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Carboxylation of Pyruvate and Acetyl Coenzyme A by Reversal of the Na$^+$ Pumps Oxaloacetate Decarboxylase and Methylmalonyl-CoA Decarboxylase[†]

Peter Dimroth* and Wilhelm Hilpert

ABSTRACT: Proteoliposomes reconstituted by detergent dialysis from purified oxaloacetate decarboxylase and phospholipids catalyzed an oxaloacetate–$^{14}CO_2$ exchange. Similarly prepared proteoliposomes containing methylmalonyl-CoA decarboxylase catalyzed the exchange between malonyl-CoA and $^{14}CO_2$. These exchange reactions were completely dependent on the Na$^+$ ion gradients established during decarboxylation of part of the substrates since no exchange took place in the presence of the Na$^+$ carrier monensin. After a large Na$^+$ concentration gradient $Na_{in}^+ > Na_{out}^+$ was applied to methylmalonyl-CoA decarboxylase containing proteoliposomes, acetyl coenzyme A (acetyl-CoA) was carboxylated to malonyl-CoA which was trapped with fatty acid synthetase. In the absence of a Na$^+$ gradient, no acetyl-CoA carboxylation occurred. When oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase were simultaneously incorporated into proteoliposomes, a transcarboxylase system was constructed. Upon decarboxylation of oxaloacetate to pyruvate, acetyl-CoA was carboxylated to malonyl-CoA and vice versa. These transcarboxylations are mediated by a Na$^+$ circuit since dissipation of the Na$^+$ gradient with monensin also abolished the transcarboxylation reactions. Disruption of the membrane potential with valinomycin or carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone severely reduced the rate of oxaloacetate decarboxylation dependent acetyl-CoA carboxylation, in accord with the electrogenic properties of the Na$^+$ pumps. These Na$^+$ pumps therefore act as reversible vectorial catalysts either by creating electrochemical Na$^+$ gradients upon decarboxylation or by CO$_2$ fixation to yield carboxylic acids at the expense of an already existing Na$^+$ gradient. This type of CO$_2$ fixation is new and basically different from the classical carboxylation reactions which require ATP hydrolysis to overcome the energetically unfavorable metabolic processes. The stoichiometry between Na$^+$ transport and malonyl-CoA decarboxylation was 2:1 in the initial phase but decreased after a Na$^+$ concentration gradient had developed over the vesicular membrane.

The free energy of decarboxylation reactions is used by certain anaerobic bacteria to drive the active transport of Na$^+$ ions through the cell membrane (Dimroth, 1982a). Three enzymes have been recognized to perform this kind of energy transduction, i.e., oxaloacetate decarboxylase (Dimroth, 1980), methylmalonyl-CoA decarboxylase (Hilpert & Dimroth, 1982) and glutaconyl-CoA decarboxylase (Buckel & Semmler, 1982). These sodium transport decarboxylases have a number of properties in common, e.g., binding to the membrane, specific activation by Na$^+$ ions, and the prosthetic group biotin. Incorporation of purified oxaloacetate decarboxylase into phospholipid vesicles led to the reconstitution of Na$^+$ transport activity (Dimroth, 1981). Subsequently, the two other Na$^+$ pumps were reconstituted in use of the same reconstitution procedure (Hilpert & Dimroth, 1984; Buckel & Semmler, 1983).

Most of our knowledge on the mechanism of the Na$^+$ pumps has come from studies of oxaloacetate decarboxylase. The first step is a transfer of the carboxyl group from oxaloacetate to the enzyme-bound biotin (Dimroth, 1982c). This reaction is catalyzed by a distinct carboxyltransferase subunit which is a soluble protein (Dimroth & Thomer, 1983) analogous to the carboxyltransferase subunits of biotin-dependent carboxylases. The carboxyl transfer is completely independent from the presence of Na$^+$ ions and is freely reversible as shown by the exchange of radioactivity between [1-^{14}C]pyruvate and oxaloacetate (Dimroth, 1982c; Dimroth & Thomer, 1983). The next step is a Na$^+$-dependent decarboxylation of the carboxybiotin enzyme intermediate, and this apparently makes the overall decarboxylation process irreversible. So far, the reversibility has only been studied with the soluble enzymes, i.e.,

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[†] From the Institut für Physiologische Chemie der Technischen Universität München, 8000 München 40, West Germany. *Received March 22, 1984.* This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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in a completely uncoupled state. It was possible, therefore, that the decarboxylations are reversible under physiological conditions where they are coupled to Na^+ transport. We report here on carboxylations of pyruvate or acetyl-CoA by applying a Na^+ gradient to proteoliposomes containing the pertinent decarboxylase. Moreover, proteoliposomes reconstituted with both decarboxylases by mediation of a Na^+ circuit catalyzed the transcarboxylations between oxaloacetate and acetyl-CoA to pyruvate and malonyl-CoA and vice versa. We show here for the first time that a Na^+ ion gradient rather than ATP hydrolysis is used in a biological system to overcome energetically unfavorable carboxylation reactions.

Experimental Procedures

Oxaloacetate decarboxylase (EC 4.1.1.3) from *Klebsiella aerogenes* (Dimroth, 1982b) and methylmalonyl-CoA decarboxylase (EC 4.1.1.41) from *Veillonella alcalescens* (Hilpert & Dimroth, 1983) were purified by affinity chromatography on avidin–Sepharose as described. Fatty acid synthetase was purified from yeast (Lynen, 1969). Lipids for the reconstitution experiments were prepared as described (Gustafson, 1965) with the following modifications: *K. aerogenes* cells (200 g wet weight) were suspended in 100 mL of 2 M NaCl and extracted overnight with 600 mL of methanol and 300 mL of chloroform. After centrifugation (2000*g*, 5 min) the extraction of the pellet was repeated with half the volume of the two solvents. After the combined extracts were washed by adding 300 mL each of water and chloroform, the organic phase was evaporated under reduced pressure. The lipids were dissolved in a small volume of $CHCl_3$ and stored in sealed glass vials under reduced pressure at −20 °C. Prior to reconstitution, the solvent was removed with a stream of nitrogen, and the lipids were dissolved in peroxide-free ether. Removal of the solvent and dissolution in ether were repeated 4 times. Finally, the lipids were dried in a vacuum (<0.5 torr) for 24 h.

Reconstitution of the Na^+ Pumps Oxaloacetate Decarboxylase and Methylmalonyl-CoA Decarboxylase. The reconstitutions were performed as described (Dimroth, 1981; Hilpert & Dimroth, 1984) modified as follows: lipids from *Klebsiella aerogenes* (80 mg) were vigorously agitated on a Vortex mixer for about 10 min under nitrogen with 2.67 mL of 3% octyl glucoside in reconstitution buffer (30 mM potassium phosphate, pH 7.0, containing 1 mM Na_2SO_4, 0.5 mM dithioerythritol, and 1.5 mM NaN_3). Depending on the experiments to be performed, oxaloacetate decarboxylase (0.08 mL, 0.35 mg, 20 units) and (or) methylmalonyl-CoA decarboxylase (0.05 mL, 0.26 mg, 7 units) were added to the lipid detergent mixture followed by dialysis against 1 L of reconstitution buffer for 3 days at 4 °C with two changes of the dialysis buffer.

Oxaloacetate–$^{14}CO_2$ Exchange. The incubation mixtures contained, in 0.5 mL at 25 °C, 0.25 mL (7.5 mg) of oxaloacetate decarboxylase containing reconstituted proteoliposomes, 30 mM potassium phosphate, pH 7,0, 1 mM Na_2SO_4, 0.5 mM dithioerythritol, 1.5 mM NaN_3, 6 mM $KH^{14}CO_3$ (3000 counts min^{-1} $nmol^{-1}$), 10 units of carbonic anhydrase, and 5 mM lithium oxaloacetate which was used to initiate the reaction. Samples (0.12 mL) were transferred after appropriate incubation periods into 110 μL of 0.2 M HCl to terminate the exchange reactions. After the solutions were neutralized with 0.2 mL of 1 M tris(hydroxymethyl)aminoethane hydrochloride (Tris-HCl) buffer, pH 8.0, oxaloacetate was reduced to malate with 0.1 mL of 10 mM NADH and 50 units of malate dehydrogenase (25 °C, 10 min). The solutions were acidified with 0.1 mL of 6 M HCl and evaporated to dryness at 95 °C. The residues were taken up in 0.2 mL of 3 M HCl, and evaporation was repeated. The acid-stable radioactivity ([^{14}C]malate) was determined by liquid scintillation counting.

Malonyl-CoA–$^{14}CO_2$ Exchange. The incubation mixtures contained, in 0.5 mL at 25 °C, 0.25 mL (7.5 mg) of methylmalonyl-CoA decarboxylase containing reconstituted proteoliposomes, 30 mM potassium phosphate, pH 7.0, 1 mM Na_2SO_4, 0.5 mM dithioerythritol, 1.5 mM NaN_3, 6 mM $KH^{14}CO_3$ (3000 counts min^{-1} $nmol^{-1}$), 10 units of carbonic anhydrase, and 4.5 mM malonyl-CoA with which the reaction was initiated. After appropriate incubation periods, samples (0.12 mL) were pipetted to 0.1 mL of 6 M HCl and 0.02 mL of 0.1 M malonic acid, and the mixture was brought to dryness at 95 °C. After 0.2 mL 3 M HCl was added, the drying was repeated, and the residual acid stable radioactivity was determined by liquid scintillation counting. This radioactivity was identified as [^{14}C]malonic acid by thin-layer chromatography (Myers & Huang, 1969).

Na^+ Ion Gradient as Energy Source for Pyruvate Carboxylation. The incubation mixtures contained, in 1.0 mL at 25 °C, 0.5 mL (15 mg) of reconstituted proteoliposomes containing oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase, 30 mM potassium phosphate, pH 7.0, 1 mM Na_2SO_4, 0.5 mM dithioerythritol, 1.5 mM NaN_3, 20 mM $KHCO_3$, 2.7 mM potassium pyruvate, 22 units of citrate synthase, and 4.5 mM malonyl-CoA. Samples (0.16 mL) were added after appropriate times to 10 μL of 2 M HCl to terminate the reactions. The citrate content of the centrifuged supernatants was determined in a coupled fluorometric assay with citrate lyase and malate dehydrogenase. NADH fluorescence was excited at 340 nm, and the emission was followed at 465 nm. The cuvette contained, in a total volume of 2.0 mL, 100 mM Tris-HCl buffer, pH 8.0, 1 mM $MgCl_2$, 0.009 mM NADH, 1.2 units of malate dehydrogenase, and the acidified sample (0.15 mL). The reactions were initiated by adding citrate lyase (2.5 units), and the change in fluorescence was determined and compared with that of a citrate standard. Under our conditions, the fluorescence change caused by 5 nmol of citrate was 31%.

Na^+ Ion Gradient as Energy Source for Acetyl-CoA Carboxylation. (*a*) *With an Artifically Imposed Na^+ Gradient.* The reconstitution of proteoliposomes containing methylmalonyl-CoA decarboxylase was performed as described above but in the presence of 0.25 M Na_2SO_4 to yield an internal Na^+ concentration of 0.5 M. The Na^+ concentration gradient was imposed by suspending the centrifuged liposomes in reconstitution buffer containing 0.25 M K_2SO_4 instead of Na_2SO_4.

The incubation mixtures contained, in 0.73 mL at 25 °C, 0.5 mL (15 mg) of the liposomes, 30 mM potassium phosphate, pH 7.0, 0.25 M K_2SO_4, 0.5 mM dithioerythritol, 1.5 mM NaN_3, 70 mM $KHCO_3$, 1.2 mM NADPH, 0.6 unit of fatty acid synthetase, and 0.74 mM [1-^{14}C]acetyl-CoA (2770 counts min^{-1} $nmol^{-1}$). In control experiments the incubation mixtures contained 0.25 M Na_2SO_4 to avoid the formation of a Na^+ gradient across the vesicular membranes. After appropriate incubation periods, the reactions were terminated in samples (0.22 mL) by placing them in a boiling water bath for 5 min. Precipitated proteins and lipids were sedimented by short centrifugation, and the supernatants were mixed with 0.2 mL of 10% KOH in methanol and 2 μmol of stearic acid in 0.1 mL of petroleum ether. The samples were refluxed for 30 min, acidified with 0.7 mL of 1.7 M H_2SO_4, and extracted 4 times with 1 mL of petroleum ether each. The petroleum ether extracts were washed with 3 mL of 3 M acetic acid and

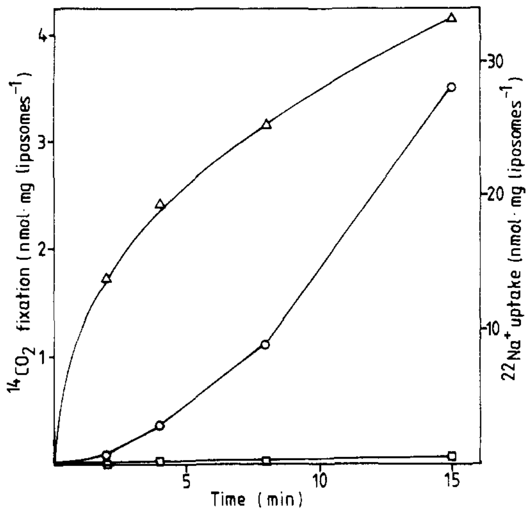


FIGURE 1: Kinetics of oxaloacetate–$^{14}CO_2$ exchange as catalyzed by reconstituted oxaloacetate decarboxylase containing proteoliposomes. The incubations of the proteoliposomes (7.5 mg) with oxaloacetate and $^{14}CO_2$ and the determinations of $^{14}CO_2$ incorporation into oxaloacetate were performed as described under Experimental Procedures in the absence (○) and presence (□) of 0.4 mM monensin. In a parallel experiment, the uptake of $^{22}Na^+$ into the proteoliposomes was determined (△).

3 × 1 mL of H_2O. The radioactivity incorporated into fatty acids were determined by liquid scintillation counting after evaporation of the solvent.

(*b*) *With a Na$^+$ Ion Gradient Generated by Oxaloacetate Decarboxylation.* The incubation mixtures contained, in 1.13 mL at 25 °C, 0.3 mL (9 mg) of proteoliposomes containing oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase, 30 mM potassium phosphate, pH 7.0, 1 mM Na_2SO_4, 0.5 mM dithioerythritol, 1.5 mM NaN_3, 40 mM $KHCO_3$, 1.1 mM NADPH, 0.6 unit of fatty acid synthetase, 0.5 mM [1-^{14}C]acetyl-CoA (2770 counts min^{-1} nmol^{-1}), and 9 mM lithium oxaloacetate which was used to initiate the reaction. The carboxylation of acetyl-CoA was determined in samples (0.33 mL) from the amount of radioactivity incorporated into fatty acids as described above under (a).

$^{22}Na^+$ Uptake into Proteoliposomes. The transport of $^{22}Na^+$ into proteoliposomes was determined as described (Dimroth, 1981; Hilpert & Dimroth, 1984). For a comparison of the kinetics of Na$^+$ transport with the exchange of $^{14}CO_2$ with oxaloacetate or malonyl-CoA, the same incubation mixtures were used, but $KH^{14}CO_3$ was replaced by the nonlabeled salt, and $^{22}NaCl$ was added to yield a specific radioactivity in the mixture of about 300–700 counts min^{-1} nmol^{-1}. The actual Na$^+$ content of the incubation mixtures was determined by atomic abosorption spectrophotometry and was used together with the radioactivity counted to calculate the specific radioactivity.

Results

Oxaloacetate–$^{14}CO_2$ Exchange and Malonyl-CoA–$^{14}CO_2$ Exchange. It has been shown previously that the decarboxylation of oxaloacetate to pyruvate and CO_2, when catalyzed by soluble oxaloacetate decarboxylase, cannot be reversed not even in the presence of an oxaloacetate trapping system (Dimroth, 1982c). An exchange between $^{14}CO_2$ and oxaloacetate was also not found. Similar observations have been made with methylmalonyl-CoA decarboxylase (Galivan & Allen, 1968), thus indicating that the decarboxylation reactions which are catalyzed by the biotin-dependent decarboxylases are irreversible.

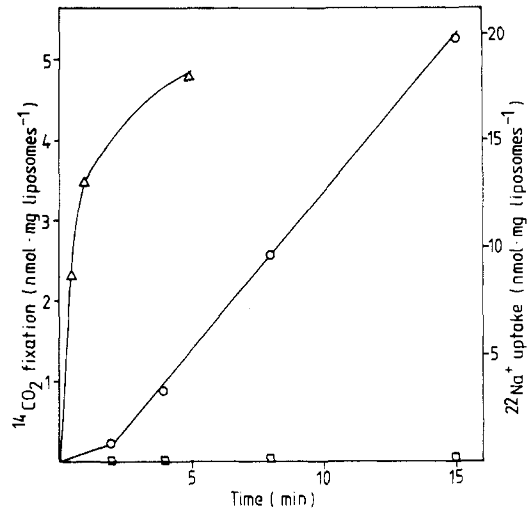


FIGURE 2: Kinetics of malonyl-CoA–$^{14}CO_2$ exchange as catalyzed by reconstituted methylmalonyl-CoA decarboxylase containing proteoliposomes. The incubations of the proteoliposomes (7.5 mg) with malonyl-CoA and $^{14}CO_2$ and the determinations of $^{14}CO_2$ incorporation into malonyl-CoA were performed as described under Experimental Procedures in the absence (○) and presence (□) of 0.4 mM monensin. In a parallel experiment, the uptake of $^{22}Na^+$ into the proteoliposomes was determined (△).

The soluble enzymes, however, do not imitate in full the physiological situation where the decarboxylations are coupled to the transport of Na$^+$ ions, and it appeared possible to reverse the decarboxylation reactions in a coupled vesicular enzyme system. The results shown in Figure 1 indicate that reconstituted proteoliposomes containing oxaloacetate decarboxylase performed an oxaloacetate-dependent accumulation of Na$^+$ ions and catalyzed the exchange between $^{14}CO_2$ and oxaloacetate. The rate of this exchange increased considerably at times where steeper Na$^+$ gradients had been developed and was negligible when the Na$^+$ gradient was dissipated with the Na$^+$ ionophore monensin. These results indicate that a gradient of Na$^+$ ions over the proteoliposomal membrane is required to catalyze the incorporation of $^{14}CO_2$ into oxaloacetate. Analogous results were obtained with reconstituted methylmalonyl-CoA decarboxylase containing proteoliposomes. The decarboxylation of malonyl-CoA generated a Na$^+$ ion gradient, and this effected the exchange between $^{14}CO_2$ and malonyl-CoA (Figure 2). The rate of this exchange was slow in the beginning but remained unchanged after a Na$^+$ gradient of sufficient magnitude had been developed. Addition of monensin completely destroyed both the Na$^+$ gradient (not shown) and exchange activity. These results thus indicate that the sequence of events by which CO_2 is released from oxaloacetate or malonyl-CoA is reversible, but only under the conditions of vectorial Na$^+$ translocation in a coupled membrane system.

Na$^+$ Concentration Gradient as Driving Force for Acetyl-CoA Carboxylation. The above results on the exchange of CO_2 with the carboxyl groups of oxaloacetate or malonyl-CoA suggested that net carboxylation of pyruvate to oxaloacetate or acetyl-CoA to malonyl-CoA may occur in the presence of an artificially imposed Na$^+$ gradient. To investigate this possibility, a Na$^+$ concentration gradient $Na^+_{in}:Na^+_{out}$ of about 30:1 was applied by reconstitution of methylmalonyl-CoA decarboxylase containing proteoliposomes in the presence of 0.25 M Na_2SO_4 followed by centrifugation and suspension of the pellet in buffer containing 0.25 M K_2SO_4 instead of Na_2SO_4. The proteoliposomes were then immediately incubated with [1-^{14}C]acetyl-CoA, CO_2, NADPH, and fatty acid synthetase as a malonyl-CoA trapping system, and the car-

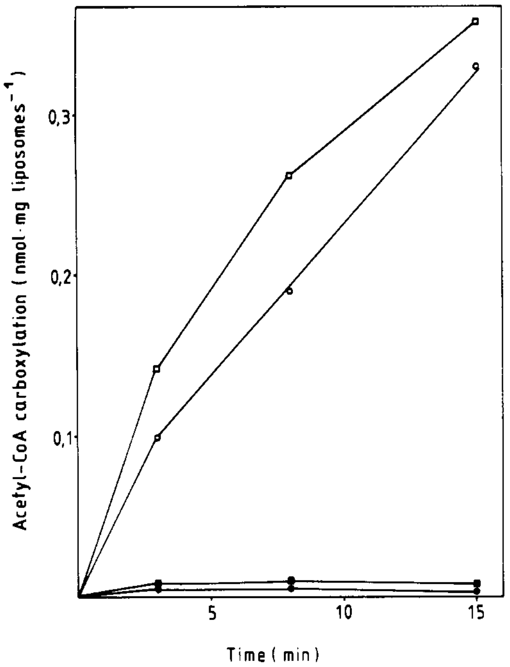


FIGURE 3: Acetyl-CoA carboxylation by methylmalonyl-CoA decarboxylase containing proteoliposomes after applying a Na$^+$ concentration gradient. Na$^+$-transporting methylmalonyl-CoA decarboxylase was reconstituted as described under Experimental Procedures but with reconstitution buffer containing 0.25 M Na_2SO_4. After dialysis against this buffer, samples of the vesicles (1.4 mL) were centrifuged at 40000*g* for 90 min. The samples were suspended in 1.0 mL each of the following buffers: (a) dialysis buffer containing 0.25 M K_2SO_4 (●), (b) dialysis buffer containing 0.25 M K_2SO_4 plus 40 μM valinomycin (■), (c) Na$^+$-free dialysis buffer containing 0.25 M K_2SO_4 (○), and (d) Na$^+$-free dialysis buffer containing 0.25 M K_2SO_4 plus 40 μM valinomycin (□). Each of these vesicle preparations was immediately incubated with [1-^{14}C]acetyl-CoA, $KHCO_3$, NADPH, and fatty acid synthetase to determine acetyl-CoA carboxylation as described under Experimental Procedures.

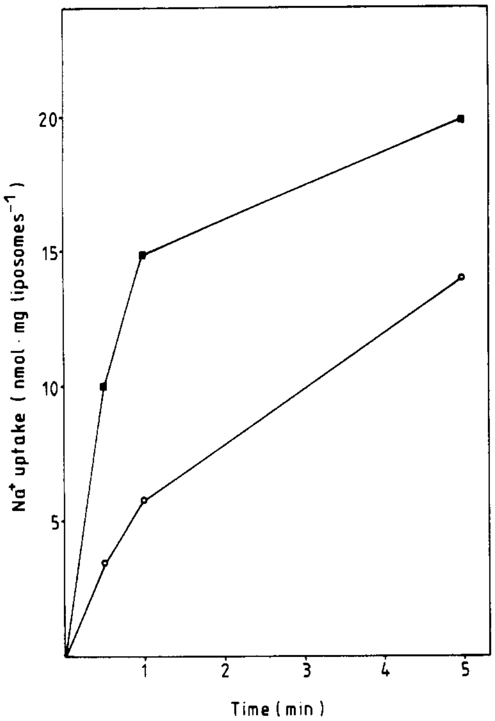


FIGURE 4: Simultaneous reconstitution of the Na$^+$ pumps oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase. The two decarboxylases were simultaneously reconstituted into proteoliposomes as described under Experimental Procedures. The incubation mixtures for determining Na$^+$ uptake contained, in 340 μL at 25 °C, 30 mM potassium phosphate buffer, pH 7.0, 0.5 mM dithioerythritol, 1.5 mM NaN_3, 1 mM Na_2SO_4, ^{22}NaCl (specific radioactivity of ^{22}Na$^+$ 360 counts min^{-1} nmol^{-1}), and proteoliposomes (9.1 mg, 4.4 μg of protein/mg of phospholipid). The transport was initiated with 1 μmol of oxaloacetate (○) or 0.6 μmol of malonyl-CoA (■) and measured as described under Experimental Procedures.

boxylation of acetyl-CoA was followed from the incorporation of radioactivity into long-chain fatty acids. The results, shown in Figure 3, indicate increasing amounts of acetyl-CoA carboxylation over a period of 15 min. The vesicles, therefore, apparently maintain a Na$^+$ concentration gradient of sufficient quantity for at least 15 min to drive the carboxylation of acetyl-CoA. As expected, no acetyl-CoA carboxylation took place in the absence of a Na$^+$ gradient under otherwise identical conditions. When an inside positive membrane potential was applied by a valinomycin-mediated K$^+$ diffusion from outside to inside in addition to the Na$^+$ concentration gradient, the rate of acetyl-CoA carboxylation increased. This observation is in accord with the electrogenic nature of the Na$^+$ pump methylmalonyl-CoA decarboxylase (Hilpert & Dimroth, 1983). The membrane potential in the absence of a Na$^+$ concentration gradient, however, was insufficient to support acetyl-CoA carboxylation. It is concluded from these results that methylmalonyl-coA decarboxylase requires a Na$^+$ ion gradient to overcome the energy barrier of the highly endergonic carboxylation of acetyl-CoA to malonyl-CoA. This is an alternative carboxylation mechanism to that of the classical biotin-dependent carboxylases where the energy for carboxylation is provided by ATP hydrolysis.

Construction of a Transcarboxylase System from Oxaloacetate Decarboxylase and Methylmalonyl-CoA Decarboxylase. The occurrence of two different Na$^+$ transport decarboxylases opened the possibility to generate the Na$^+$ gradient with one enzyme and use it as driving force for carboxylation on the other enzyme. Proteoliposomes containing oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase were prepared by a simultaneous reconstitution with the two decarboxylases. These proteoliposomes accumulated Na$^+$ ions when energized with either oxaloacetate or malonyl-CoA (Figure 4). Upon generation of an electrochemical gradient of Na$^+$ ions with one of the substrates, the carboxylation of the decarboxylated second substrate was determined. Trapping systems for the carboxylated products were provided to protect these from a renewed decarboxylation. Oxaloacetate was therefore converted into citrate and malonyl-CoA into fatty acids.

The results of Figure 5 indicate the carboxylation of pyruvate in response to the decarboxylation of malonyl-CoA. The concentration of oxaloacetate trapped as citrate continuously increased over a period of 40 min during which the rate of pyruvate carboxylation increased slightly in the beginning and decreased after about 20 min. These rate differences probably reflect variations of the sodium motive force which increases until a steady state is reached and decreases when the decarboxylation slows down due to consumption of most of the substrate (Dimroth, 1982d). There was no or negligible carboxylation of pyruvate in the absence of malonyl-CoA or in the presence of monensin which abolishes the generation of a Na$^+$ gradient (Hilpert & Dimroth, 1984). The total amount of pyruvate carboxylation was about $^1/_{50}$ that of malonyl-CoA decarboxylation. Probably, irreversible steps such as the dissipation of the membrane potential by secondary ion fluxes or decarboxylation uncoupled from transport consume most of the energy before it can be used to drive the carboxylation of pyruvate.

The carboxylation of acetyl-CoA in response to the decarboxylation of oxaloacetate is shown in Figure 6. The amount

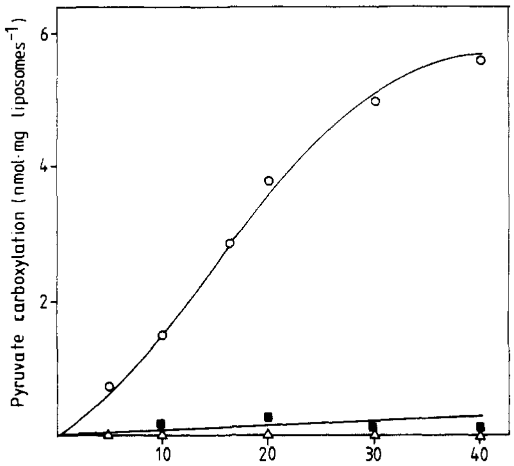


FIGURE 5: Carboxylation of pyruvate by a Na^+ ion gradient generated from the decarboxylation of malonyl-CoA. With oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase reconstituted proteoliposomes (15 mg, containing 4.4 μg of protein/mg of phospholipid) were incubated at 25 °C with malonyl-CoA, pyruvate, $KHCO_3$, Na_2SO_4, and citrate synthase. Generated oxaloacetate which is unstable in the presence of the decarboxylase was thus converted into stable citrate. Citrate formation was determined in samples taken after the indicated incubation times from the fluorescence decrease of NADH in a coupled assay system with citrate lyase and malate dehydrogenase (○). Parallel experiments were performed in the presence of 0.4 mM monensin (■) and in the absence of malonyl-CoA (△). For details see Experimental Procedures.

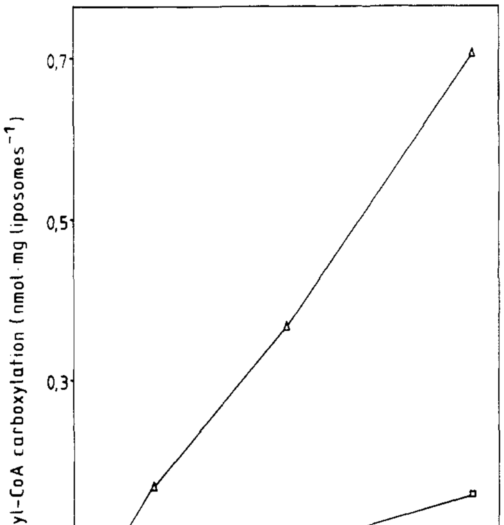


FIGURE 6: Carboxylation of acetyl-CoA by a Na^+ ion gradient generated from the decarboxylation of oxaloacetate. Reconstituted proteoliposomes (9 mg) containing oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase (4.4 μg of protein/mg of phospholipid) were incubated at 25 °C with oxaloacetate, [1-^{14}C]-acetyl-CoA, Na_2SO_4, $KHCO_3$, NADPH, and fatty acid synthetase. Acetyl-CoA carboxylation was determined from the incorporation of radioactivity into long-chain fatty acids (△). Parallel experiments were performed in the presence of 80 μM each of valinomycin (○), carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (□), and monensin (●). For details see Experimental Procedures.

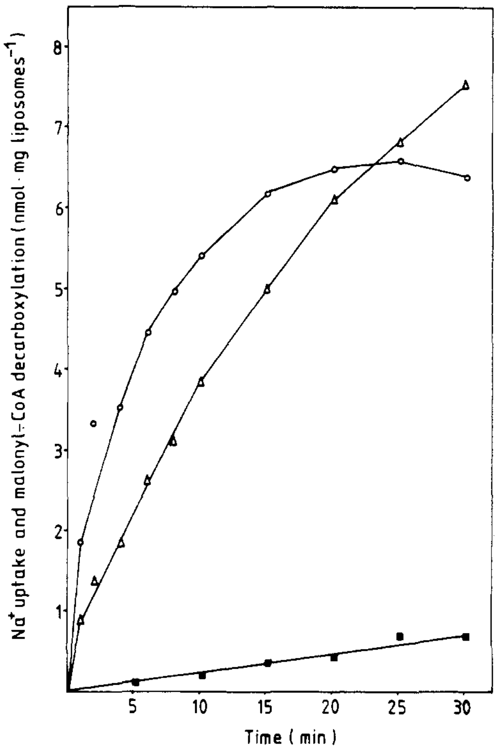


FIGURE 7: Coupling of Na^+ transport and malonyl-CoA decarboxylation. Na^+ uptake (○) and malonyl-CoA decarboxylation (△) were determined in parallel incubation mixtures containing, in a total volume of 1.15 mL at 25 °C, 30 mM potassium phosphate buffer, pH 7.0, 0.5 mM dithioerythritol, 20 mM $KHCO_3$, 2.5 μg of carbonic anhydrase, 1 mM $^{22}Na_2SO_4$ [620 counts min^{-1} (nmol of Na^+)$^{-1}$] in the transport assay and 1 mM unlabeled Na_2SO_4 in the decarboxylation assay, 28 mg reconstituted methylmalonyl-CoA decarboxylase containing proteoliposomes, and 1.7 mM malonyl-CoA which was used to initiate the reaction. The uptake of $^{22}Na^+$ was determined as described under Experimental Procedures, and malonyl-CoA decarboxylation was determined from the amount of acetyl-CoA formed by a coupled fluorometric assay with malate dehydrogenase and citrate synthase (Hilpert & Dimroth, 1984). Na^+ uptake in the absence of malonyl-CoA (■).

of oxaloacetate added was sufficient for a decarboxylation period of about 90 min, and acetyl-CoA was thus continuously carboxylated during the 15-min incubation time. When the Na^+ gradient was disrupted with monensin, acetyl-CoA carboxylation was also abolished. The uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone and the K^+ carrier valinomycin considerably reduced the rate of acetyl-CoA carboxylation but did not abolish it. This effect is probably due to dissipation of the membrane potential by these ionophores. Since decarboxylation creates an electrochemical Na^+ gradient (Dimroth, 1982d; Hilpert & Dimroth, 1983), the reversal of this process would have to be electrogenic, as well, and should be severely impaired by dissipation of the membrane potential.

In summary, these results show that a transcarboxylase can be constructed from oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase which mediated by Na^+ transport catalyzes the carboxylation of one substrate in response to the decarboxylation of the other.

Stoichiometry between Decarboxylation and Na^+ Transport. The kinetics of malonyl-CoA decarboxylation and Na^+ uptake by reconstituted methylmalonyl-CoA decarboxylase containing proteoliposomes are shown in Figure 7. In the initial phase of the transport, close to 2 mol of Na^+ ions was pumped into the vesicles by decarboxylation of 1 mol of malonyl-CoA. Later, when steeper Na^+ concentration gradients had developed, the stoichiometry changed, since decarboxylation continued even after a constant Na^+ concentration gradient was generated. However, partial coupling remained under these conditions since the rate of malonyl-CoA decarboxylation decreased in response to the increasing Na^+

concentration gradient and since the decarboxylation rate was stimulated about 2-fold by monensin (not shown). Similar results were obtained with oxaloacetate decarboxylase containing proteoliposomes, indicating that the stoichiometry between Na^+ transport and decarboxylation reaction, as determined from initial rates, was 2:1. The sodium motive force generated by the Na^+ pump oxaloacetate decarboxylase is about 110 mV (Dimroth, 1982d) which is equivalent to 2.5 kcal/mol of transported ion. The free energy of the decarboxylation reaction is about 6–7 kcal/mol and is therefore sufficient for the transport of 2 mol of Na^+ ions against the electrochemical gradient. The observed stoichiometry is thus in the range which can be expected from bioenergetics.

Discussion

Reversibility is recognized as one of the fundamental features of coupled vectorial transport systems (Tanford, 1983; Jencks, 1980). All different kinds of ATP-driven pumps (Ca^{2+}-, Na^+/K^+-, and H^+-ATPases) can perform active transport of the appropriate cation by ATP hydrolysis or ATP synthesis at the expense of the cation gradient, the direction of operation depending on the free energy change under the conditions of the physiological steady state. The only known exception to the rule of reversibility is bacteriorhodopsin in which the energy absorbed from light far exceeds the requirements of the transport process.

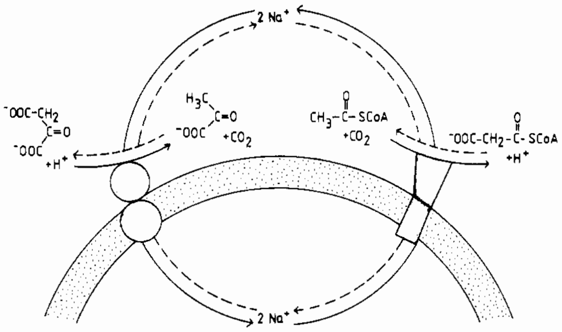
Other examples of reversible coupled transport systems are the biotin-dependent sodium transport decarboxylases, as described in this paper. The mechanism of free energy coupling in active transport may therefore be analogous for ATPases and decarboxylases. From the results presented here and elsewhere (Dimroth, 1982c; Hilpert & Dimroth, 1983; Hilpert, 1983) the decarboxylation reactions can be divided into two distinctive steps: the transfer of the carboxyl group form the substrate to enzyme-bound biotin (eq 1) and the

$$\text{R–COO}^- + \text{E–biotin} \rightleftharpoons \text{RH} + \text{E–biotin–COO}^- \quad (1)$$

$$\text{E–biotin–COO}^- + \text{H}^+ + 2\text{Na}^+_{\text{in}} \rightleftharpoons \text{E–biotin} + \text{CO}_2 + 2\text{Na}^+_{\text{out}} \quad (2)$$

decarboxylation of carboxybiotin enzyme to CO_2 and the free biotin enzyme (eq 2). The first partial reaction is catalyzed by a carboxyltransferase which is a distinct subunit (α) in case of oxaloacetate decarboxylase (Dimroth & Thomer, 1983). This subunit can be readily dissociated from the membrane and purified without detergent in its catalytically active state, free from the membrane-bound subunits β and γ. The carboxyltransferase-catalyzed reaction is reversible and completely independent from the presence of Na^+ ions. Although our knowledge on the second part of the reaction sequence is far more remote, the following statements can be made. Protons are used as substrate and CO_2 arises as the reaction product (Hilpert & Dimroth, 1983; Dimroth & Thomer, 1983). The decarboxylation of the carboxybiotin enzyme specifically depends on the presence of Na^+ ions, suggesting that coupling to Na^+ translocation is connected to this part of the reaction sequence. Since translocation of Na^+ notoriously requires binding to the membrane-bound parts of the enzyme, the specific protection of the membrane-bound β subunit from tryptic hydrolysis by Na^+ ions (Dimroth & Thomer, 1983) deserves special attention. The stoichiometry of decarboxylation to Na^+ transport was 1:2 in coupled reconstituted transport systems with methylmalonyl-CoA decarboxylase or oxaloacetate decarboxylase. A strong argument to place Na^+ translocation in the second half of the reaction (eq 2) comes from the present studies on the reversal of oxaloacetate and malonyl-CoA decarboxylation. Since these reversions require a Na^+ ion gradient in a coupled membrane transport system whereas the carboxyl transfer (eq 1) is freely reversible in solution, the coupling apparently is connected to the second part of the sequence. This reaction almost certainly is composed of several distinct steps involving conformational changes of the enzyme to account for the coupling of decarboxylation and transport.

Scheme I: Na^+ Circuit Mediating the Transcarboxylation from Oxaloacetate and Acetyl-CoA to Pyruvate and Malonyl-CoA and Vice Versa



This report describes for the first time that substrate carboxylations which are energetically unfavorable can be driven by coupling to a Na^+ gradient. In contrast, the biotin-dependent carboxylases derive the energy required for carboxylation from ATP hydrolysis. Transcarboxylase is a special carboxylase which gains the free energy for carboxylation from direct chemical coupling to a decarboxylation reaction (Wood & Barden, 1977). The transcarboxylase system constructed from oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase catalyzes the same overall reaction as the classical transcarboxylase, i.e. carboxylation of one substrate by decarboxylation of the second substrate. Carboxyltransferase and biotin carboxyl carrier protein components participate in both processes, but the coupling mechanism is completely different. Soluble transcarboxylase catalyzes a direct CO_2 transfer from the carboxylated to the decarboxylated substrate via a carboxybiotin enzyme intermediate. The coupled vectorial transcarboxylase system, however, depends on a Na^+ circuit as shown in Scheme I. A Na^+ ion gradient is established during the decarboxylation part of the pathway and drives carboxylation in the second half. Coupling by energy-rich chemical bonds in soluble transcarboxylase is thus opposed to vectorial energy coupling in the membrane-linked transcarboxylase system. These two carboxylation mechanisms parallel the two well-established ways of ATP synthesis either by substrate level phosphorylation or by the coupled vectorial phosphorylation reactions.

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Registry No. Na, 7440-23-5; oxaloacetate decarboxylase, 9024-98-0; methylmalonyl-CoA, 37289-44-4; pyruvic acid, 127-17-3; acetyl-CoA, 72-89-9.

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Effect of Surface Composition on Triolein Hydrolysis in Phospholipid Vesicles and Microemulsions by a Purified Acid Lipase[†]

Robert E. Burrier[‡] and Peter Brecher*

ABSTRACT: Sonicated dispersions of egg yolk phosphatidylcholine and triolein as vesicles and microemulsions have been used as substrates for the assay of a purified acid lipase. Previous studies have also shown that triolein localized in the surface phase of emulsions is the preferred substrate. In this study, we examined enzyme activity following several surface modifications using both vesicles and microemulsions. When the acidic phospholipids phosphatidylserine and phosphatidic acid were incorporated into both vesicles and microemulsions at up to 10 mol % of the total phospholipid, a dose-dependent reduction in the apparent K_m was observed. Using the vesicles as substrate, a dose-dependent decrease in V_{max} was also observed. Agarose gel electrophoresis was used to verify suspected changes in net particle charge. Analogous inclusion of phosphatidylethanolamine, sphingomyelin, or cholesterol did not affect kinetic parameters. Addition of oleic acid to sonication mixtures produced vesicles with a decreased apparent K_m and V_{max}, but triolein hydrolysis in microemulsions was not significantly altered. Triolein-containing vesicles prepared by using dimyristoyl- or dipalmitoylphosphatidylcholine were hydrolyzed maximally at the gel ↔ liquid-crystalline transition temperatures of the appropriate phospholipid. Differential scanning calorimetry was used to verify the temperatures of transition in these vesicles. The results indicate that acid lipase activity is influenced by the charge or physical state of the surface phase of model substrates and suggest that degradation of core components of naturally occurring substrates such as lipoprotein may be influenced by chemical changes on the surface of these particles.

Acid lipase is a lysosomal enzyme involved in the intracellular degradation of triglycerides and cholesteryl esters associated with lipoproteins. Studies on the mechanism of action for the enzyme have been hampered by methodological problems including low yields, instability of the purified enzyme, and poorly characterized substrate preparations. In a previous study (Burrier & Brecher, 1983), we isolated a form of an acid lipase from rat liver using a modification of a procedure for purification of the human enzyme (Warner et al., 1981). The purified enzyme preparation was characterized by using miceoemulsions and vesicles containing triolein as substrates. Both substrate types were readily hydrolyzed by the enzyme, and kinetic parameters suggested that triolein was preferentially hydrolyzed when incorporated into the phospholipid surface layer.

The action of lipases on membrane bilayers or microemulsions is thought to involve an initial binding to the enzyme to the lipid surface followed by localization of the substrate molecule to the active site where hydrolysis then occurs. It was suggested that alterations in the composition, charge, or structure of the substrate cause changes in "the quality of the interface", thus affecting the ability of the enzyme to interact with the substrate (Verger, 1980). Previous studies using lipases which act on cholesteryl esters or triglycerides have utilized several types of substrates including lipid monolayers (Demel et al., 1982; Pieroni & Verger, 1979), phospholipid vesicles (Brecher et al., 1976), and micelles or emulsions containing detergents or emulsifiers (Mahadevan & Tappel, 1968; Brown & Sgoutas, 1982).

The present work examines the activity of the rat liver acid lipase toward triolein-containing microemulsions and vesicles prepared with egg yolk lecithin in which the surface phase of the particles has been altered. The effect on enzymatic activity of the incorporation of the acidic phospholipids, phosphatidylethanolamines, sphingomyelin, cholesterol, and oleic acid into both substrate types was investigated. Triolein-containing vesicles of either dimyristoyl- or dipalmitoylphosphatidylcholine also were characterized and used as substrates at temperatures below and above the transition temperatures.

Materials and Methods

Materials. Tri[1-^{14}C]oleoylglycerol (99.8 mCi/mmol), [1-^{14}C]oleic acid (54.5 mCi/mmol), and [*2-palmitoyl*-9,10-^{3}H]dipalmitoylphosphatidylcholine (60 Ci/mmol) were purchased from New England Nuclear Corp., Boston, MA. Liquiscint was purchased from National Diagnostics, Som-

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[†] From the Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118. *Received December 27, 1983.* This work was supported by a grant from the U.S. Public Health Service (HL 25157).

[‡] Present address: Department of Comparative Medicine, Bowman Gray School of Medicine, Winston-Salem, NC 27103.

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