4\). [ring-\(^{14}\)C\(_4\)]Benzoate was converted largely into \(^{14}\)CO\(_2\).

(iii) Propionate, acetate and formate were detected in the steam-volatile fatty acid fraction of the fermentation liquor. The propionate was labelled when [ring-\(^{14}\)C\(_4\)]benzoate was used as substrate, but not with [ring-\(^{14}\)C\(_1\)]benzoate or [carboxyl-\(^{14}\)C]-benzoate.

(iv) \(\alpha\)-Chlorobenzoate inhibited benzoate utilization without affecting methane production from acetate (Ferry, 1974).

No experimental evidence existed regarding the nature of the biochemical transformations of the aromatic ring before the production of the aliphatic acids in this consortium.

Anaerobic inocula cultures from sheep rumen liquor (5 litres and 1 litre) at 37°C in benzoate/mineral-salts media (Ferry, 1974) were established. It took about 2 months for them to reach a steady state in C balance, i.e. approximating to the stoichiometry demanded by the above equation; this time-lag could be shortened to 1 month by the inclusion of acetate in the initial medium. Gas production was measured continuously and its composition monitored occasionally in a suitable g.l.c. apparatus fitted with a katharometer detector; it had a composition of methane (54\%) and CO\(_2\) (46\%) with slight variations. No H\(_2\) or any other gas was detected. The rate of benzoate utilization was 6mg/h in the 5-litre cultures; at this stage, periodic sampling (100ml) of the fermentation liquor followed by exploration of the acid/diethyl ether-soluble components revealed the following.