# PROTON MOVEMENT ACCOMPANYING MONOCARBOXYLATE PERMEATION IN HEMOGLOBIN-FREE PERFUSED RAT LIVER

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## 1. Introduction

While considerable information has accumulated recently from studies with isolated liver mitochondria on transport systems for di- and tricarboxylates (cf. reviews [1, 2]), comparatively little is known on the properties of uptake and release by the liver cell of the major metabolic fuels which are either uncharged (e.g. glucose) or weak monocarboxylic acids (fatty acids, lactic and pyruvic acids). The experiments to be reported here represent attempts to follow the rate and the extent of monocarboxylic acid uptake and release by the liver cell. With the help of a 'model monocarboxylic acid', benzoic acid (pK = 4.2), which is considered metabolically inert when compared to the compounds named above, a simple and continuous method to follow monocarboxylic acid movements into and out of the hepatocyte will be outlined. The experimental system is that of the hemoglobin-free perfused rat liver [3, 4].

The underlying idea is based on the concept of Overton [5], elaborated and applied by Conway to the yeast cell [6, 7] and by Hohorst, Kreutz and Bücher to the liver cell [8], that permeant monocarboxylates are passively transported across biological membranes in the form of the uncharged acids. Therefore, *net* monocarboxylate transport should be coupled to *net* proton transport, no matter whether the mechanism is 'proton symport' or 'hydroxyl antiport'. In other words, the compartment from which monocarboxylic acid is leaving is expected to be rendered more alkaline, and vice versa. On this basis, extracellular pH is explored as an indicator of monocarboxylate gradient equilibration in an open (non-recirculating) system of perfusion.

## 2. Materials and methods

Hemoglobin-free perfusion of livers from female Wistar rats, 140–180 g weight, fed on stock diet, was performed with bicarbonate-buffered solution consisting of 115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $NaH_2PO_4$, 1.2 mM $Na_2SO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, equilibrated with an oxygen–carbon dioxide mixture (95/5, v/v) in a temperature-regulated (33° on liver surface) rotating cylinder oxygenator [4]. The perfusion fluid passed through the liver via vena portae and vena cava at a rate of 3–3.5 ml per min per gram of liver; it was not recirculated (open system). Stepwise additions of substances were made by infusion of neutralized stock solutions into the perfusion fluid entering the liver using precision micropumps.

Perfusate leaving the liver was continuously monitored for pH and for $[O_2]$. pH was detected by an Ingold Ag 7.0 glass electrode with a Knick pH meter 291. $[O_2]$ was measured with a Teflon-shielded Ag–Pt (20 $\mu$m) microelectrode. The signals were fed to compensation recorders (Servogor, Metrawatt) for registration against time.

$\Delta[H^+]$ calibrations were performed by infusion of standardized HCl into the entering perfusate at different concentrations. $\Delta pH/\Delta[H^+]$ were found to be constant over the small pH range under study.

L-Lactate (Roth, Karlsruhe) and pyruvate (Boehringer, Tutzing) were infused as sodium salts to give concentrations of 0.8 and 0.2 mM, respectively. All other chemicals were from Merck, Darmstadt.



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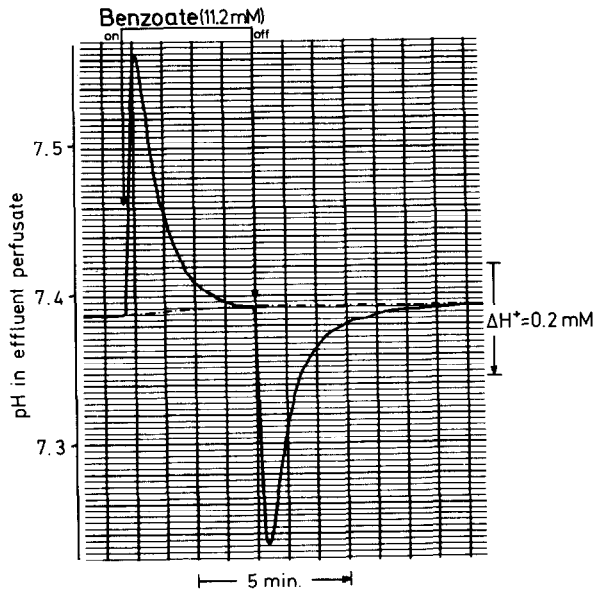


Fig. 1. Response of pH in the effluent perfusate to infusion of sodium benzoate into the entering perfusate in hemoglobin-free perfused rat liver. $[H^+]$ calibration at right is obtained by HCl infusion. Dotted line indicates the area used for planimetry. $H^+$ uptake and $H^+$ release are 1.51 and 1.55 μmoles $H^+$ per g wet weight liver, respectively.

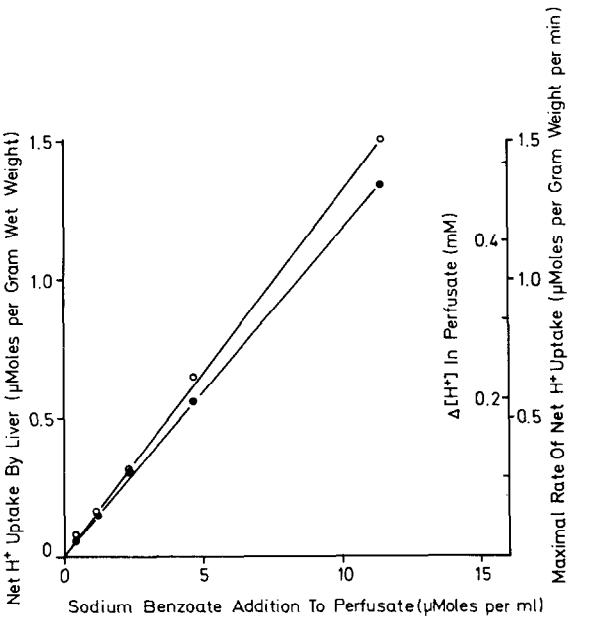


Fig. 2. Dependence of net $H^+$ uptake (left ordinate, ○) and of maximal observed rate of net $H^+$ uptake (right ordinate, ●) upon sodium benzoate concentration added to entering perfusate. Results are obtained from the integrated area and from the peak height, respectively, of the pH excursion such as that shown in fig. 1.

## 3. Results

### 3.1. *Response of perfusate pH to stepwise increase of monocarboxylate concentration*

From what is outlined in the introduction, it is expected that in hemoglobin-free perfused liver a stepwise increase of monocarboxylate concentration in the entering perfusate is followed by a transient alkalinization of the pH in the perfusate leaving the liver as long as there is net transport. In the experiment shown in fig. 1, benzoic acid neutralized to pH 7.4 with NaOH was infused from a stock solution to give a perfusate concentration of 11.2 mM over the period indicated on the top of the figure. There is an initial steep rise of the pH by about 0.16 and a subsequent return of the pH to the original level. When the benzoate infusion is stopped, the reverse process is observed: an initial acidification by about 0.16 pH and subsequent return to the baseline. In the absence of a liver in the system, the final benzoate concentration was shown to be attained within about 5 sec, causing no detectable pH change.

Since the system of perfusion is open, concentration changes in the perfusate may be regarded directly to signify rates. The curve shown in fig. 1 may, therefore, be considered as the time derivative of net proton transport during the process of equilibration of benzoate with the intracellular space. Two features of the pH excursion are evaluated; first, the integrated area under the trace is expressed in terms of μmoles $H^+$ taken up or released per gram liver wet weight, and second, the peak height is expressed in terms of μmoles $H^+$ taken up or released per gram liver wet weight per min, giving a maximal observed rate of uptake.

For the experiment shown in fig. 1, $H^+$ uptake is 1.51 μmoles per gram, and $H^+$ release is 1.55 μmoles per gram. In other words, all of the protons taken up during the increase of benzoate concentration are released from the intracellular space when the gradient is reversed. $H^+$ uptake calculated from the area under the pH trace is linearly dependent upon the concentra-

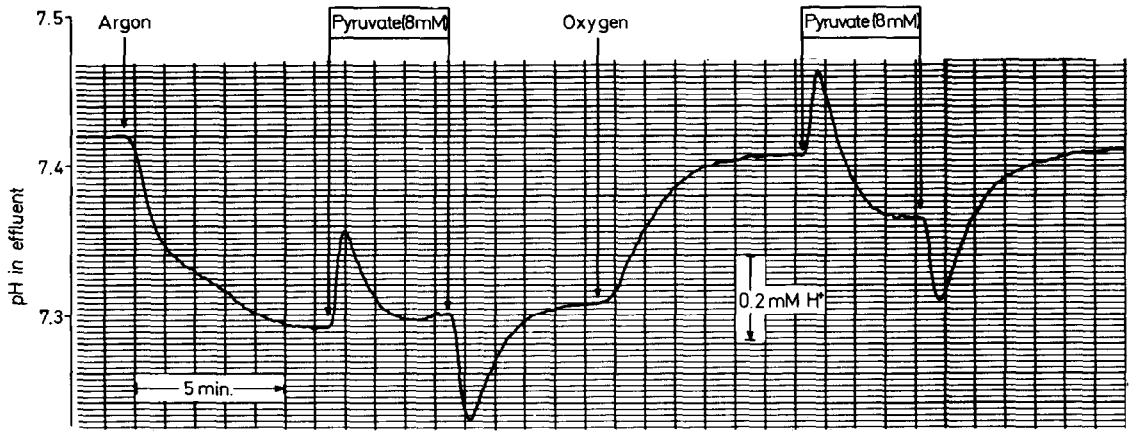


Fig. 3. Response of pH in the effluent perfusate to infusion of sodium pyruvate into the entering perfusate in hemoglobin-free perfused rat liver. At the left hand side, anoxic conditions are provided by exchange of oxygen by argon in the gas mixture. At the center, re-supply of oxygen is indicated.

tion of added benzoate, as is shown in fig. 2 (left ordinate, open circles).

The maximal observed rate of net $H^+$ uptake also is linearly dependent upon the concentration of added benzoate, demonstrated in fig. 2 (right ordinate, full circles). It is about 0.12 μmoles $H^+$ taken up per gram per min when the perfusate concentration of benzoate is raised by 1 mM. It is evident that these rates merely reflect the lower limit of those occurring at the cellular level. Clearly, the bulk rate measured after the liver is dependent on the effectiveness of perfusion.

### 3.2. *The 'metabolic response' observed in effluent pH*

Due to the constant respiration-linked production of carbon dioxide and other acids such as ketone bodies, there is an entering-leaving difference in the perfusate pH. Roughly estimated, 1 g of perfused liver produces 1.4 μmoles $H^+$ per min at a respiration rate of 1.8 μmoles $O_2$ per min.

When monocarboxylates other than an inert substance like benzoate are added to the perfusate, a 'metabolic response' may occur, e.g. an increased production of carbon dioxide. As shown in fig. 3, the infusion of 8 mM neutralized sodium pyruvate into the perfusate entering the liver is followed by a pH excursion of the type demonstrated for benzoate in fig. 1. However, the initial baseline in the effluent pH is reached only in the left part of the figure where the liver is kept anoxic by perfusion with argon-saturated medium. In the right part of the figure, where oxygen supply is restored, there is a new steady state in the effluent pH in the presence of pyruvate, it being 0.04 units more acidic. On a wet weight basis, this corresponds to 0.4 μmoles of extra net $H^+$ production per g liver per min. The integrated areas under the pH trace, however, are similar under normoxic and anoxic conditions.

Simple transitions from one steady state pH to another are observed when metabolically active substances carrying no net charge are infused. Two examples may be briefly indicated, fructose and ethanol, each leading to an acidification. The extra acid production by fructose (fig. 4) is half-maximal at 2.8 mM fructose in the entering perfusate; the maximal rate is 1.5 μmoles $H^+$ per g wet weight per min. This considerable acidification is attributable mainly to the measured extra lactate and pyruvate release from the liver. Glucose, in contrast, has no effect on effluent pH. The extra acid production caused by infused ethanol is partially sensitive to the alcohol dehydrogenase inhibitor, pyrazole.

## 4. Discussion

The monocarboxylate gradient equilibration between extracellular and intracellular spaces of rat liver

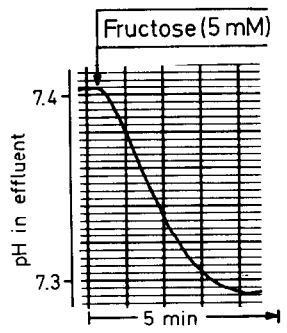


Fig. 4. Response of pH in the effluent perfusate to infusion of fructose into the entering perfusate in hemoglobin-free perfused rat liver. In this experiment, the new steady state pH corresponds to an extra production of acid equivalents of 1.2 μmoles $H^+$ per g liver per min.

is directly demonstrated to be accompanied by proton movement. Continuous measurement of this process is afforded by sensitive measurement of extracellular pH in an open perfusion system. The process is fully reversible (cf. fig. 1) and is linearly dependent upon the monocarboxylate concentration in the range examined (cf. fig. 2). This implies a strict stoichiometry between net monocarboxylate transport and net $H^+$ transport. However, this stoichiometry factor must not necessarily be equal to unity. For example, simultaneously there may be equally strictly coupled proton–cation or monocarboxylate–anion exchanges. Another possibility would be that only a constant fraction of the intracellular space would reveal net $H^+$ changes whereas other compartments respond differently. Recently, a correlation between $H^+$ and $P_i$ and malate movement in isolated rat liver mitochondria gave a stoichiometry factor of unity [9]. Preliminary results on the stoichiometry for benzoate and $H^+$ movement in the perfused liver indicate that it may be below unity. While 0.13 μmoles $H^+$ per g liver are transported (net) at a gradient of 1 mM benzoate in the perfusate (fig. 2), the $^3H$-benzoate space was found higher than the $^3H_2O$ space in perfused liver.

Nevertheless, the strict relationship between monocarboxylate gradient and observed $\Delta H^+$ qualifies sensitive extracellular pH measurement as a suitable tool to follow monocarboxylate transport. A useful example is given in a recent study on the flow-equilibrium between the extracellular monocarboxylate redox couple lactate/pyruvate and the intracellular cytosolic coenzyme couple NADH/$NAD^+$ [4]. Upon a stepwise increase of lactate concentration, the integrated extracellular pH increments and the intracellular NADH-specific fluorescence increments during the equilibration gave a practically identical time response, suggesting that the permeation of lactate into the cell was rate-limiting.

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