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Research Article

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Hepatic Oleate Uptake

Electrochemical Driving Forces in Intact Rat Liver

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Abstract

Recent observations suggest that the hepatic uptake of oleate may be sodium coupled. To assess the electrochemical forces driving fatty acid uptake, we used microelectrodes to monitor continuously the electrical potential difference across the plasma membrane in the perfused rat liver while simultaneously monitoring the rate of tracer [^{3}H]oleate uptake from 1% albumin solutions. Isosmotic cation or anion substitution was used to vary the potential difference over the physiologic range. Depolarization of cells from −29 to −19 mV by substituting gluconate for chloride reduced steady-state oleate uptake by 34%. Conversely, hyperpolarization of cells to −52 mV by substituting nitrate for chloride increased uptake by 41%. Replacement of perfusate sodium with choline depolarized the cells to −18 mV and reduced uptake by 58%, an amount greater than expected from the degree of depolarization alone. Oleate in higher concentrations (1.5 mM in 2% albumin) depolarized cells by 3 mV in the presence of sodium, but had no effect in sodium-free buffer. These results suggest that a portion of oleate uptake in the intact liver occurs by electrogenic sodium cotransport. Uptake appears to be driven by both the electrical and sodium chemical gradients across the plasma membrane.

Introduction

Long-chain fatty acids are essential intermediates in triglyceride metabolism and provide a major source of metabolic energy during fasting and exercise (1). In the liver, fatty acids are also used for VLDL and phospholipid synthesis. Hepatic uptake of unesterified fatty acids from plasma proceeds rapidly despite binding of > 99.98% of plasma fatty acids to serum albumin. It has been suggested that this high efficiency reflects a direct interaction of the albumin–fatty acid complex with the liver cell membrane (2–4), although we no longer favor this view (5). On the other hand, the uptake mechanism would need to be extraordinarily efficient to account for observed uptake rates if cellular uptake draws only on the small unbound fatty acid pool in plasma (3).

The mechanisms whereby fatty acids cross the plasma membrane remain incompletely defined. It has been proposed that fatty acids cross biological membranes by diffusion through the lipid bilayer (6–8); however, this simple mechanism may be too slow to account for highly efficient uptake (9). Recent studies have implicated a specific membrane protein in the transport of fatty acids. Immunologically cross-reacting 40-kD membrane fatty acid–binding proteins have been isolated from plasma membrane in liver (10), heart (11–13), jejunum (14), and adipocytes (11, 15). Their relationship to the 85-kD protein earlier linked to fatty acid uptake by adipocytes (16) is uncertain. Evidence that the 40-kD protein may serve as a receptor for fatty acids is provided by the observation that antibody to this protein specifically inhibited uptake of fatty acid but not of bile acids or other organic anions by isolated liver cells (17). Moreover, saturable fatty acid uptake has been demonstrated in hepatocytes (17), cardiac cells (12, 18–21), and adipocytes (16, 22). Saturation in liver cells appears to reflect membrane transport events rather than subsequent metabolism (23); however, the opposite conclusion was reached by one group for saturation in cardiac cells (20, 21). Other mechanisms may also contribute substantially to fatty acid uptake, as not all studies support a saturable membrane transport process for fatty acids (2, 20, 21, 24), and nonsaturable uptake components have been identified in certain nonhepatic cells (18, 19, 20, 22).

Studies in isolated liver cells and liver plasma membrane vesicles have suggested that fatty acid uptake is actively driven by cellular energy metabolism. Stremmel and co-workers have reported that uptake of oleate is reduced by ouabain, metabolic inhibitors, and removal of extracellular sodium (17), suggesting a sodium-coupled active transport mechanism. Studies in basolateral membrane vesicles further suggest that fatty acid uptake is voltage sensitive and driven by transmembrane sodium gradients (25). However, recent reports have found no consistent effect of sodium removal on fatty acid uptake by hepatocytes (26), adipocytes (15, 16), or myocardium (12, 26), and it has been proposed that the apparent sodium dependence is an indirect effect of changes in the transmembrane electrical potential difference (PD)[1] caused by sodium replacement rather than a direct result of blocking sodium cotransport (26), or that it reflects reduced viability of cells in sodium-free media (12). A recent model has also been proposed in which hepatic fatty acid uptake proceeds spontaneously, and is regulated by the physical and chemical properties of fatty acids, albumin, and plasma membrane lipids (27). This persisting uncertainty regarding the driving forces for hepatic fatty acid uptake reflects, at least in part, that previous studies have not directly measured the effects of cation and anion substitution on the PD.

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1. *Abbreviations used in this paper:* PD, transmembrane electrical potential difference.

This study uses a newly developed experimental system to provide direct, quantitative correlations between PD and fatty acid uptake rates. To accomplish this, we modified the single-pass isolated perfused rat liver model to allow continuous monitoring of PD while simultaneously monitoring steady-state oleate uptake rates. We postulated that if oleate uptake occurs by electrogenic sodium cotransport, then uptake rates should be sensitive to changes in PD induced by ion substitution. Moreover, the decrease in uptake rate after sodium replacement should be greater than for a similar change in PD produced by other methods. Finally, high uptake fluxes should cause sodium-dependent depolarization of liver cells, as has been reported for other sodium-coupled solutes (28–30). Each of these predictions was confirmed. The results suggest that a portion of oleate uptake by the intact liver is due to a sodium-coupled, electrogenic transport process.

Methods

Sources of chemicals. Sodium oleate, BSA (fraction V, essentially fatty acid free), choline chloride, choline bicarbonate, sodium gluconate, potassium gluconate, sodium nitrate, and potassium nitrate were from Sigma Chemical Co. (St. Louis, MO); Optifluor scintillant was from Packard Instrument Co. (Downers Grove, IL). [9,10-^{3}H]Oleic acid in ethanol (2–10 Ci/mmol) was obtained as the citrate from New England Nuclear (Boston, MA). All other chemicals were of the highest grade commercially available.

Solution preparation and validation. Preparation of radiolabeled oleate solutions for liver perfusion has been previously described (5). The basic perfusion solution was a Krebs-Henseleit bicarbonate (Krebs) buffer containing 131 mM Na^+, 118 mM Cl^-, 5.9 mM K^+, 1.2 mM Mg^{2+}, 2.5 mM Ca^{2+}, 1.2 mM phosphate, 1.2 mM sulfate, 20 mM bicarbonate, and 11.5 mM glucose in deionized water, and was prepared daily from concentrated stock solutions. Where indicated, this solution was modified by isosmotic replacement of either sodium or chloride with an ion of the same charge. Replacement of Na^+ with choline resulted in solutions containing 131 mM choline. Chloride replacement resulted in solutions containing 116 mM gluconate or nitrate and 1.2 mM Cl^-. All solutions had a pH of 7.4 when gassed with 95% O_2, 5% CO_2 at 37°C.

Liver perfusion. Liver perfusion was performed as previously described (31) except as noted below. Briefly, livers from 220–260-g adult male Sprague-Dawley rats were surgically removed under ether anaesthesia and perfused via the portal vein with 50 ml of recirculating fluorocarbon emulsion (Oxypherol FC-43; Alpha Therapeutics, Los Angeles, CA) at 22–26 ml/min for a 30-min stabilization period. The perfusion system (Fig. 1) was modified to allow placement of the liver in a Faraday cage next to the perfusion cabinet by inserting 30 cm of electrically grounded stainless steel tubing (2.5 mm i.d.) in the fluid path leading to the liver. This was followed by 60 cm of polyethylene tubing (1.5 mm i.d.) that was jacketed along most of its length with 6-mm i.d. latex tubing. Liver temperature was maintained at 37±0.5°C by perfusing the outer tubing with water at 37–39°C using a circulating water bath (Haake FE-2, Federal Republic of Germany). Perfusate then passed through an inverted "Y" tube bubble trap/pressure gauge before entering the liver. The liver was suspended on a 10-cm glass dish fitted with a central 2.5-cm glass tube to accommodate the outflow catheter (12 cm of 3 mm o.d. glass tubing). A watertight seal was achieved between this catheter and the dish using a one-hole rubber stopper. During recirculating perfusion, the fluorocarbon emulsion was collected in a small glass beaker and returned by suction to the perfusion pump through a sealed 20-ml bubble trap. The deadspace volume of this system (including tubing, pump, lung, pH probe, filter, and switches) was ~ 30 ml.

After 30 min, the system was changed to single-pass perfusion with Krebs buffer equilibrated with 95% O_2, 5% CO_2 and a short length of tubing attached to the outflow catheter to carry effluent from the Faraday cage for sampling. The distal end of this tubing was positioned at approximately the level of the liver to provide the lowest outflow pressure compatible with smooth flow. Flow rate was adjusted to 25–30 ml/min at this time, and the lung, in-line filter and pH probe were switched out of the circuit to minimize the delay between selection of a new perfusion solution and that solution reaching the liver (typically 12–15 s).

Changing the perfusate solution from recirculating Oxypherol to single-pass Krebs perfusion was associated with a transient hyperpolar-

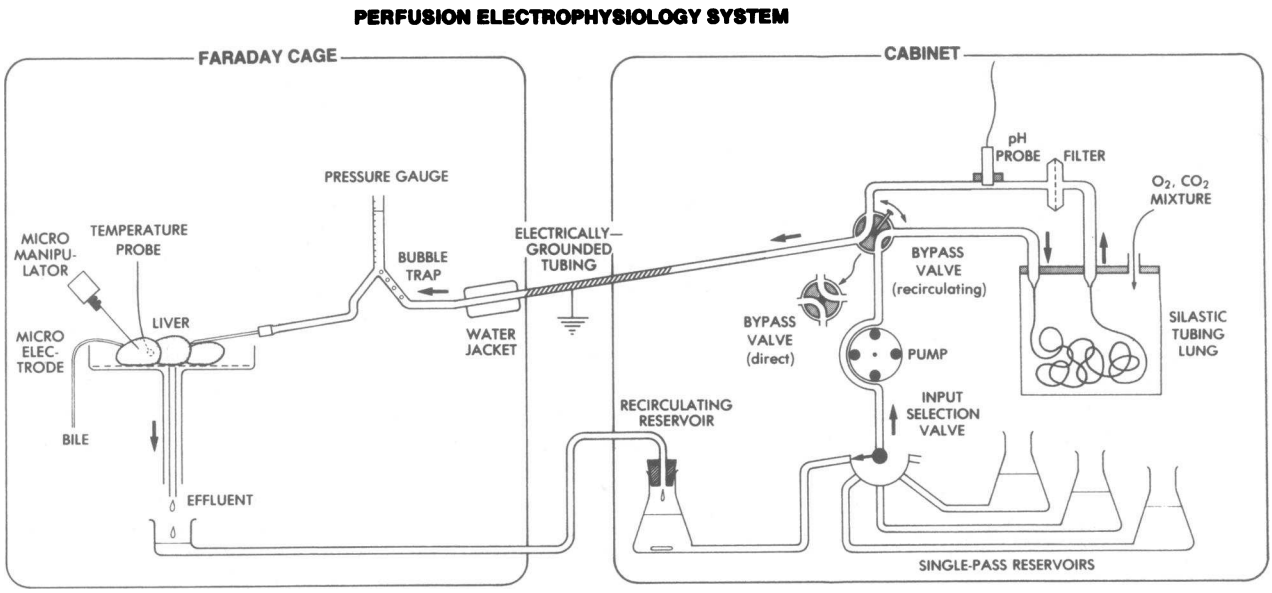

Figure 1. Schematic diagram of perfusion electrophysiology system. Livers were perfused single-pass with solutions containing radiolabeled oleate in Krebs-albumin buffer. Membrane PD was monitored with conventional microelectrodes while the net oleate uptake rate was simultaneously monitored from the concentration drop across the liver. Ion substitution was used to transiently change the membrane potential difference or extracellular sodium concentration, and these changes then correlated with changes in the hepatic transport of oleate.

ization. When membrane potential had stabilized (usually 6–8 min), the perfusate was changed to the same buffer containing 1% defatted bovine albumin plus radiolabeled oleate as specified (usually 1 μM). This solution was perfused for 5 min to allow further stabilization of the membrane potential and to achieve steady-state oleate uptake. The sample clock was then started (defined as zero time) and sampling of the effluent perfusate begun at 15–60-s intervals as described below to determine the baseline oleate uptake rate. In most experiments, a different perfusate in which chloride or sodium had been isosmotically replaced with an ion of the same charge was selected at 1 min. At 6 min, the original perfusate was selected again and sampling was continued until the PD had returned to its starting level (10–12 min). Experiments designed to determine the effects of ion substitution on hepatic viability were done within the perfusion cabinet as previously described (31) without electrophysiologic monitoring, and the baseline observation period was increased to 5 min.

Sample processing and data analysis. To remove radiolabeled metabolites, [^{3}H]oleate was extracted from 1-ml samples of effluent and perfusate solutions by the method of Dole (35) before radioactivity was determined by scintillation counting. The fraction of the oleate extracted in a single pass through the liver (extraction) was calculated as $(C_{in}\text{-}C_{out})/C_{in}$, where C_{in} and C_{out} are the concentrations of oleate entering and leaving the liver, respectively. The calculated net uptake rate across the organ (uptake velocity) was determined from the product of the total oleate concentration, the extraction, and the flow rate per gram of drained liver weight, and was assumed to reflect steady-state conditions when unchanged for more than three successive 15-s intervals. Changes in uptake rate resulting from ion substitution were calculated relative to the basal uptake rate for the same liver. All studies were repeated four to six times and data averaged to give the results shown. Statistical significance was assessed by the student t test with a value of $P < 0.05$ considered significant.

Kinetic interpretation. As used in this paper, the term "uptake" refers to the net removal of oleate from the extracellular fluid by the liver, and is therefore not synonymous with influx. Uptake for a given concentration of oleate was assumed to be determined by the rate constants for influx (k_1), efflux (k_2), and metabolism (k_3) of oleate as described by Goresky (36). Under steady-state conditions, these rate constants may be combined to give a single composite rate constant (k_{ss}) (5, 36):

$$k_{ss} = \frac{k_1 \cdot k_3}{(k_2 + k_3)} \quad (1)$$

This rate constant defines the uptake of oleate by a tubular hepatic sinusoid according to the relationship:

$$C_{out} = C_{in}e^{-k_{ss}t} \quad (2)$$

where t is the time required for perfusate to transit the sinusoid, which varies inversely with the flow rate. From Eq. (1), it is apparent that k_{ss} is proportional to the influx rate constant, regardless of the values of the other rate constants. Changes in the influx rate thus will result in proportional changes in overall uptake, even if metabolism is rate limiting. A more complete discussion of the role of the influx rate constant in determining steady-state removal rates has appeared (34).

This approach assumes that plasma flow and dissociation of oleate from albumin do not limit oleate uptake. Plasma flow need not be considered when the single-pass extraction, is low as is true for oleate when albumin is present (2). However, dissociation has been shown to limit oleate uptake by the perfused rat liver at very low albumin concentrations (5). We therefore selected an albumin concentration (1%) sufficiently high to avoid dissociation-limited uptake under the conditions of our study. As discussed elsewhere (34), binding equilibrium may be assumed to be present in the sinusoidal space when the uptake rate is much less than the unidirectional rate of dissociation of oleate from albumin within this space (5). The latter rate is given by the product of the dissociation rate constant under the conditions of our study (0.14 s^{-1}, or 8.4 min^{-1}, reference 5), the bound oleate concentration (1 μM, or 1 nmol $\cdot$ cm^{-3}), and the volume of the sinusoids 0.15 $cm^3 \cdot g^{-1}$ (32), and was $\sim$ 1,260 pmol $\cdot$ $min^{-1} \cdot g$ $liver^{-1}$. As this value is considerably larger than the uptake rates reported here, binding equilibrium within the hepatic sinusoids should not be greatly disturbed by uptake.

Measurement of membrane potentials. The method for monitoring membrane potentials has been described in detail (33). Briefly, microelectrodes were prepared from 1-mm borosilicate glass (Omega-Dot, Glass Co. of America, Millville, NJ) and had a tip resistance of 80–100 megohms when filled with 3 M potassium acetate. The microelectrode was connected to a preamplifier via a Ag/AgCl wire and after further amplification was monitored on an oscilloscope and recorded using a chart recorder. Krebs solution surrounding the liver was used as the electrical reference. The input resistance of the microelectrode was monitored by passing a current pulse through the microelectrode (20-ms duration every 3 s). Microelectrodes were positioned using a micromanipulator and the hepatocyte was impaled using an hydraulic microdrive. Criteria for acceptable impalement were as previously described (33) and included an abrupt negative deflection on cell penetration, a stable intracellular potential for 20 s or more, and return to baseline potential upon withdrawal from the cell. Using this technique, the PD of a single cell could be monitored for as long as 5–10 min. Because tight electrical coupling between different cells in the liver results in little cell to cell variability (33), measurements in a single cell are representative of the PD throughout the entire liver.

Liver weight. To evaluate the possibility that fluid shifts caused by ion substitution might affect uptake rates through solvent drag, separate experiments were performed to determine the effect of ion substitution on liver weight (an indirect measure of cell volume). Liver weight was monitored by suspending the dish holding the liver from one end of a 30-cm brass beam supported by a central pivot. A lead counterweight sufficient to lift the dish by 5–10 mm above its usual position was suspended from the opposite end of the beam. Care was taken to support the portal vein catheter so that vertical movement of the dish was not restricted. The lead weight was supported on a sensitive top-loading balance, which thus registered the mass of the weight minus the mass of the liver and associated apparatus. Indicated weight was recorded at 30–60-s intervals during the entire perfusion. At the end of the experiment the liver was drained and weighed separately, and this weight was used to back calculate the absolute weight of the liver at each time point during the perfusion. This method allowed changes in liver weight of as little as 10 mg to be detected.

Electron microscopy. In selected experiments, livers were postperfused with 2.7% glutaraldehyde plus 0.8% paraformaldehyde in Krebs buffer (2 min at 8–10 cm H_2O pressure) and prepared for electron microscopy using standard methods. At least 10 areas from each liver were examined at a magnification of 9–30,000 for evidence of toxicity.

Hepatic viability. Viability of each liver was assessed by bile flow, perfusion pressure, and return of PD and oleate extraction to initial values after ion substitution. In selected experiments, the effects of ion substitution on oxygen consumption, total liver weight, and ultrastructure as assessed by electron microscopy were also measured. Oxygen tension in the effluent was continuously monitored with a flow-through oxygen probe (Yellow Springs Instrument Co., Yellow Springs, CO). Prior studies have confirmed viability of livers for at least 30 min under these conditions (14).

Validation of method

In preliminary experiments, membrane potential and oleate extraction (measured using 1% albumin and 1 μM [^{3}H]oleate) remained constant for at least 30 min of single-pass perfusion. Similar PD values were obtained when cells were sampled from separate lobes of the liver either sequentially or simultaneously with two microelectrodes, suggesting that tight electrical coupling exists between hepatocytes in the perfused liver, as has been reported in vivo (28, 33). Measurements of basal membrane potential in Krebs buffer were highly reproducible, with an SD of 3.4% for experiments performed on the same day and 3.8% for experiments performed on different days. Variation in the

basal oleate uptake velocity under these conditions was 8.5 and 11%, respectively.

Liver weight. We were initially concerned that ion substitution might affect uptake rates by inducing fluid shifts across the plasma membrane. However, ion substitution caused only minor changes in liver weight (Fig. 2). Liver weight increased by a maximum of only 1% during nitrate substitution, while gluconate and choline substitution caused liver weight to decline by no more than 1.9 and 2.4%, respectively. These changes were gradual, did not follow the time course of the changes in PD or extraction, and tended to reverse when the liver was perfused with normal Krebs buffer. To document the sensitivity of our system to osmotically generated fluid shifts, livers were perfused with standard buffer containing 20, 60, or 180 mM added sucrose (an impermeant solute), resulting in decreases in liver weight of 1.4±0.8, 3.8±1.8, and 10±4%, respectively, within 1 min. Together, these data suggest that changes in uptake rate are not attributable to changes in hepatocyte volume resulting from ion replacement.

Oxygen consumption. Oxygen consumption from Krebs buffer averaged 2.24±0.14 $\mu mol \cdot min^{-1} \cdot g\ liver^{-1}$, similar to values previously reported for well-oxygenated livers (37). Replacement of chloride with nitrate or gluconate had no measurable effect on oxygen consumption (Fig. 2). However, replacement of sodium with choline caused a transient 10% drop in oxygen consumption that was followed by a transient increase in oxygen consumption when sodium was restored to the medium.

Perfusion pressure and perfusate flow rate. Basal indicated perfusion pressure was 7.2±0.3 cm H_2O at the flow rates used (2.6–2.9 $ml \cdot min^{-1} \cdot g\ liver^{-1}$). Perfusion pressure was unaffected by nitrate or gluconate substitution. However, replacement of sodium with choline caused a reversible increase in the perfusion pressure to ~ 15 cm H_2O (Fig. 2). This increase in vascular resistance is worrisome because it could affect uptake if resulting flow rates were reduced or if hepatic perfusion became highly uneven. However, flow rates are fixed by the perfusion pump in our system and were unaffected, and there is no reason to believe that the effects of ion substitution on vascular resistance are not similar for all sinusoids.

Bile flow. Basal bile flow was 0.94±0.05 $\mu l \cdot min^{-1} \cdot g\ liver^{-1}$, similar to the value of 0.80±0.22 $\mu l \cdot min^{-1} \cdot g\ liver^{-1}$ reported by Van Dyke and co-workers for bile acid depleted livers (38). Bile flow increased by 58±6% for the 150-s period immediately after nitrate substitution, whereas it virtually ceased with gluconate or choline substitution (Fig. 2). Similar results have been reported previously for choline (38).

Microscopy. Electron microscopy of livers fixed immediately after completion of selected experiments showed no significant morphologic or ultrastructural changes for any of the three ion substitutions used (Fig. 3). In particular, there was no evidence of cell blebbing, vacuolization, canalicular dilatation, cell lysis, or disruption of the endothelium.

Reversibility. In most experiments, the effects of ion substitution on membrane potential and oleate uptake rates were fully reversed within 4 min after returning to Krebs buffer (Figs. 4 and 5). Although recovery of the PD to basal values required up to 6 min in a few cases, it was always complete.

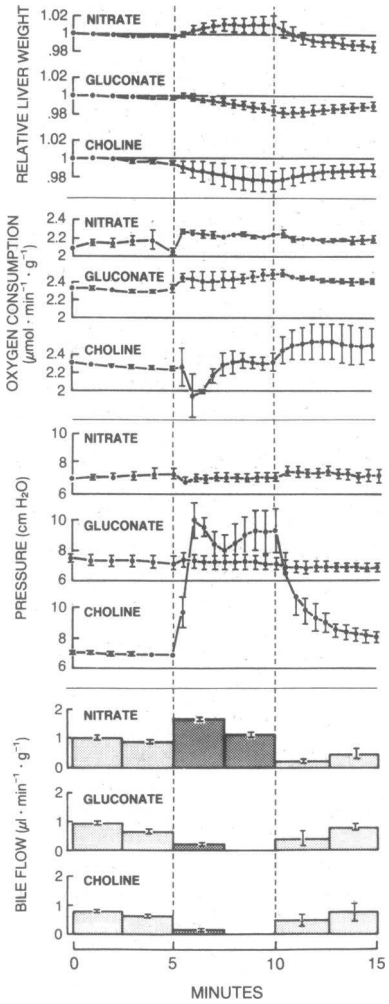

Figure 2. Effects of ion substitution on liver viability. Isolated rat livers were perfused single-pass with Krebs bicarbonate buffer containing 1% bovine albumin. After 5 min, either chloride or sodium was isosmotically replaced with an ion of like charge as indicated for an additional 5 min before returning to the original buffer. Resulting changes in weight were monitored using an integral balance while changes in oxygen consumption were monitored using a flow-through oxygen electrode. Changes in perfusion pressure and bile flow were monitored directly. Changes produced by ion substitution were generally small and reversible, indicating little or no measurable toxicity. Each panel shows mean data±SEM for three livers. Experimental details are given in the text.

Results

Membrane PD. Membrane PD could be followed in a single cell for extended periods during ion substitution (Fig. 5). In most experiments, however, we sampled multiple cells to minimize the effects of cell variation and baseline drift. The basal potential difference across the plasma membrane averaged −29.0±0.5 mV in Krebs buffer (mean±SEM, n = 16 livers) and was similar for all experimental groups (range: from −28.5 to −29.8 mV). Substitution of gluconate for chloride or of choline for sodium resulted in a sustained 35–38% depolarization of the cells (Table I, Fig. 4). In contrast, nitrate substitution caused a transient 80% hyperpolarization that returned to baseline within 3–5 min, despite continued perfusion with nitrate buffer (Table I, Fig. 5). Reintroduction of chloride after 5 min of perfusion with nitrate buffer caused a transient depolarization that was maximal after ~ 45 s (−10.8±2.4 mV, n = 5). In all cases the potential difference returned to presubstitution values within 5–7 min after reperfusion with Krebs buffer.

Oleate uptake rate. The net rate of oleate uptake was uniformly increased by ion substitutions that caused hyperpolarization, and decreased by substitutions that caused depolarization (Table I, Fig. 5). However, the decrease in uptake was greater in choline buffer (58%) than in gluconate buffer (34%) despite a similar degree of depolarization. Uptake increased 41% in nitrate buffer and transiently fell 12% below baseline when the perfusate was switched back to Krebs buffer. This latter effect was associated with transient depolarization of the liver.

Whereas the membrane PD rapidly returned to baseline after replacement of chloride with nitrate, oleate uptake remained significantly elevated until nitrate was removed from the perfusate (Fig. 5). To determine whether nitrate substitution had any effect on albumin binding, 20-ml perfusion buffers were incubated overnight with a small amount (2 ml) of heptane and the partition of oleate between the heptane and

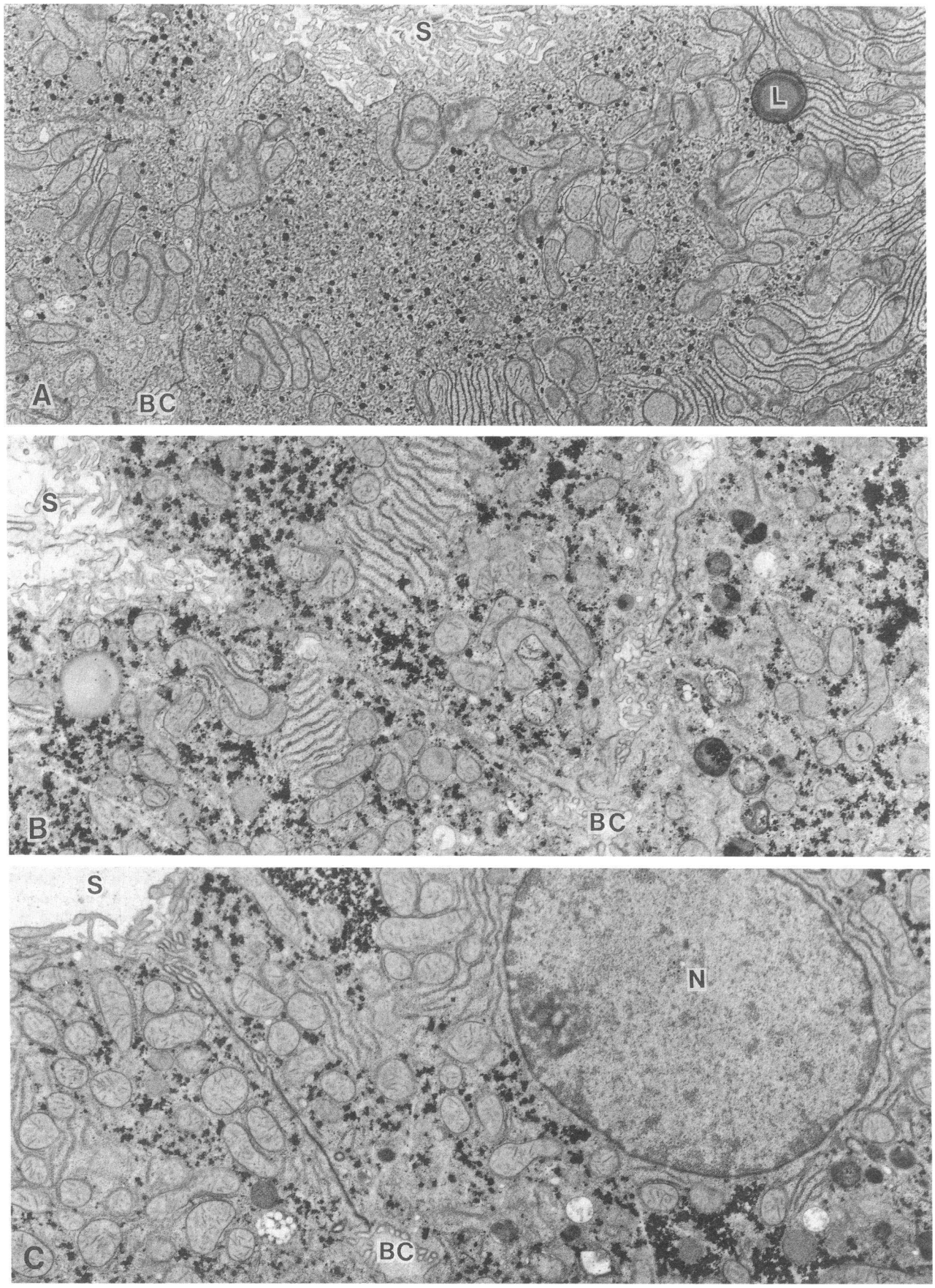

Figure 3. Effect of ion substitution on liver morphology and ultrastructure. Livers were perfusion fixed and prepared for electron microscopy immediately after completion of typical experiments involving (*A*) nitrate substitution, (*B*) gluconate substitution, and (*C*) choline substitution. At least 10 blocks from each liver were examined. Representative sections are shown for each liver at a magnification of 9,000. No morphologic or ultrastructural evidence of toxicity was apparent for any ion substitution used. Symbols: S, sinusoid; BC, bile canaliculus; N, nucleus; L, lipid droplet.

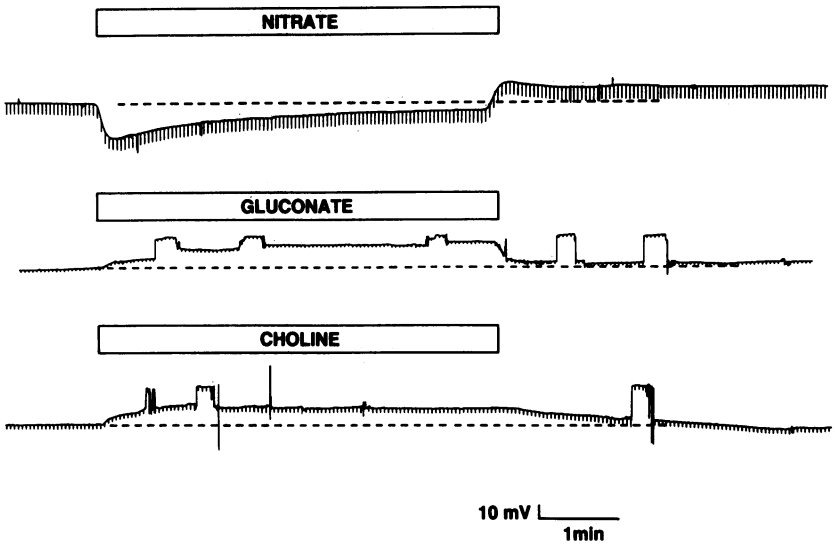

Figure 4. Effects of ion substitution on PD. Representative tracings are shown for isosmotic substitution of nitrate for chloride, gluconate for chloride, or choline for sodium. Although it was possible to monitor a single cell for the entire experiment (*top tracing*), most experiments sampled multiple cells to minimize error due to cell variation and baseline drift.

perfusate determined by the method of Spector (39). To avoid oxidation of the oleate, buffers were equilibrated with 5% CO_2, 95% N_2. Gluconate and choline substitution had no effect on albumin binding when compared with control buffers, whereas nitrate substitution increased the partition into heptane by 18–22% (n = 2). These results indicate that high concentrations of nitrate (> 100 mM) may produce slight displacement of oleate from albumin. Despite this, a clear correlation between PD and oleate uptake is present during the transient phases of nitrate introduction and withdrawal that cannot be accounted for by altered albumin binding (Fig. 5).

Effect of oleate on membrane potential. Abrupt exposure of the liver to higher oleate concentrations caused depolarization. Perfusion with 1.5 mM oleate in Krebs buffer containing 2% albumin caused an immediate decline in membrane potential that was maximal within 60 s after the new perfusate reached the liver (Fig. 6). Mean PD fell from −31.3±0.6 to −28.4±0.4 mV (n = 4, $P < 0.02$). Depolarization was not seen when sodium was replaced with choline (Fig. 5), indicating it was sodium dependent. Similar hepatic depolarization has been reported in association with uptake of such well-documented sodium-coupled solutes as taurocholate and alanine (28).

Discussion

The remarkable efficiency with which the liver removes fatty acids from plasma suggests that uptake is driven by a large electrochemical gradient across the plasma membrane. However, available data suggest that fatty acids do not themselves display such a gradient. The fatty acid chemical gradient is determined by the difference in concentration of the transportable form of the fatty acid across the plasma membrane. Because fatty acids are extensively bound to plasma and cytosolic proteins and the relative contributions of bound and unbound forms to uptake remain uncertain, this gradient is not precisely known. However, available data (discussed later) suggest that it is small. Influx of fatty acids does not appear to be electrically favored either. Because the cell interior is electrically negative with respect to the exterior and fatty acids do not carry a positive charge, influx of fatty acids cannot be directly driven by the transmembrane electrical gradient.

Efficient uptake might be explained if influx of fatty acids were coupled to movement of a second solute down its electrochemical gradient. Active transport of small molecules by mammalian cells is typically coupled to influx of sodium, which is favored both electrically and chemically (40). Recent reports by Stremmel and co-workers have suggested that fatty acid uptake by plasma membrane vesicles and isolated hepatocytes is sodium dependent (17, 25). Interpretation of these studies is hampered, however, because the same manipulations used to alter the sodium chemical gradient may also affect the electrical gradient. Indeed, Sorrentino and coworkers have recently suggested that the reported sensitivity of fatty acid uptake to extracellular sodium concentrations in a variety of cell types does not reflect sodium-coupled transport, but rather changes in the PD induced by ion substitution (26). However, the PD was not measured in these studies.

This report directly relates changes in the PD across the plasma membrane to changes in fatty acid uptake rates in the perfused rat liver. This approach offers several advantages. First, use of an intact liver preparation preserves normal polarity of the tissue, permitting a more direct extrapolation to in vivo conditions. More importantly, however, it permits direct, simultaneous measurement of hepatocyte PD and fatty acid uptake in the same preparation on a continuous basis. This is particularly relevant to the study of electrochemical driving forces, because the effects of ion substitution on PD and uptake rates may be transient. This method also makes it easier to distinguish primary and secondary effects of ion substitution. Although ion substitution has proven quite useful for studying electrochemical driving forces in a variety of cell types including hepatocytes (41–45), it almost certainly has secondary effects on other cell processes that could influence transport. By limiting observations to early time points, such secondary effects are presumably minimized. For example, changes in PD and uptake in our study were effectively simultaneous and immediate, occurring within 15 s after the new solution reached the liver. Because this is too early for appreciable changes in intracellular electrolyte composition to take place (41, 43, 46), the observed changes very likely reflect direct modulation of transport rates by the PD rather than an indirect effect of ion substitution on cellular metabolism.

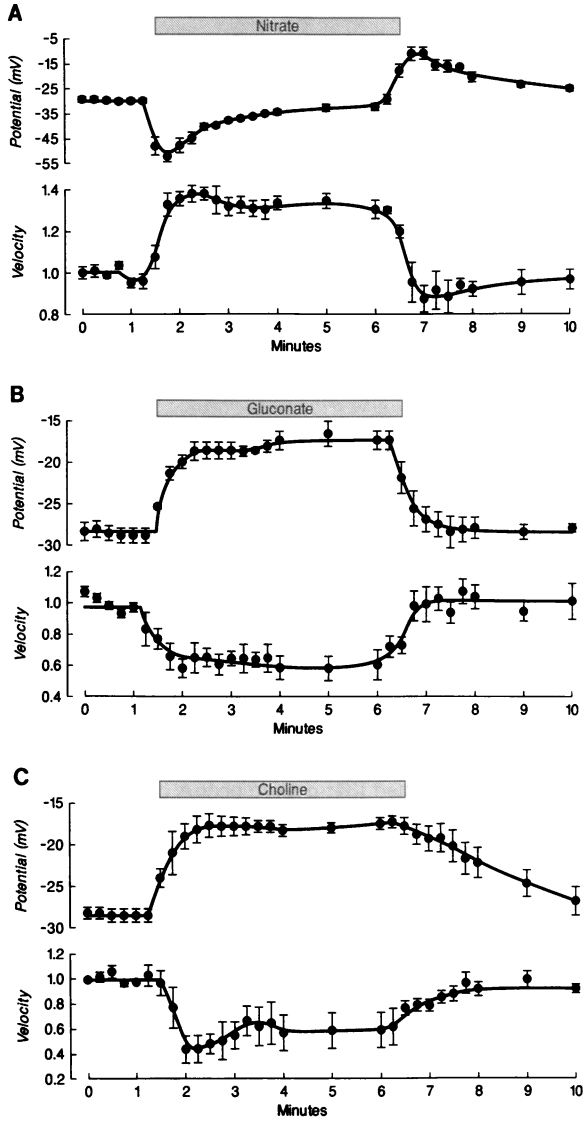

Figure 5. Effects of ion substitution on net oleate uptake rate and PD. The transmembrane potential difference and net oleate uptake rate were simultaneously monitored during isosmotic ion substitution of (*A*) nitrate for chloride, (*B*) gluconate for chloride, or (*C*) choline for sodium. Changes in potential were accompanied by corresponding changes in uptake velocity suggesting that the PD is a significant driving force for oleate uptake. Maximal inhibition of uptake by choline (58%) was greater than for gluconate (34%) despite a similar effect on the potential difference, suggesting that the sodium gradient also drives oleate transport. All changes were reversible. Mean data are shown for five or more perfusions in each group.

Although ion substitution is widely used to study electrochemical driving forces, the potential toxicity of this maneuver has never been systematically evaluated. The current data suggest that ion substitution is remarkably well tolerated by liver cells. Electron microscopy failed to detect any changes in hepatic morphology or ultrastructure after a 5-min period of sodium or chloride replacement (Fig. 3). Although we could not measure cell volume directly, changes in liver weight caused by ion substitution were small and followed a different time course than the changes in PD and oleate uptake velocity (Fig. 2). Oxygen consumption was unaffected by nitrate or gluconate substitution, and was transiently reduced by < 15% with choline substitution. A similar fall in oxygen consumption has been reported for replacement of sodium with lithium (47), and may reflect reduced cellular utilization of ATP due to inhibition of the Na^+/K^+ ATPase. Sodium replacement had a significant effect on vascular resistance as evidenced by doubling of the indicated perfusion pressure. To our knowledge, this effect has not previously been reported. We speculate that increased pressure reflects activation of poorly defined vasoconstrictive mechanisms in the liver by sodium replacement. Whatever its basis, this effect was reversible and did not affect perfusion rates (which are fixed in our system). The effects of ion substitution on bile flow were likewise reversible, indicating no toxicity.

Our results support complementary roles for the electrical and sodium chemical gradients in driving fatty acid uptake. Because more negative PD values were associated with more rapid uptake rates and more positive PD values with less rapid rates, these results are compatible with an electrogenic transport mechanism in which fatty acid enters as a positively charged complex. This positive charge appears to be provided at least partly by sodium, since removal of sodium from the medium reduced uptake by a greater amount than expected from the degree of depolarization alone.

To test this hypothesis, we looked for direct evidence of electrogenic sodium cotransport. High uptake fluxes of known sodium-coupled solutes, such as alanine, have been reported to cause cellular depolarization due to the associated influx of positive charge (28, 30, 48). We therefore examined the effect of perfusion with 1.5 mM oleate on PD. Although this concentration is within the physiologic range, it was necessary to increase the albumin concentration to 2% to avoid exceeding the binding capacity of albumin. Perfusion with fatty acid caused a rapid depolarization of ~ 3 mV. Depolarization has also been reported to result from transport of alanine and taurocholate (28, 48, 49), both known sodium-dependent solutes. When the experiment was repeated in sodium-free medium, no depolarization was seen. This result confirms that depolarization was caused by a specific, sodium-dependent process rather than by a nonspecific effect of oleate on PD. Although we cannot exclude the possibility that depolarization is due to an indirect effect of the fatty acid on electrogenic sodium pumping, there is no precedent for such a mechanism. The data thus provide evidence that at least a portion fatty acid uptake occurs by electrogenic, sodium-dependent transport.

Our results are consistent with modulation of the oleate influx rate constant by ion substitution. However, the method used for this study does not specifically measure oleate influx, but rather gives the steady-state uptake velocity, which also depends on the rate constants for efflux and metabolism as defined by Eq. 1. Determination of each of these rate constants is possible using the indicator dilution method as described by Goresky (36). This method was not used in this study, however, because it does not permit continuous monitoring of transport during rapid changes in PD. We must therefore consider whether the effects of ion substitution on oleate extraction could possibly reflect changes in intracellular fatty acid metabolism rather than changes in plasma membrane transport. The rate constant for metabolism of palmitate by dog liver is slower than the rate constant for efflux (50). If a similar situation exists for oleate in rat liver, then metabolism will be rate limiting to uptake at steady state and the cytoplasmic and

Table I. Perfusion Parameters Showing Effect of Ion Substitution on Membrane PD and Hepatic Oleate Uptake Velocity

	PD	Uptake rate	Extraction	Flow	Weight	*n*
	mV	*pmol·min⁻¹·g⁻¹*	*%*	*ml·min⁻¹·g⁻¹*	*g*	
Control	−29.0±0.5	545±23	19.6±1.2	2.85±0.12	9.41±0.18	16
Nitrate	−52.2±2.2	769±46	30.2±2.5	2.61±0.25	9.75±0.08	5
Gluconate	−18.6±1.1	360±54	11.7±2.1	3.14±0.11	9.43±0.33	5
Choline	−18.2±1.6	230±58	8.7±2.3	2.81±0.15	9.10±0.39	6

* Net oleate uptake rate was calculated for each liver from the product of extraction, oleate concentration (1 μM) and flow rate per gram liver weight, and these results were averaged to give the velocities shown. Control data are mean values ± SEM for the 60-s period before ion substitution. Other values are reported at the time of maximum perturbation of the PD, which occurred within 45 s of ion substitution. Liver weight was determined at the end of the experiment. All differences in PD, uptake rate, and extraction were highly significant ($P < 0.005$ vs. control).

extracellular fatty acid activities will adopt values close to the equilibrium distribution determined by the ratio of the influx and efflux rate constants (34). Because the equilibrium distribution is determined by the Donnan relationship (40), it will be sensitive to changes in PD if membrane transport is electrogenic. In other words, any change in PD will result in a change in the intracellular fatty acid concentration driving metabolism at steady state whether or not metabolism is rate limiting. The changes in extraction observed upon manipulation of PD in this study, however, are not those predicted for an anion whose distribution reflects a Donnan electrochemical potential (40). Rather, they are most consistent with transport of oleate as a positively charged species, presumably reflecting sodium coupling.

Several lines of reasoning suggest that the effects of ion substitution on hepatic extraction reflect changes in membrane transport rather than metabolism. First, the sodium-dependent depolarization produced by abrupt exposure to oleate fluxes is strong evidence for electrogenic transport and is difficult to ascribe to changes in oleate efflux or metabolism. Second, modulation of metabolism by ion substitution would not be expected to result in such rapid changes in fatty acid extraction because of the large buffering effect of cytoplasmic proteins and membranes. Stremmel and co-workers have shown that the initial rate of fatty acid uptake by isolated hepatocytes is maintained for at least 30 s (17), even though most fatty acid remains unesterified over this period (23). This result suggests that equilibration of the intracellular fatty acid pool with the extracellular space requires much longer than 30 s. Finally, changes in extraction associated with changes in PD are more readily explained by modulation of an electrogenic transport mechanism in which influx of oleate is associated with entry of positive charge into the cell than by effects of ion substitution on metabolism.

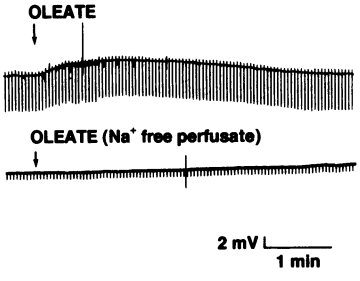

Figure 6. Effect of oleate on transmembrane PD. Infusion of 1.5 mM oleate in 2% albumin (*arrow*) depolarized the liver by 3 mV when sodium-containing Krebs bicarbonate buffer was used (*top tracing*), but had no effect on the potential difference when sodium was replaced with choline (*bottom tracing*). These results support an electrogenic sodium cotransport mechanism for hepatic oleate uptake. Representative data are shown from one of four perfusions performed. The periodic deflections result from current pulses used to monitor microelectrode resistance. Although these appear larger in the upper plot, this reflects the magnitude of the current pulse selected rather than a difference in resistence. Baseline deflections cannot be shown on this plot because of the high sensitivity used.

Previous studies of fatty acid uptake by adipocytes and cardiac cells have suggested that uptake occurs by both saturable and nonsaturable mechanisms (12, 18, 19, 22). The nonsaturable mechanism is thought to reflect simple diffusion of protonated fatty acid molecules across the lipid bilayer. In contrast, fatty acid influx into isolated hepatocytes (17) and hepatocyte plasma membrane vesicles (25) did not display a nonsaturable component, although these studies were not specifically designed to address this question. In this study, removal of sodium inhibited uptake of oleate by 58%, suggesting that a portion of fatty acid uptake occurs by a sodium-independent mechanism. This mechanism could be simple diffusion; however, we cannot exclude the possibility that another ion may substitute for sodium during uptake. Uptake of fatty acids by prokaryotes is known to be driven by proton cotransport, and a recent preliminary report suggests a similar mechanism may exist in the rat kidney (51). Alternatively, efflux of sodium from intracellular stores might possibly provide sufficient extracellular sodium to permit uptake at a lower level. This possibility appears unlikely, however, because single-pass perfusion would rapidly remove any effluxed sodium before it could accumulate.

If the sodium-independent pathway is simple diffusion, it may explain why some investigators have failed to detect sodium-dependent fatty acid uptake using much higher concentrations of fatty acids than those used here. Saturation of the sodium-dependent carrier at higher oleate concentrations would tend to increase the relative importance of the diffusion pathway, possibly obscuring sodium-dependent uptake. For similar reasons, we cannot estimate the relative importance of the sodium-dependent pathway to total oleate uptake under in vivo conditions without further data.

We have recently argued that uptake of fatty acids requires that they first enter the unbound pool by spontaneous dissociation from albumin, and have shown that at lower albumin concentrations dissociation may limit the uptake rate by limit-

ing the availability of unbound fatty acid within the hepatic sinusoids (5). Because unbound fatty acids make up < 0.02% of the total fatty acid in plasma, such a sequence requires an extraordinarily efficient cellular mechanism for removing unbound fatty acids from the sinusoids. Sodium-dependent, potential driven fatty acid uptake appears capable of accounting for this efficiency. These results describing transport across the plasma membrane thus are consistent with earlier results describing transfer of fatty acids from albumin to the membrane.

In conclusion, these studies indicate that a portion of oleate uptake by the intact rat liver occurs by an electrogenic, sodium-coupled transport mechanism. These results are compatible with a carrier system for mediating fatty acid transport across the hepatic plasma membrane. Because fatty acids are also able to cross phospholipid membranes by passive permeation (9), the physiologic role of this mechanism remains speculative. During periods of starvation, however, large fluxes of fatty acids are carried by the plasma. These fatty acids have very limited solubility in water and are potentially toxic to several organ systems (1). It is likely that the multiple high affinity binding sites on serum albumin have evolved to permit high plasma concentrations without toxicity, but at the cost of reducing the unbound concentration of fatty acids in plasma. Active fatty acid transport may serve to maintain fatty acid uptake rates at optimum levels despite avid albumin binding.

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