Biochimica et Biophysica Acta 814 (1985) 23–34
Elsevier

BBA 72509

Mechanisms of uptake of ketone bodies by luminal-membrane vesicles

Karl Evald Jørgensen and M. Iqbal Sheikh

Institute of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C (Denmark)

(Received August 7th, 1984)

Key words: Ketone body transport; Membrane potential; Na^+ dependence; (Rabbit kidney)

The energetics and location of renal transport of acetoacetate, β-hydroxybutyrate, α-hydroxybutyrate and γ-hydroxybutyrate by luminal-membrane vesicles from either whole cortex or pars convoluta or pars recta of rabbit proximal tubule were studied. Addition of either acetoacetate or β-hydroxybutyrate or its analogues to dye-membrane-vesicle suspensions in the presence of Na^+ gradient (extravesicular > intravesicular) resulted in absorbance changes indicative of depolarizing event(s). Valinomycin enhanced the Na^+-dependent uptake of monocarboxylic acids, provided a K^+ gradient (intravesicular > extravesicular) was present. By contrast, Na^+-dependent uptake of these compounds was nearly abolished by ionophores that permit Na^+ to pass through the luminal-membrane via another channel, either electrogenically (e.g. gramicidin D) or electroneutrally (e.g. nigericin). These results established that the Na^+-dependent transport of ketone bodies and analogues by luminal-membrane vesicles is an electrogenic process. Eadie-Hofstee analysis of saturation kinetic data suggested the presence of multiple transport systems in vesicles from whole cortex for these compounds. Tubular localization of the transport systems was studied by the use of vesicles derived from pars convoluta and pars recta. In pars recta uptake of all these compounds was mediated by means of a single high affinity common transport system. Uptake of these compounds by vesicles from pars convoluta was carried out via a relatively low affinity but common transport system. The physiological importance of the transport systems is discusssed.

Introduction

Monocarboxylic acids especially L-lactate, pyruvate, acetate and long chain fatty acids under normal physiological conditions, and ketone bodies during fasting and postnatal development are among the major metabolic fuels of the mammalian kidney (for review, see Ref. 1). Recently, renal transport of monocarboxylic acids has been studied by luminal-membrane vesicles prepared from whole renal cortex. These membrane vesicle preparations have shown to possess a Na^+-dependent transport system for monocarboxylic acids [2–5]. The transport of these compounds is relatively insensitive to the presence of di- and tricarboxylic acids, glucose, alanine and a number of other compounds, leading to the suggestion that the system is specific for the reabsorption of monocarboxylic acids. However, the results reported in the literature on the Na^+-dependent mechanism of a wide variety of monocarboxylic acids are controversial. Thus, Barac-Nieto et al. [2] found that the presence of monocarboxylic acids such as acetate, pyruvate and propionate (5 mM) in the incubation medium did not significantly inhibit the Na^+-dependent uptake of L-lactate by luminal-membrane vesicles derived from whole renal cortex, suggesting the existence of various transport systems for different monocarboxylic acids. By contrast, Ullrich et al. [6–8] in microper-

fusion experiments found that the Na^+-dependent uptake of D-lactate is inhibited by other monocarboxylic acids.

Garcia et al. [3] have recently studied the mechanism of excretion of ketone bodies (acetoacetate and β-hydroxybutyrate), which is also the subject of the present communication of this series. They found that the uptake of ketone bodies by luminal-membrane vesicles from whole renal cortex was an electroneutral process in contrast to electrogenic transport of L-lactate demonstrated by Barac-Nieto et al. [2].

The primary purpose of this series of studies is to clarify the main characteristics of renal transport system(s) of various monocarboxylic acids. We have recently developed methods for this purpose, based on the use of highly purified membrane vesicles from rabbit kidney cortex [9,10]. The strategy is based on the rapid screening procedures to measure electrogenic transport of solutes by the use of potential-sensitive dye [10,11], in conjunction with conventional transport studies by the Millipore filtration technique [12,13]. With this methodology differences in monocarboxylic acid transport systems are readily shown to exist, which can be characterized in terms of differences in affinity, specificity and cation dependence. By contrast to the previous findings we present clear evidence in this paper for the existence of two different Na^+-dependent electrogenic transport systems for acetoacetate, α-hydroxybutyrate, β-hydroxybutyrate and γ-hydroxybutyrate, localized in luminal-membrane vesicles from two different regions of kidney cortex, namely pars recta and pars convoluta of proximal tubule.

Materials and Methods

Materials

Lithium acetoacetate, sodium salts of α-hydroxybutyrate, β-hydroxybutyrate and γ-hydroxybutyrate together with valinomycin and gramicidin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Nigericin was bought from Calbiochem-Behring Corp., La Jolla, CA, U.S.A. 3,3′-Diethyloxadicarbocyanine iodide was supplied by Eastman Kodak Co., Rochester, NY, U.S.A. Radioactive ethyl[3-^{14}C]acetoacetate (spec. radioactivity 8.5 mCi/mmol) and D-(-)-3-hydroxy[3-^{14}C]butyrate (spec. radioactivity 55 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [3-^{14}C]Acetoacetate was prepared by hydrolysis of ethyl [3-^{14}C]acetoacetate according to the method of Edmon [14].

Isolation of luminal-membrane vesicles

Luminal-membrane vesicles were isolated from either whole renal cortex or from pars convoluta or pars recta of proximal tubule of rabbit kidney as recently reported from this laboratory [11,15]. Vesicles were suspended in a solution containing 310 mM mannitol and 15 mM Hepes/Tris buffer (pH = 7.5), and used within 6 h after the preparation. Vesicles were preloaded by first removing the extravesicular mannitol/Hepes/Tris solution by centrifugation (25 000 × g for 30 min at 2°C) and afterwards resuspending the loose pellet in an isoosmolar loading medium as mentioned in the legends to the figures. The purity of the membrane preparations was examined by electron microscopy and by measuring specific activities of various enzyme markers as previously described [9,11,15]. The amount of protein in different fractions was determined by the method of Lowry et al. [16] as modified by Petersen [17].

Transport studies

The uptake of monocarboxylic acids by luminal-membrane vesicles from whole cortex or pars convoluta or pars recta was studied both by Millipore filtration technique and spectrophotometry as recently described [10,11,13,15]. The details of the individual experiments are given in the legends to the figures. In short, the uptake of radioactive acetoacetate and β-hydroxybutyrate was studied as follows. 50 μl of luminal-membrane vesicle suspension were added at the time zero to 100 μl of incubation medium containing 310 mM mannitol, or 155 mM KCl, or 155 mM NaCl, or 155 mM sodium gluconate, or 103 mM Na_2SO_4 dissolved in 15 mM Hepes/Tris buffer (pH 7.5). Transport of radioactive compounds in vesicles was stopped by addition of 850 μl ice-cold stop buffer containing 155 mM NaCl and 15 mM Hepes/Tris buffer (pH 7.5). The resulting suspension was rapidly filtered through a Sartorius membrane filter (0.45 μM, type SM 11106, Göttingen, F.R.G.) that was washed twice with 2.5 ml of ice-cold stop

buffer. The filter was dried overnight and the radioactivity was counted in a liquid scintillation counter (Wallac LKB 1210 Ultrabeta) in Luma gel (Lumac, The Netherlands). Correction for non-specific binding to the filter and membrane vesicles was made by subtracting from all uptake data the value of a blank obtained by filtering denatured membranes (boiled for 2 min) added to an incubation tube containing radioactive acetoacetate or β-hydroxybutyrate. The rate of uptake of non-radioactive compounds by luminal-membrane vesicles was investigated by the use of potential-sensitive dye, namely 3,3'-diethyloxadicarbocyanine iodide (for details see Refs. 10, 11, 15).

Calculations

The results of the saturation experiments were analyzed by using Michaelis-Menten kinetics. When data of the filtration experiments indicated uptake by more than one transport system the results were analyzed according to the following equation

$$\text{Uptake} = \frac{V^1_{max}[S]}{K^1_m + [S]} + \frac{V^2_{max}[S]}{K^2_m + [S]}$$

where K_m represents the substrate concentration that gives half-maximal uptake, V_{max} denotes maximal uptake and [S] indicates initial concentration of substrate. Index 1 and 2 refer to the first and the second transport system, respectively. In case of transport via a single pathway the same equation without the second fraction was used. The spectrophotometric data were analyzed using an analogue relationship.

$$\Delta A = \frac{\Delta A^1_{max}[S]}{K^1_A + [S]} + \frac{\Delta A^2_{max}[S]}{K^2_A + [S]}$$

where ΔA is the absorbance change measured by addition of solute at concentration [S], ΔA_{max} is the maximal absorbance change and K_A is the monocarboxylic acid concentration producing a half-maximal absorbance. The various kinetic parameters for both types of uptake studies were calculated using iteration computer programs [18].

Results

Time-course of ketone bodies uptake

Figs. 1A and 1B describe the uptake of radioactive acetoacetate and β-hydroxybutyrate (50 μM) by the membrane vesicles prepared from whole kidney cortex during incubation for different lengths of time in various incubation media as measured by Millipore filtration. The presence of sodium salt gradient between the external medium and the intravesicular medium stimulated uptake of both acetoacetate and β-hydroxybutyrate. However, both the initial and maximal uptake values were dependent on the type of sodium salt anion present and increased in the following order; gluconate$^-$ < SO_4^{2-} < Cl^-. In the presence of Na^+-gradient accumulation of acetoacetate and β-hydroxybutyrate into the membrane vesicles was maximal at 1 min. Afterwards the amount of ketone bodies in the vesicles decreased, indicating efflux of these compounds. Replacing sodium salts by KCl or mannitol completely abolished the overshoot. The final level of uptake of these organic anions in the presence or abscence of the Na^+ gradient was approximately the same, suggesting that equilibrium was approached after 1 h of incubation.

Spectrophotometric measurements

By contrast to the previous findings of Garcia et al. [3] the results presented in Figs. 1A and 1B suggest that the uptake of ketone bodies by the luminal-membrane vesicles from whole kidney cortex is dependent on membrane potential (see Discussion). In order to study the importance of the membrane potential for the transport process spectrophotometric measurements using the potential-sensitive dye, 3,3'-diethyloxadicarbocyanine iodide were carried out. Figs. 2A and 2B depict the pattern of uptake of 5 mM acetoacetate and β-hydroxybutyrate, respectively. Curves 1, 2, and 3 in Figs. 2A and 2B show the absorbance changes caused by the addition of ketone bodies in the presence of NaCl, Na_2SO_4 and sodium gluconate, respectively. It is seen that addition of acetoacetate or β-hydroxybutyrate to the membrane-dye suspension in the presence of various sodium salt gradients depolarizes the membrane vesicles and resulted in spectral curves of a form resembling

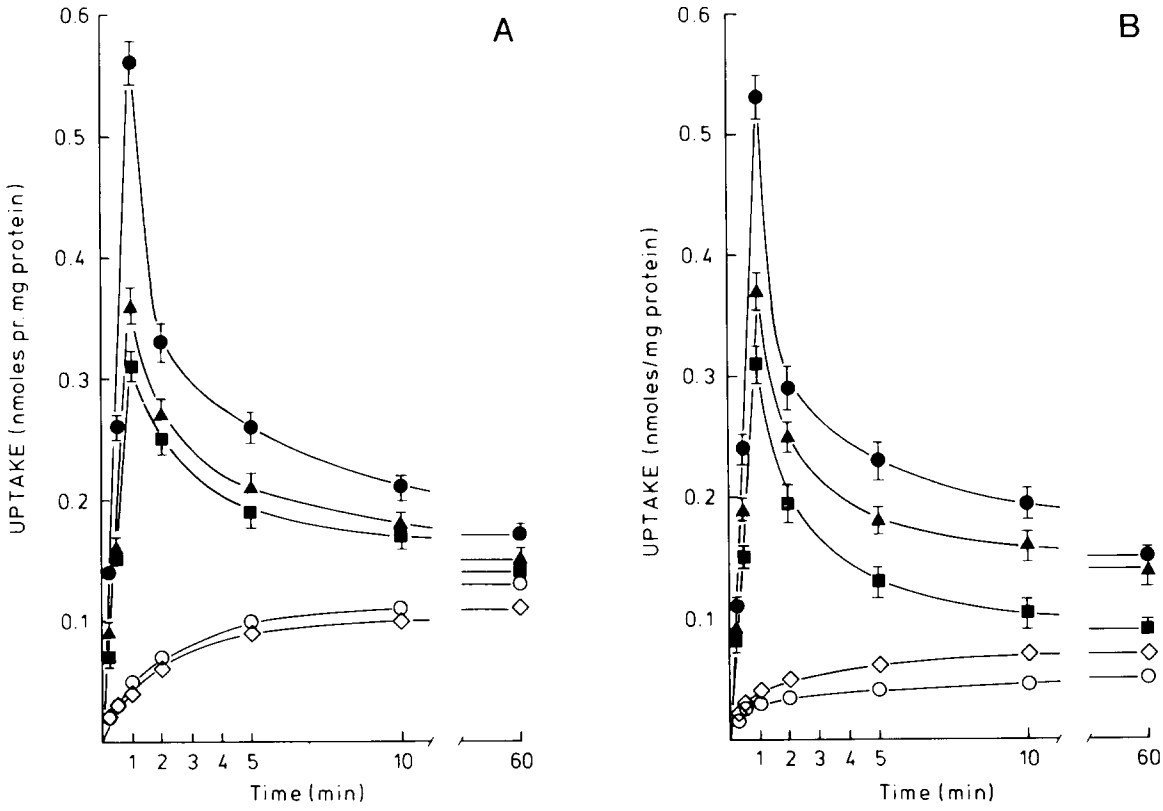

Fig. 1. Uptake of 50 μM [^{14}C]acetoacetate (panel A) and 50 μM β-hydroxy[^{14}C]butyrate (panel B) by luminal-membrane vesicles prepared from whole cortex. Common experimental conditions: protein concentration 2.0 mg/ml; pH 7.5; temperature 20°C. The intravesicular medium was 310 mM mannitol, whereas the external media were 155 mM NaCl (●———●), 103 mM Na_2SO_4 (▲———▲), 155 mM sodium D-gluconate (■———■), 155 mM KCl (◇———◇) or 310 mM mannitol (○———○). In both intravesicular and extravesicular media 15 mM Hepes-Tris was used as buffer system. Results are given as mean values ± S.D. of three experiments.

the Millipore filtration curves shown in Figs. 1A and 1B. The magnitude of the spectral changes ('overshoot') varies with the type of sodium salt anion in the same direction as seen in Figs. 1A and 1B. Replacing sodium salt anion by either KCl, K_2SO_4 or potassium gluconate abolished the overshoot (not shown). Therefore, the observations that penetration of ketone bodies follows $Cl^- > SO_4^{2-} >$ gluconate$^-$ can not be accounted for by the possible alteration in the degree of Ca^{2+} ionization that may occur by the presence of these anions.

Substrate specificity of ketone bodies' transport system

To investigate the structural specificity of the Na^+-dependent ketone bodies' transport pathway in luminal-membrane vesicles from whole renal cortex the spectrophotometric measurements were extended to include α- and γ-isomers of hydroxybutyrate. Introduction of both α- and γ-hydroxybutyrate to the membrane-dye suspension in the presence of sodium salt gradient depolarizes the luminal-membrane vesicles in a similar way as observed in the case of the acetoacetate and β-hydroxybutyrate (not shown).

Effect of ionophores on the uptake of ketone bodies

The question whether the Na^+-dependent transport of ketone bodies is electrogenic or electroneutral was further probed by studying the effect of various ionophores on the uptake of these

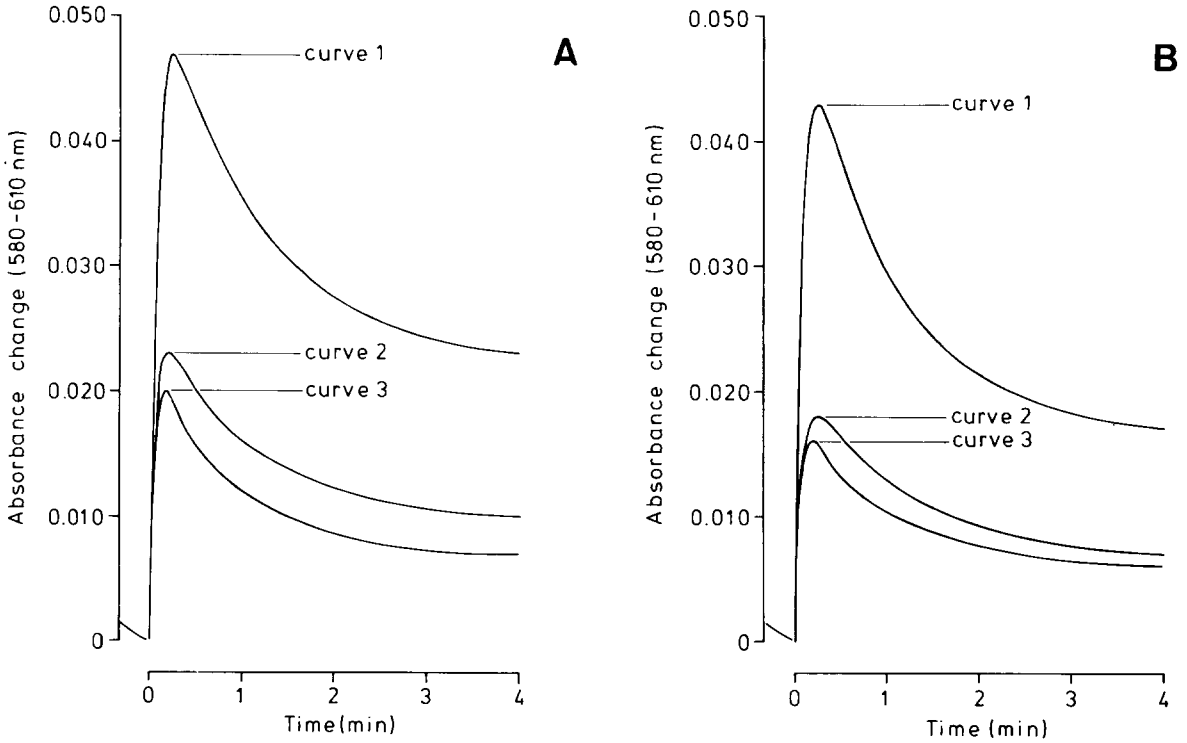

Fig. 2. Uptake of 5 mM acetoacetate (panel A) and 5 mM β-hydroxybutyrate (panel B) by luminal-membrane vesicles prepared from whole cortex as studied by spectrophotometry. Common experimental conditions: protein concentration 0.25 mg/ml; pH 7.5; temperature 20°C; dye concentration 15 μM. The intravesicular medium was 310 mM mannitol, whereas the external media were 155 mM NaCl (curves 1), 103 mM Na_2SO_4 (curves 2) or 155 mM sodium D-gluconate. In both intravesicular and extravesicular media 15 mM Hepes-Tris was used as buffer system. The break in the curves, at 0 min, indicates addition of solute. All the spectral curves were corrected for the effect of adding a small volume of 15 mM Hepes-Tris buffer alone (the medium of the solute's stock solutions). The spectrophotometer was operated in the dual wavelength mode with 580 nm and 610 nm (reference wavelength).

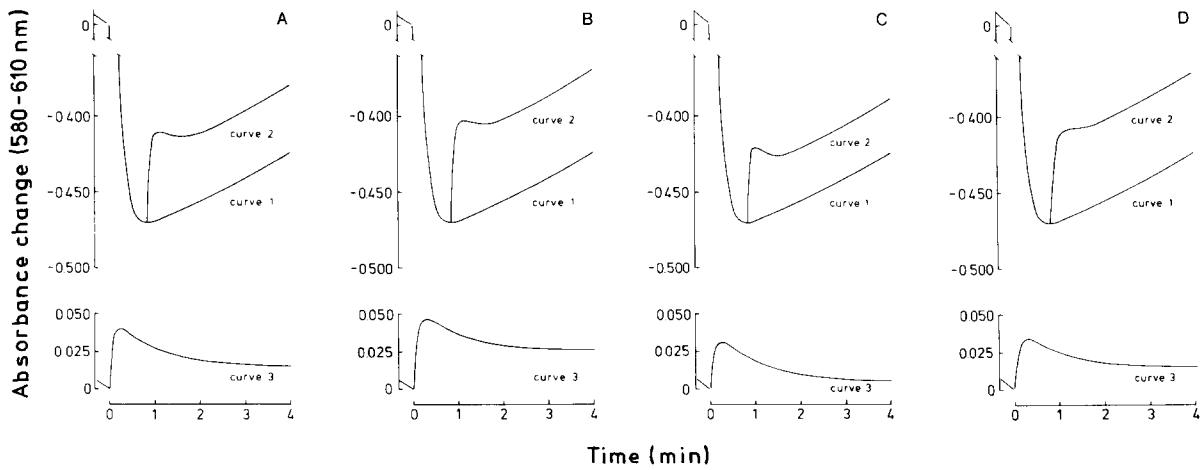

Fig. 3. Effect of valinomycin on the uptake of acetoacetate (panel A), β-hydroxybutyrate (panel B), α-hydroxybutyrate (panel C) and γ-hydroxybutyrate (panel D) by luminal-membrane vesicles prepared from whole cortex. The membrane vesicles were preloaded for 1 h in 103 mM K_2SO_4, and the spectrophotometric experiments were carried out with an external medium of 103 mM Na_2SO_4. Curve 1 in each panel, addition of 5 μg of valinomycin per mg of protein. Curve 2 in each panel, addition of valinomycin and, about 50 s later, 10 mM of the various monocarboxylic acids. Curve 3 in each panel, addition of solute in the absence of valinomycin. For further details see Fig. 2 legend.

compounds. Figs. 3A, B, C and D show the influence of K^+ diffusion potentials generated by valinomycin on the uptake of acetoacetate, β-, α- and γ-hydroxybutyrate, respectively. In these experiments the luminal-membrane vesicles from whole renal cortex were preloaded with K_2SO_4 and suspended in Na^2SO_4. This resulted in an outwardly directed K^+ gradient and an inwardly directed Na^+ gradient. According to expectation addition of valinomycin under these conditions resulted in approx. 2-fold increase in dye response caused by monocarboxylic acids (compare the difference between curves 1 and 2 with curve 3 in Figs. 3A, B, C and D).

The question whether the depolarization caused by the uptake of ketone bodies is obligatorily coupled with the influx of Na^+ in membrane vesicles from whole renal cortex was examined by studying the effect of gramicidin D or nigericin plus valinomycin on the optical response associated with uptake of monocarboxylic acids. The effect of gramicidin D on the rate of uptake of acetoacetate, β-, α, - and γ-hydroxybutyrate was studied as follows. These experiments were carried out with the same initial concentration of Na^+ inside and outside the vesicles in the absence or in presence of gramicidin D. It was seen that in all cases pretreatment of the membrane vesicles with gramicidin nearly abolished the overshoot (not shown). The effect of simultaneous addition of nigericin and valinomycin on the optical response associated with uptake of acetoacetate, β-, α- and γ-hydroxybutyrate was studied as follows. These experiments were carried out with the same initial concentration of Na^+ and K^+ inside and outside the vesicles in the absence or in the presence of nigericin and valinomycin. It was found that pretreatment of the membrane vesicles with these ionophores drastically reduced the optical response (not shown).

Kinetics of ketone bodies' transport

The uptake of acetoacetate and various isomers of hydroxybutyrate in the presence of sodium chloride gradient at different concentrations of these organic compounds by luminal-membrane vesicles derived from either whole renal cortex or from pars convoluta or from pars recta or proximal tubule were studied. Figs. 4A, B, C and D show the absorbance changes measured at the peak of the 'overshoot' (30 s uptake values) induced by addition of increasing concentrations of monocarboxylic acids to membrane vesicle-dye suspension from whole renal cortex. The Na^+-dependent uptake of monocarboxylic acids shows a rapid increase at low medium concentrations (< 2 mM). In the range of 2–6 mM the increase in the uptake of these compounds was less pronounced, and the optical response was almost constant at higher concentrations suggesting that the renal transport of monocarboxylic acids approached saturation. Insets show Eadie-Hofstee analysis of the experimental data presented in Figs. 4A, B, C and D. Curvilinear plots were obtained in all cases, which suggests the presence of multiple transport systems in luminal-membrane vesicles from whole renal cortex for the transport of monocarboxylic acids. The apparent K_A values (i.e. substrate concentration that gives half maximal uptake) for Na^+-dependent renal uptake of moncarboxylic acids are given in Table I (for calculation see Methods). We have also determined the K_A values for these compounds by using the initial rate of uptake (4 s uptake value) instead of measuring the peak value of the 'overshoot'. This resulted in more scattered data, but to very similar K_A values.

To test the validity of the kinetic parameters obtained by spectrophotometric measurements the saturation experiments were repeated by using the Millipore filtration technique. For this purpose radioactive β-hydroxybutyrate was chosen. Fig. 5 shows the Na^+-dependent initial uptake (5 s uptake in the presence of sodium chloride gradient) of β-hydroxy[^{14}C]butyrate at increasing medium concentrations by membrane vesicles from whole cortex. It is immediately apparent from the figure that the uptake of β-hydroxybutyrate in the membrane vesicle preparation is rapid at relatively low concentrations and the renal accumulation of this compound slowly but steadily increases at higher concentrations approaching saturation. Inset in Fig. 5 illustrates Eadie-Hofstee plot of the same experimental data. In accordance with the results obtained by spectrophotometric method a curvilinear relationship between V and V/S is observed, indicating the existence of multiple transport systems in vesicles from whole renal cortex.

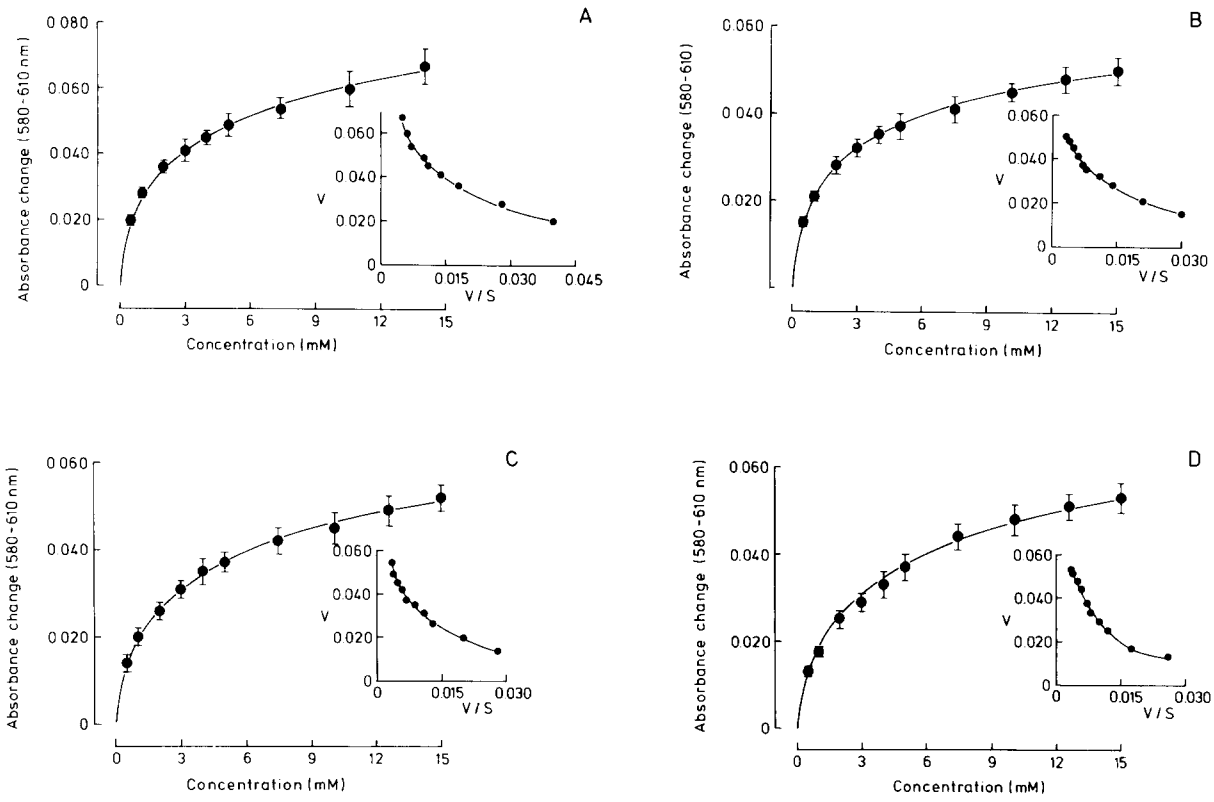

Fig. 4. Uptake of increasing concentrations of acetoacetate (panel A), β-hydroxybutyrate (panel B), α-hydroxybutyrate (panel C) and γ-hydroxybutyrate (panel D) by luminal-membrane vesicles prepared from whole cortex as studied by spectrophotometry. At time zero 100 μl membrane vesicle suspension (7–10 mg protein/ml) was added to 3.0 ml of 155 mM NaCl and dye. After equilibrium was obtained 100 μl of stock solution of the monocarboxylic acid in question was added. In the inset the results are shown in an Eadie-Hofstee plot. V represents the absorbance change at substrate concentration S. For further details see Fig. 2 legend. Results are given as mean values ± S.D. of four experiments. Statistical analysis of the experimental data showed that least-squares analysis give the best fit to the data assuming two transport systems (e.g. S.D. for K_A values for the four monocarboxylic acids assuming one transport system were in the range 3.6–4.4, and S.D. for K_A values for two transport systems were in the range 1.2–1.6).

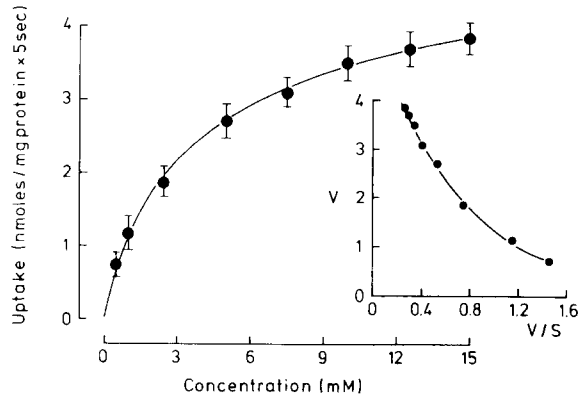

Fig. 5. Uptake of increasing concentrations of β-hydroxy[^{14}C]butyrate by luminal-membrane vesicles prepared from whole cortex. At time zero 20 μl of a concentrated membrane vesicle suspension (16–23 mg protein/ml) was added to 100 μl of uptake buffer containing unlabelled β-hydroxybutyrate, β-hydroxy[^{14}C]butyrate and various concentrations of NaCl in order to maintain a constant Na^+ gradient of 150 mM and a constant isoosmolarity of 341 mosM. The intravesicular medium was 326 mM mannitol and 15 mM Hepes-Tris (pH 7.5). After 5 s the uptake was stopped by adding 1 ml of ice-cold stop buffer consisting of 163 NaCl in 15 mM Hepes-Tris (pH 7.5). In the inset the results are shown in an Eadie-Hofstee plot. V represents the rate of transport at substrate concentration S. Results are as mean values ± S.D. of three experiments.

The K_m and V_{max} values for transport systems in vesicles from whole renal cortex are given in Table I. It should be noted that K_M values for low-affinity and high-affinity transport systems present in

TABLE I

KINETIC PARAMETERS FOR KETONE BODIES' TRANSPORT

K_A^1, K_m^1 and K_A^2, K_m^2 describe substrate concentration (mM) that gives half maximal uptake for high-affinity and low-affinity transport systems, respectively. V_{max}^1 and V_{max}^2 describe rate of maximal transport nmol·(mg protein)$^{-1}$·5 s^{-1}) for high-affinity and low-affinity systems, respectively. The values given are means ± S.D. of four experiments. Statistical analysis of the experimental data

Compound	Vesicles					
	Whole cortex					
	K_A^1	K_A^2	K_m^1	K_m^2	V_{max}^1	V_{max}^2
Acetoacetate	0.98 ± 0.30	6.56 ± 0.50	–	–	–	–
β-Hydroxybutyrate	0.88 ± 0.13	6.91 ± 0.68	1.39 ± 0.19	5.44 ± 0.67	2.03 ± 0.35	3.97 ± 0.18
α-Hydroxybutyrate	1.06 ± 0.22	6.44 ± 0.86	–	–	–	–
γ-Hydroxybutyrate	2.65 ± 0.29	8.03 ± 0.95	–	–	–	–

membrane vesicles from whole renal cortex are in reasonably good agreement with values of this kinetic parameter obtained by spectrophotometric method (compare columns 2 and 3 with columns 4 and 5 in Table I). Therefore, these findings establish that the two different methods used in this study are equally good for the determination of kinetic parameters.

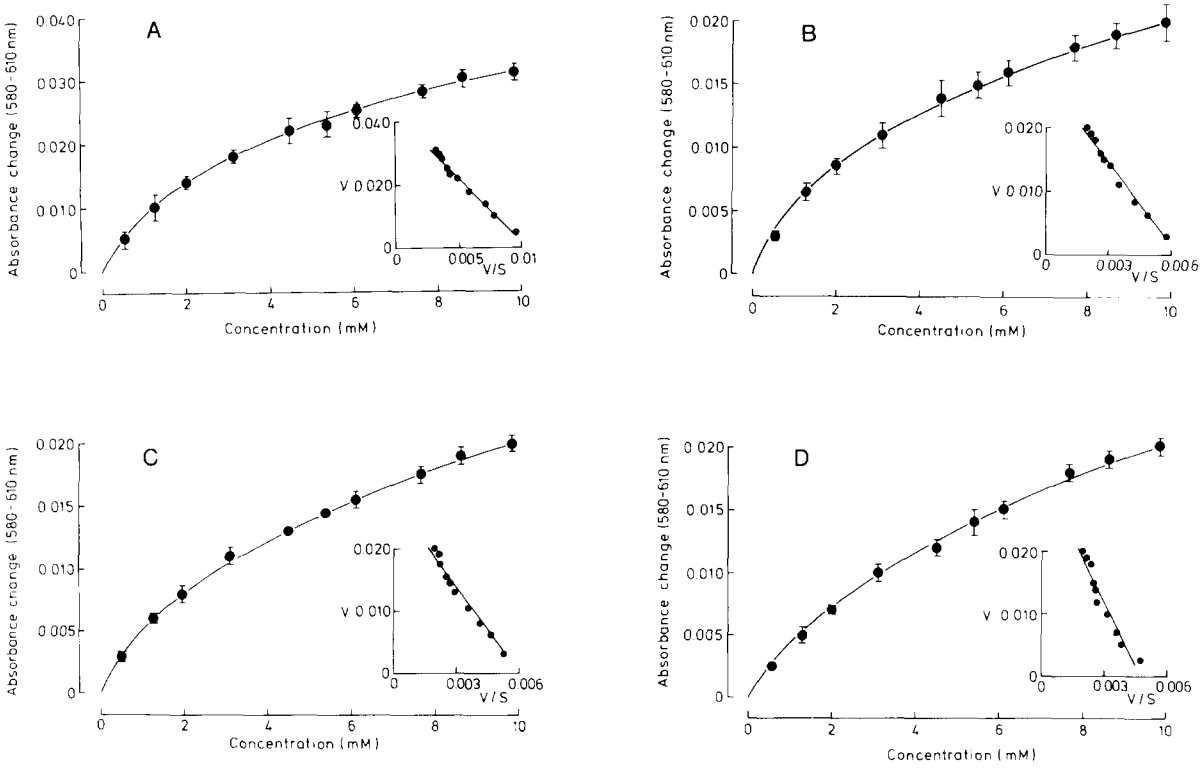

Fig. 6. Uptake of increasing concentrations of acetoacetate (panel A), β-hydroxybutyrate (panel B), α-hydroxybutyrate (panel C) and γ-hydroxybutyrate (panel D) by luminal-membrane vesicles prepared from pars convoluta of the proximal tubules as studied by spectrophotometry. In the inset the results are shown in an Eadie-Hofstee plot. For further details see Fig. 4 legend. Results are given as mean values ± S.D. of four experiments.

showed that K_A values for acetoacetate and β-hydroxybutyrate are not significantly different in the case of vesicles from pars convoluta and pars recta. They are nominally significant in the case of α-hydroxybutyrate when compared to acetoacetate and β-hydroxybutyrate. But K_A values for γ-hydroxybutyrate are significantly higher than that of the other three monocarboxylic acids.

Pars convoluta		Pars recta	
K_A	P	K_A	P
4.55 ± 0.76		1.13 ± 0.15	
4.94 ± 0.86	$0.6 < P < 0.7$	1.03 ± 0.17	$0.4 < P < 0.5$
5.69 ± 0.60	$0.1 < P < 0.2$	1.30 ± 0.12	$0.2 < P < 0.3$
8.85 ± 0.56	$P < 0.01$	2.49 ± 0.11	$P < 0.001$

Tubular localization of ketone bodies' transport systems

In the following we report the results obtained on the tubular localization of monocarboxylic acid transport systems by using luminal-membrane vesicles prepared from pars convoluta and pars recta according to the method described from this laboratory [15]. Figs. 6A, B, C and D show the absorbance changes produced by Na^+-dependent uptake of increasing concentration of acetoacetate, β-, α-, and γ-hydroxybutyrate in luminal-membrane vesicles prepared from pars convoluta. The insets show Eadie-Hofstee analysis of the experimental data. It is apparent from this plot that the renal uptake of monocarboxylic acids by pars convoluta of proximal tubule occurred via a single and a low-affinity transport system. K_A values calculated for these compounds are given in Table I. It is of interest to note that these K_A values are in fairly good agreement with the K_A values for a low-affinity transport system obtained in the case of membrane vesicles prepared from whole renal cortex (for comparison see column 3 and column 8 in Table I). The uptake of increasing concentrations of these monocarboxylic acids were also studied by using membrane vesicles from pars recta. The experimental data are analyzed in a similar way as in the case of vesicle data from pars convoluta. Eadie-Hofstee analysis of the data reveals that the uptake of monocarboxylic acids by membrane vesicles from pars recta occurred also via a single but high-affinity transport system (not shown). These K_A values are in good agreement with the K_A values for the high-affinity transport system observed in the case of vesicles derived from whole kidney cortex (compare K_A values given in column 2 with column 9 in Table I).

Evidence for common transport system for ketone bodies

The results presented in the foregoing sections showed that the characteristics of the uptake of ketone bodies and derivatives by luminal-membrane vesicles are very much alike. In this section we examine the question of how many transport systems there exist for various monocarboxylic acids. We have attempted to answer this question by studying additivity of the potential response to different compounds as previously described from this laboratory [10,11]. Two substrates 1 and 2 were added in saturating concentrations either separately or jointly to vesicle-dye suspension and the magnitude of the dye response was compared. Ideally if the transport of substrates 1 and 2 are completely independent of each other, the optical response should be the sum of their individual response according to the following equation:

$$\Sigma \Delta A = \Delta A_1 + \Delta A_2$$

where $\Sigma \Delta A$ is the maximal adsorbance change observed by simultaneous addition of saturating concentrations of two substrates, and ΔA_1 and ΔA_2 are the maximal absorbance changes induced by individual application of these compounds. However, in reality $\Sigma \Delta A$ should be less than the

sum of ΔA_1 and ΔA_2, since both compounds are driven by the same electrochemical Na^+-gradient and each substrate lowers this gradient resulting in decreased membrane potential across the luminal-membrane vesicles. To test this concept a series of competition experiments was carried out with β-hydroxybutyrate and D-glucose, which are shown to be transported by different carrier systems [3]. Figs. 7A, B and C show the absorbance changes induced either by individual application of β-hydroxybutyrate (Fig. 7A) and D-glucose (Fig. 7B) or simultaneous addition of these compounds (Fig. 7C) to vesicle suspension from pars convoluta. It is seen that both substrates when added alone induced maximal absorbance changes by 0.037 and 0.096, but when applied together the maximal absorbance change observed is approx. 0.124 instead of 0.133. Identical experiments were performed by using luminal-membrane vesicles prepared from pars recta of proximal tubule (not shown). The results of these experiments are similar to those found in the case of vesicle preparations from pars convoluta.

Alternatively, if the two substrates are transported by the same common transport system the magnitude of $\Sigma\Delta A$ should be the same as ΔA_1, or ΔA_2 obtained when substrates 1 and 2 are introduced alone in saturating concentrations. In reality, however, the situation may be slightly different because, when dealing with two different compounds 1 and 2, we have to take into account differences in the affinities and maximal transport rates of these substances. A test with ketone bodies, which presumably use the same transport routes present in luminal-membrane of renal cells from pars convoluta, is depicted in Figs. 8A, B, C and D. It can be seen that the magnitude of the maximal optical response induced either by simultaneous addition of β-hydroxybutyrate and acetoacetate (Fig. 8B) or β-hydroxybutyrate and α-hydroxybutyrate (Fig. 8C) or β-hydroxybutyrate and γ-hydroxybutyrate (Fig. 8D) is approximately the same as caused by the addition of β-hydroxybutyrate (Fig. 8A) alone. Again identical experiments were performed by using luminal-membrane vesicles derived from pars recta (see Figs. 9A, B, C and D). It can be seen that the magnitude of maximal optical response induced by simultaneous addition of two compounds is approximately the same as observed by the application of β-hydroxybutyrate alone (compare Fig. 9A with Figs. 9B, C and D). These results established that various monocarboxylic acids studied in this paper are transported via a low-affinity common transport system localized in vesicles from pars convoluta and a high-affinity common transport system present in vesicles from pars recta of rabbit proximal tubule.

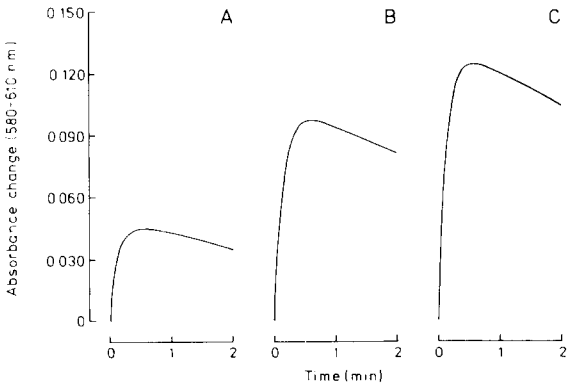

Fig. 7. Absorbance changes caused by addition of 25 mM β-hydroxybutyrate (A), 25 mM D-glucose (B) or 25 mM β-hydroxybutyrate plus 25 mM D-glucose (C) to luminal-membrane vesicles prepared from pars convoluta of the proximal tubules. The intravesicular medium was 310 mM mannitol and 15 mM Hepes-Tris (pH 7.5), whereas the external medium was 155 mM NaCl and 15 mM Hepes-Tris (pH 7.5). For further details see Fig. 2 legend.

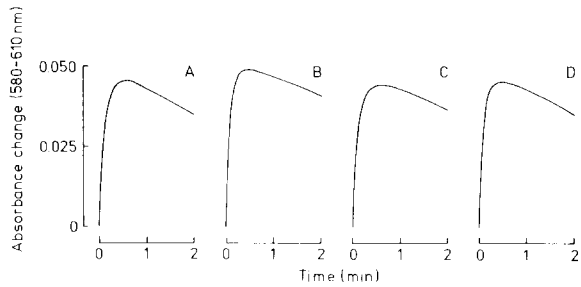

Fig. 8. Absorbance changes caused by addition of 25 mM β-hydroxybutyrate (A), 25 mM β-hydroxybutyrate plus 25 mM acetoacetate (B), 25 mM β-hydroxybutyrate plus 25 mM α-hydroxybutyrate (C) or 25 mM β-hydroxybutyrate plus 25 mM γ-hydroxybutyrate to luminal-membrane vesicles prepared from pars convoluta of the proximal tubules. For further details see Fig. 7 legend.

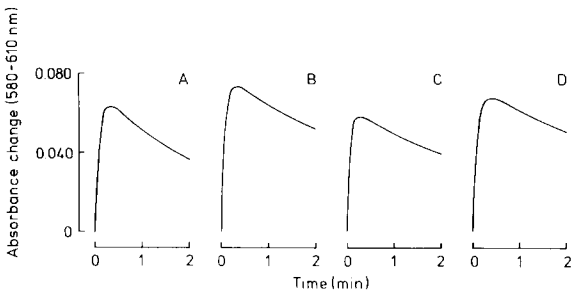

Fig. 9. Absorbance changes caused by addition of 10 mM β-hydroxybutyrate (A), 10 mM β-hydroxybutyrate plus 10 mM acetoacetate (B), 10 mM β-hydroxybutyrate plus 10 mM α-hydroxybutyrate (C) or 10 mM β-hydroxybutyrate plus 25 mM γ-hydroxybutyrate (D) to luminal-membrane vesicles prepared from pars recta of the proximal tubules. For further details see Fig. 7 legend.

Discussion

By contrast to the previous findings [3] the results presented in this paper showed that the Na^+-dependent transport of ketone bodies into renal luminal-membrane vesicles is an electrogenic process and the driving force is provided by the electrochemical potential across the membrane. This interpretation is supported by several lines of evidence. The rates of uptake of radioactive acetoacetate and β-hydroxybutyrate in vesicles were enhanced by the presence of Na^+-salt anions of increasing permeabilities in the following order: gluconate$^-$ < SO_4^{2-} < Cl^-. These anions penetrate the renal luminal-membrane vesicles more rapidly than Na^+ and generate an electrochemical potential, negative on the inside (for a review, see Ref. 19), that permits the uphill Na^+ dependent transport of ketone bodies in a similar way as found in the case of D-glucose [20], L-phenyl alanine [21,15], L-serine [22], L-proline, hydroxy-L-proline, 5-oxoproline [23] and various di- and tricarboxylic acids [11,13,24]. These findings are further supported by spectrophotometric experiments, using potential-sensitive carbocyanine dye. We found that addition of either acetoacetate or β-hydroxybutyrate or its analogues to membrane vesicle-dye suspension in the presence of various Na^+-salt gradients results in a rapid and pronounced increase in the absorbance change, indicative of a depolarizing event(s) associated with the electrogenic transport of these compounds (for details see Ref. 10). These results established that the ketone bodies and analogues are taken up by the luminal-membrane vesicles in an electrically positive form (i.e. Na^+/substrate coupling ratio probably 2 : 1). In this connection is it of interest to note that Mengual and co-workers have recently studied in detail the mechanism of Na^+/L-lactate cotransport by brush-border membrane vesicles prepared from horse kidney [5,25]. Analysis of their experimental data suggested that Na^+ and lactate interact sequentially with the carrier and 2 Na^+/ions are required to transport one molecule of lactate (i.e. coupling ratio of Na^+: lactate of 2 : 1) in brush-border membrane vesicles. Whether similar mechanisms operate in the case of ketone bodies' transport, however, remains to be investigated.

The role of the electrochemical membrane potential was examined additionally by studying the effect of various ionophores on the absorbance changes associated with ketone bodies' transport. According to the expectations, valinomycin enhanced the Na^+-dependent uptake of monocarboxylic acids, provided a K^+ gradient (intravesicular > extravesicular) was present. By contrast, Na^+-dependent uptake of these compounds was nearly abolished by ionophores that permit Na^+ to pass through the luminal-membrane via another channel, either electrogenically (e.g. gramicidin D) or electroneutrally (e.g. nigericin).

Garcia et al. [3] reported that acetoacetate and β-hydroxybutyrate transport in brush-border membrane vesicles derived from whole rat kidney cortex occurred by means of a single Na^+-specific system with K_m values of 4 mM and 10 mM, respectively. In contrast to their findings our experiments clearly showed that uptake of acetoacetate, β-hydroxybutyrate and its analogues in vesicles from whole rabbit kidney cortex was mediated by dual but common transport systems, viz. a high-affinity system and a relatively low affinity system (see Table I). Na^+-dependent uptake of ketone bodies and analogues by vesicles from pars convoluta occurred via a single low-affinity system. Similarly, we found that membrane vesicles from pars recta possessed a distinct Na^+-dependent high affinity transport system for these compounds. Recently, Sheikh and co-workers

[15,22,23] have studied the characteristics of Na^+-dependent transport of various organic compounds by rabbit luminal-membrane vesicles prepared from two regions of the proximal tubule, namely pars convoluta (S_1-segment) and pars recta predominantly consisting of S_2- and S_3-segments. The results of these experiments indicated the existence of multiple Na^+-coupled solutes transport systems in these vesicle preparations: generally a low affinity system localized in pars convoluta and a high-affinity system characteristic of the luminal-membrane vesicles obtained from pars recta. In conclusion, the results presented in this paper on the uptake of acetoacetate, β-hydroxybutyrate and its analogues as well as our recent studies on the mechanism of various organic compounds transport strongly suggest that the high-affinity transport system for ketone bodies, localized in pars recta is mainly responsible for efficient reabsorption of very low luminal concentrations of these important metabolites that exist in this region of proximal tubule (for further discussion see Kragh-Hansen et al. [15]).

Acknowledgements

This work was supported in part by the Danish Medical Research Council, Aarhus Universitets Forskningsfond, P. Carl Petersens Fond, Fogh-Nielsens legat and NOVO Foundation.

References

1 Cohen, J.J. and Kamm, D.E. (1981) in The Kidney, 2nd Edn. (Brenner, B.M. and Rector, F.C., eds.), Vol. 1, pp. 144–248, W.B. Saunders Company, Philadelphia
2 Barac-Nieto, M., Murer, H. and Kinne, R. (1980) Am. J. Physiol. 239 (Renal Fluid Electrolyte Physiol. 8), F496–F506
3 Garcia, M.L., Benavides, J. and Valdivieso, F. (1980) Biochim. Biophys. Acta 600, 922–930
4 Nord, E., Wright, S.H., Kippen, I. and Wright, E.M. (1982) Am. J. Physiol. 243 (Renal Fluid Electrolyte Physiol. 12), F456–462
5 Mengual, R. and Sudaka, P. (1983) J. Membrane Biol. 71, 163–171
6 Ullrich, K.J., Rumrich, G. and Klöss, S. (1982) Pflügers Arch. 395, 212–219
7 Ullrich, K.J. Rumrich G. and Klöss, S. (1982) Pflügers Arch. 395, 220–226
8 Ullrich, K.J., Rumrich, G., Klöss, S. and Fasold, H. (1982) Pflügers Arch. 395, 227–231
9 Sheikh, M.I., Kragh-Hansen, U., Jørgensen, K.E. and Røigaard-Petersen, H. (1982) Biochem. J. 208, 377–382
10 Kragh-Hansen, U., Jørgensen, K.E. and Sheikh, M.I. (1982) Biochem. J. 208, 359–368
11 Kragh-Hansen, U., Jørgensen, K.E. and Sheikh, M.I. (1982) Biochem. J. 208, 369–376
12 Hopfer, U., Nelson, K., Perotto, I. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32
13 Jørgensen, K.E., Kragh-Hansen, U., Røigaard-Petersen, H. and Sheikh, M.I. (1983) Am. J. Physiol. 244 (Renal Fluid Electrolyte Physiol. 13), F686–F695
14 Edmon, J. (1974) J. Biol. Chem. 249, 72–80
15 Kragh-Hansen, U., Røigaard-Petersen, H., Jacobsen, C. and Sheikh, M.I. (1984) Biochem. J. 220, 15–24
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
17 Peterson, G.L. (1977) Anal. Biochem. 83, 346–356
18 Jacobsen, C., Frich, J.R. and Steensgaard, J. (1982) J. Immunol. Methods 50, 77–88
19 Sacktor, B. (1977) Curr. Top. Bioenerg. 6, 39–81
20 Beck, J.C. and Sacktor, B. (1975) J. Biol. Chem. 250, 8674–8680
21 Evers, J., Murer, H. and Kinne, R. (1976) Biochim. Biophys. Acta 426, 598–615
22 Kragh-Hansen, U. and Sheikh, M.I. (1984) J. Physiol. London, 354, 55–67
23 Røigaard-Petersen, H. and Sheikh, M.I. (1984) Biochem. J. 220, 25–33
24 Wright, S.H., Krasne, S., Kippen, I. and Wright, E.M. (1981) Biochim. Biophys. Acta 640, 767–778
25 Mengual, R., Leblanc, G. and Sudaka, P. (1983) J. Biol. Chem. 258, 15071–15078