Membrane lipids and sodium pumps of cattle and crocodiles: an experimental test of the membrane pacemaker theory of metabolism

B. J. Wu, A. J. Hulbert, L. H. Storlien, and P. L. Else

Departments of Biomedical Science and Biological Science, Metabolic Research Centre, University of Wollongong, Wollongong, New South Wales, Australia 2522

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Wu, B. J., A. J. Hulbert, L. H. Storlien, and P. L. Else. Membrane lipids and sodium pumps of cattle and crocodiles: an experimental test of the membrane pacemaker theory of metabolism. Am J Physiol Regul Integr Comp Physiol 287: R633–R641, 2004; 10.1152/ajpregu.00549.2003.—The influence of membrane lipid composition on the molecular activity of a major membrane protein (the sodium pump) was examined as a test of the membrane pacemaker theory of metabolism. Microsomal membranes from the kidneys of cattle (Bos taurus) and crocodiles (Crocodylus porosus) were found to possess similar sodium pump concentrations, but cattle membranes showed a four- to fivefold higher enzyme (Na⁺-K⁺-ATPase) activity when measured at 37°C. The molecular activity of the sodium pumps (ATP/min) from both species was fully recoverable when delipidated pumps were reconstituted with membrane from the original source (same species). The results of experiments involving species membrane crossovers showed cattle sodium pump molecular activity to progressively decrease from 3,245 to 1,953 (P < 0.005) to 1,031 (P < 0.003) ATP/min when subjected to two cycles of delipidation and reconstitution with crocodile membrane as a lipid source. In contrast, the molecular activity of crocodile sodium pumps progressively increased from 729 to 908 (P < 0.01) to 1,476 (P = 0.01) ATP/min when subjected to two cycles of delipidation and reconstitution with cattle membrane as a lipid source. The lipid composition of the two membrane preparations showed similar levels of saturated (~31–34%) and monounsaturated (~23–25%) fatty acids. Cattle membrane had fourfold more n-3 polyunsaturated fatty acids (11.2 vs. 2.9%) but had a reduced n-6 polyunsaturate content (29 vs. 43%). The results support the membrane pacemaker theory of metabolism and suggest membrane lipids and their polyunsaturates play a significant role in determining the molecular activity of the sodium pump.

endotherms; ectotherms; sodium-potassium-adenosinetriphosphatase; [³H]ouabain binding; reconstitution; metabolism

The ability to change the mass-specific rate of metabolism is likely to be a major factor in allowing organisms to 1) adapt to changing environments, 2) evolve into either a larger or a smaller species, and 3) accommodate changes in body size associated with growth. Because organisms can differ enormously in their mass-specific metabolic rate, mechanisms must exist to allow for such changes to occur. To understand fundamental aspects of metabolism, we have previously used a number of comparisons of animal models. The models compared have included endothermic (“warm blooded”) with ectothermic (“cold blooded”) vertebrates (15, 16, 19, 25, 26), vertebrate species that vary in body mass (11, 12, 14, 32), and mammals at different stages of development (13, 27). Within each of these diverse comparisons there exists substantial variation in mass-specific metabolic rate (from 2- to 100-fold), yet there also exists a number of shared common features.

The features of metabolism commonly shared by organisms include 1) a limited number of cellular processes consuming most of the energy used by organisms, 2) these energy-consuming cellular processes representing a relatively similar proportion of metabolism irrespective of differences in metabolic rate, and 3) that most of these energy-consuming cellular processes are either directly or indirectly linked to membranes. Based on these basic observations we developed the “membrane pacemaker theory of metabolism” (27, 28). This theory proposes that variation in animal metabolism is due to membrane composition determining the overall pace of the common energy-consuming cellular processes. The theory suggests that the metabolic rate of a species is determined largely by the activities of the membrane-bound proteins associated with the energy-consuming processes of cells. Furthermore, the theory suggests that membrane-bound proteins are influenced by changes in their membrane environment, especially changes in lipid composition that act to alter the physical properties of the membrane and thus serve as a common underlying mechanism that largely determines the differences in the metabolic rate between species (28).

Comparison of the membrane lipid composition of a wide range of species (4, 7, 11, 19, 24, 29, 30, 32) has shown that most species share similar types and proportion (in the same tissues) of lipid head groups, similar percentages of saturated fatty acids, and an average acyl chain length of 18–19 carbons. Differences in membrane lipid composition are predominantly due to different proportions of unsaturated fatty acids (mono- and polyunsaturates) and in particular the amount of docosahexaenoic acid (DHA; 22:6 n-3) in the membrane lipids (see Ref. 17 for further details).

Our previous work, examining the influence of membrane lipids on the activity of membrane-linked processes, has concentrated primarily on the sodium pump (18, 19) and proton pumping and proton conductance of mitochondrial membranes (3, 5, 29). These two cellular processes can account for up to one-half of the resting metabolic rate of species (45). These two membrane-linked processes have often shown positive correlations between their activity and the levels of membrane polyunsaturation, especially DHA content (4–6, 52).

In the case of the sodium pump, the differences in Na⁺-K⁺-ATPase activity between species were often associated with changes in the rate of substrate turnover (i.e., molecular activity) rather than changes in pump concentration (18). The
molecular activity of the sodium pump was found to vary from 1,000 to 20,000 ATP/min when measured under ideal conditions at the same temperature in a broad range of species (52). Because the lipid composition of the membranes also varied in these comparisons, this model was used to test how changes in membrane lipid composition could modify the molecular activity of this intrinsic membrane protein.

We previously investigated this possibility with an experiment that partly exchanged membrane lipids between species while measuring the molecular activity of the sodium pump (19). In this membrane lipid crossover study, membranes with high sodium pump molecular activity and lipid unsaturation (as found in the rat) were exchanged with membranes with low molecular activity and low lipid unsaturation (as found in the toad). This previous study used detergent treatment to disperse the membrane lipids in both species, a process that resulted in an ~90% reduction in the molecular activity of the sodium pumps. Enzyme activities were then reconstituted by the addition of heat-inactivated membrane (i.e., membrane that had been heated to destroy innate sodium pump activity but not alter fatty acid composition) from either the original source or from that of the other species (19). The results of this experiment showed that reconstitution with original membrane returned the molecular activity of the sodium pump to its original level.

However, when toad membrane lipids were added to delipidated rat pumps, the molecular activity of the sodium pumps was significantly decreased compared with normal. In contrast, when rat membrane was added to delipidated toad pumps the molecular activity of the sodium pumps was significantly increased compared with normal. These changes were repeatable in both kidney and brain membrane preparations (19). The results from this study suggested that membrane lipid composition was a significant and predictable determinant of the molecular activity of sodium pumps.

In these experiments, the changes in sodium pump molecular activities were significant but still less than those naturally occurring between the species. The question as to the full extent that membrane lipids can influence the molecular activity of the sodium pump remained. If during these reconstitutions only small changes of membrane lipid occurred, then addition of the same membrane might be expected to restore molecular activity to original levels. However, reconstitutions involving membrane lipids of the other species would likely result in only small changes in the molecular activity of the sodium pump. Limited lipid exchange is a probable consequence of the technique as excess delipidation of membrane proteins can result in conformational changes and the permanent loss of enzymatic activity. Therefore detergent treatment is normally limited to preserve the ability of proteins to regain activity after reconstitution.

The present study was designed to confirm and further address the role of membrane lipid composition in the molecular activity of the sodium pump and thus experimentally test the membrane pacemaker theory of metabolism (28). In the current study a second membrane reconstitution cycle was added to the original technique to facilitate lipid exchange. To obtain the quantities of membrane required for these experiments, previously reported research on crocodile liver mitochondria (29) allowed us to also collect kidneys from these animals to use in the current experiments. Because crocodiles are a large ectothermic species, it was envisaged that their sodium pump molecular activities might be lower than other ectotherms that we have measured (18). To provide the quantity of membrane required, domestic cattle were chosen as the endotherm. This contribution reports on the results of these delipidation-relipidation experiments.

**MATERIALS AND METHODS**

*Materials.* [3H]ouabain (0.54 TBq/mmol, 98.3% purity) in ethanol was obtained from Dupont NEN. [3H]ouabain was resuspended in pure water at 7.4 MBq/ml after the removal of ethanol under a light stream of nitrogen gas. Scintillation cocktail (Hionic-Fluor) was obtained from Packard Chemicals; ATP (special quality) was from Boehringer Mannheim; nontriitiated ouabain was from ICN; and sodium deoxycholate (DOC: C24 H39 O4 Na, 98.5%) was from BDH Chemicals. All standard reagents used were of analytical grade.

*Animals, organs, and ethics.* Kidneys from cattle (*Bos taurus*, body wt ~500 kg) and saltwater crocodiles (*Crocodylus porosus*, body wt ~90 kg) were used in all experiments. The kidney was chosen for use in these experiments due to its high sodium pump concentration and presence of a single α-isofrom (i.e., α1). Cattle kidneys were collected immediately after the death of each animal at a commercial abattoir (Parish Meat Supplies, Yallah, NSW, Australia). Crocodile kidneys were collected from a commercial abattoir specializing in crocodile meats and products (Janamba Crocodile Farm, Darwin, NT, Australia). Crocodiles were killed, the carcasses were skinned and defleshed, and kidneys were subsequently collected. Crocodile kidneys were packed and maintained in dry ice (~78°C) during transport back to the University of Wollongong where they were subsequently maintained at ~82°C. All experiments were approved by the University of Wollongong Animal Ethics Committee.

*Microsomal membrane preparation.* To provide both high enzyme activity and membrane yield, microsomal membrane preparations were used in all experiments. Microsomal membranes were prepared using a standard procedure (36). Samples of frozen kidneys were thawed, weighed, cut into small pieces, and homogenized (10 g/100 ml of 0.25 M sucrose, 0.1 mM EDTA, pH 7.4 at 25°C) on ice using a glass-glass tissue homogenizer. Because reptilian kidneys lack a true medullary region, cortex samples only were used from cattle and whole kidney from crocodiles.

Tissue homogenates were filtered through a 250-μm nylon mesh, and the extract was centrifuged at 10,000 g at 4°C for 30 min (Beckman L-80 Ultracentrifuge). Supernatants were decanted and recentrifuged at 98,000 g for 35 min. Pellets were resuspended in a Tris-ATP-EDTA buffer (25 mM Tris base, 3 mM ATP, 2 mM EDTA, pH 7.6 at 25°C). Protein content was determined using the Lowry method (38), with BSA as a standard. Membranes were diluted (with Tris-ATP-EDTA buffer) to final protein concentrations of 1 and 2 mg of protein/ml and stored in 5-ml aliquots at ~82°C until required.

**Sodium pump activity and concentration.** Maximal Na\(^+\)-K\(^+\)-ATPase activity was measured at 37°C using a standard phosphate liberation assay as previously described (18, 19). Sodium pump density of microsomal membranes was determined using a modification of the [*H]ouabain binding method described by Tobin and Sen (51). Microsomal membranes (20 μl at 1 mg protein/ml) were incubated for 40 min at 37°C in 200 μl of [*H]ouabain binding buffer (5 mM MgCl\(_2\) and 10 mM K\(_2\)HPO\(_4\), pH 7.4 at 25°C) containing 7.4 x 10\(^3\) Bq of [*H]ouabain at a final ouabain concentration of 10\(^{-6}\) M in Millipore Ultrafree-MC 30,000-nominal molecular weight limit Eppendorf filters. Parallel ouabain incubations with equivalent specific [*H]ouabain activity but with a final ouabain concentration of 10\(^{-2}\) M were used to determine nonspecific binding. Labeled microsomal membranes were separated from the incubation (and wash) media by centrifugation at 5,000 g (Eppendorf 5417R) at 4°C for 40 min including 6 x 50-μl washes of ice-cold binding buffer (minus...
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ouabain). The number of washes used was based on previous characterization of washout curve profiles over 10 washes using brain and kidney from a number of species (55). Filters with radiolabeled membranes were removed from their plastic housing to minimize any carryover of trapped residual medium and placed in vials with scintillant and the tritium activity counted on a Wallac 1409 beta counter with disintegrations per minute (DPM) correction. Sodium pump density was determined by subtracting 3H activity held at nonspecific sites in excess ouabain (10−9 M) from total 3H binding at 10−8 M and converting the remaining activity to pmoles of sodium pump per milligram of membrane protein (assuming a 1:1 stoichiometry between ouabain and the sodium pump). All measurements were carried out in duplicate. Sodium pump concentration and Na+/K+-ATPase activity were measured on the same microsomal preparations to allow determination of the relationship between detergent (sodium deoxycholate, SE) double reconstitution technique. The double membrane reconstitution technique used mild detergent treatments to disperse membrane lipids (yet retain the ability of the sodium pump to recover activity) and allow lipid exchange to occur twice in the presence of new lipid sources. The double reconstitution procedure was built on earlier work using a single reconstitution step (19).

To perform the reconstitution procedure, it was first necessary to determine the relationship between detergent (sodium deoxycholate, DOC) concentration and sodium pump activity (Na+/K+-ATPase). This relationship was determined by adding 0.5 ml of cattle or crocodile kidney microsomal membrane (at 1 mg protein/ml) to 0.5 ml of DOC buffer of varying detergent concentration (DOC, 0–8 mg/ml; Tris, 25 mM; EDTA, 2 mM; ATP, 3 mM, pH 7.6 at 25°C) for 15 min followed by measurement of Na+/K+-ATPase activity (Fig. 1). Based on these DOC-activity profiles [and similar experiments performed on other species (19)], microsomal membranes used in the reconstitution were subject to an initial detergent treatment of 3.1 ± 0.1 DOC: protein (mg:mg) or 1.55 mg DOC/ml. This DOC treatment reduced sodium pump molecular activity to 14 and 24% of maximal activity for cattle and crocodiles, respectively.

After this delipidation treatment, the 1 ml of treated microsomal membrane had its detergent content reduced by dilution with either 2 ml of buffer or 2 ml of 2 mg protein/ml of heated microsomal membrane (i.e., a one-third dilution). The use of this volume was based on previous work (19, 55) showing that further increases in buffer volume (within the range of 1.5–8 ml) does not further increase reconstituted activity. The use of 2 mg protein/ml of heat-inactivated microsomal membrane was based on work using kidney and brain from two other species that showed that no further enhanced sodium pump activity occurred after reconstitution once within the range of 1–4 mg protein/ml (19, 55). The presence of heat-inactivated heated microsomal membranes in these experiments acted as a source of lipid available for exchange during the reconstitution process. Heat inactivation of microsomal membrane involved incubating the membrane at 95°C for 5 min, a procedure that destroyed Na+/K+-ATPase activity (normally within 2–3 min) but did not alter the acyl composition of the membrane lipids (19).

Subsequently, the first lipid reconstitution involved incubating 1 ml of the detergent-treated microsomal membrane (0.5 mg of total protein) with either 2 ml of buffer (used as a control) or 2 ml of the heat-inactivated microsomal membrane at 2 mg protein/ml (4.5 mg of total protein) at room temperature. The addition of the heat-inactivated microsomal membrane provided a source of excess lipid while at the same time reducing the detergent concentration by one-third (i.e., to 0.517 mg DOC/ml, although higher concentrations are likely to be experienced in the membranes due to detergent partitioning). After being left to stand for 5 min, the mixture was centrifuged (150,000 g for 90 min at 2°C), and sodium pump activity was determined from the pellets resuspended in 0.5 ml of Tris-ATP-EDTA.

The sodium pump content (Lowry method) and the number of ouabain binding sites of the microsomal membranes with active sodium pump were determined using the detergent-treated buffer controls. The use of the buffer controls for the determination of active microsomal protein yield was based on previous work radiolabeling (Bolton Hunter method; Ref. 2) kidney microsomal membrane. These preparations showed that the same amount of labeled membrane protein (i.e., DPM) was present in the final membrane pellets after the addition of either buffer or heat-inactivated membrane (19, 55). These experiments show that the protein yield of microsomal membrane with the active sodium pumps was the same irrespective of the presence or absence of heat-inactivated membrane. The use of buffer controls to determine sodium pump concentration per milligram of microsomal membrane protein with active sodium pumps was again based on direct experimentation. The number of ouabain binding sites (per mg of membrane protein) in cattle and crocodile membrane was measured before and after the first and second detergent treatments. Experiments showed that there were no significant differences in sodium pump concentration per milligram of active protein between the different preparations (results not shown).

The second lipid reconstitution involved a second detergent treatment and reconstitution cycle. To conduct the second delipidation-reconstitution, a second DOC:activity profile was determined for each preparation using a parallel set of microsomal membranes that had undergone the initial reconstitution process. The DOC:protein ratios used to determine the detergent-activity profile for the second detergent treatment ranged from 0.21 to 1.55 (mg DOC:mg protein). These ratios where significantly lower than those originally used due to the presence of the heat-inactivated membrane protein from the first reconstitution. Based on the detergent-activity profile for each membrane preparation, the final DOC:protein ratio (i.e., mg DOC:mg total protein, including the added heat-inactivated membrane protein) used for the delipidations was 1.02 ± 0.15 for cattle and 0.69 ± 0.12 for crocodile membrane. These detergent concentrations were more variable between the species than those used in the first delipidation. However, these treatments resulted in the molecular activities of the preparations falling to 19 ± 2 and 28 ± 4% of the initial reconstituted activity for cattle and crocodile, respectively. These values compare favorably with the reductions in molecular activities produced in the first detergent treatment for each species.

After the second detergent treatment, all preparations were diluted with 2 ml of buffer (Tris-ATP-EDTA, final DOC concentrations of 1.53 ± 0.23 and 1.05 ± 0.18 mg of DOC/ml for cattle and crocodile preparations, respectively). Experiments showed that the addition of more heat-inactivated microsomal membranes at this stage yielded
identical results to buffer dilution alone in the second reconstitution. Because the preparation already contained microsomal membrane from the initial detergent treatment, this result was as anticipated as excess lipid is already available for exchange. The addition of further heat-inactivated membrane at this stage simply made it more difficult to measure sodium pump activity in the larger pellet. After reconstitution the membrane mixture was let to stand for 5 min at room temperature before being centrifuged (150,000 g for 90 min at 2°C), and sodium pump activity was measured in the pellets resuspended in 0.5 ml of Tris-ATP-EDTA buffer. Protein yield and sodium pump concentration were again determined from parallel incubations that had undergone both detergent treatments and buffer dilution.

Fatty acid and lipid analyses. The analysis of microsomal membrane fatty acids composition was conducted using gas chromatography as previously described (19, 30, 56). Phospholipid content was measured by determining the phosphorus content of the microsomal membranes. Microsomal membrane (10 μl) was digested in 10% magnesium nitrate (~3 drops dissolved in ethanol), heated over a Bunsen flame (until brown fumes ceased) followed by the additions of distilled water (100 μl) and 0.5 M HCl (300 μl), and kept at 100°C for a further 15 min. Phosphate content of the final hydrolyzed solution was determined using a standard ammonium molybdate-based color reagent [1 g of (NH₄)₆Mo₇O₂₄•4H₂O in 70 ml of distilled water with 3.3 ml of concentrated H₂SO₄ a n d 4 g of FeSO₄•7H₂O added under constant stirring and used within 2 h] with absorbance measured at 750 nm on a Shimadzu UV-visible 1601 spectrophotometer with 1 M Na₂HPO₄ used as a phosphate standard. Cholesterol content of the microsomal membranes was measured using a Sigma diagnostic kit (no. 352–20) with a cholesterol calibration standard. Statistical analyses were performed using Statview. All percentage and proportional data were compared after being transformed (arcsin-square root).

RESULTS

Table 1 shows the sodium pump activities (Na⁺-K⁺-ATPase) measured in homogenates and sodium pump activities and pump concentrations measured in microsomal membranes of cattle and crocodile kidneys. In both preparations the cattle kidney displayed Na⁺-K⁺-ATPase activities that were several-fold higher (5.7× in homogenates and 4.2× in microsomal membranes per mg of protein) than those of the crocodile preparation measured under identical ideal conditions at the same temperature (37°C). The concentration of sodium pumps in the microsomal membrane preparations of both cattle and crocodile were nearly identical. Dividing maximal enzyme activity by pump concentration produced the maximal molecular activity for the sodium pump (i.e., the rate of substrate turnover in ATP/min per pump). In cattle kidney membranes the molecular activity of the sodium pump was 4.3-fold higher than that present in the crocodile membrane measured at the same temperature (Table 1).

Figure 1 shows the profiles of sodium pump molecular activity in microsomal membranes of cattle and crocodile relative to increasing detergent concentration. The profiles show an initial increase in activity (up to 70 and 108% for cattle and crocodile, respectively) followed by decrease in molecular activity for both cattle and crocodile. These results are similar to those previously reported for microsomal membranes from kidney and brain of rat and toad (19). In all species, measured maximal activity occurred at a detergent concentration of ~1 mg DOC/ml or at a DOC:protein (mg:mg) ratio of ~2. This initial increase in activity has previously been described and attributed to the permeabilization of microsomal membrane vesicles by detergent (47) although alternative explanations involving interactions between subunits of the sodium pump (i.e., protein:protein interactions) influenced by detergent also exist (9). The decrease in the molecular activity of the sodium pumps, from both species, with increasing detergent concentration is presumed to be due to the increasing replacement of the natural membrane lipids by the detergent. Although higher detergent concentrations further reduced molecular activity and therefore were likely to remove and displace more membrane lipids, they were not used due to the increased failure of the sodium pumps to reconstitute full activity in the presence of the heat-inactivated membrane (19, 55). The ability of microsomal membrane to reconstitute sodium pump activity after detergent treatment was routinely assessed using buffer dilution (i.e., addition of an equal volume of buffer without heat-inactivated membrane). In the first reconstitution, buffer addition reconstituted ~60 and 81% of the original maximal activity compared with 102 and 90% of maximal activity reconstituted in the presence of heat-inactivated membrane in cattle and crocodile preparations, respectively. In similar experiments on other preparations from various tissues of other species (results not shown), we have found a similar trend with buffer addition averaging ~70% of full activity. Buffer additions in the initial reconstitution served as a good indicator of the potential of a preparation to recover full activity on addition of the heat-inactivated membrane but could not reconstitute full activity in the absence of a lipid source.

The difference in the ability of heat-inactivated membrane vs. buffer addition to reconstitute activity in the first reconstitution was assumed to be due to lipid loss. Buffer alone lacked the ability to replace any lipids lost as lipid-detergent micelles on reconstitution. The presence of the heat-inactivated membrane, however, was able to compensate for this lipid loss. This ability to compensate for lipid loss through lipid exchange allowed for potential changes in the molecular activity of the sodium pump to occur. In other words buffer addition alone would require the presence of heat-inactivated membrane to compensate for the potential of lipid loss through micelle

### Table 1. Na⁺-K⁺-ATPase activity, sodium pump concentration, and molecular activity in cattle and crocodile kidney microsomal membranes at 37°C

<table>
<thead>
<tr>
<th></th>
<th>Cattle (n = 8)</th>
<th>Crocodile (n = 9)</th>
<th>Significance (P Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺-K⁺-ATPase activity, μmol P₇₅₅₅ protein⁻¹·h⁻¹</td>
<td>8.12±0.33</td>
<td>1.42±0.09</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Homogenate</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Microosomal membrane</td>
<td>27.81±2.03</td>
<td>5.38±0.33</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Maximal microsomal membrane (detergent treatment)*</td>
<td>47.19±2.60</td>
<td>11.22±0.57</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Sodium pump concentration of microsomal membrane, pmoles/mg protein</td>
<td>232±13</td>
<td>239±18</td>
<td>NS</td>
</tr>
<tr>
<td>Sodium pump molecular activity, ATP/min</td>
<td>3,390±187</td>
<td>782±40</td>
<td>P &lt; 0.0001</td>
</tr>
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</table>

Values are means ± SE; n is the no. of microsomal membrane preparations used. Molecular activity in ATP/min was calculated by dividing Na⁺-K⁺-ATPase activity by sodium pump number from the same preparation. *Maximal microsomal membrane Na⁺-K⁺-ATPase activities (maximal activity associated with detergent treatment) were determined using detergent concentrations ranging from 0.5 to 4 mg sodium deoxycholate (DOC)/ml or ratios of 1–8 mg DOC/mg of protein, respectively. In both cattle and crocodile microsomal membranes, optimal activity occurred at ~1 mg of DOC/ml or a ratio of 2 mg DOC/mg protein. NS, not significant.
formation. This conclusion is supported by the results of the second reconstitution experiment. In the second reconstitution, buffer addition alone in the presence of the heat-inactivated membrane from the first reconstitution facilitated the full recovery of original activity equal to a second addition of heat-inactivated membrane in both the cattle-cattle and crocodile-crocodile preparations. Therefore the second reconstitution required only buffer addition as an alternative to the further addition of heat-inactivated membrane to reconstitute activity in both the original and other species membrane crossover experiments.

Figure 2 shows the results of the double reconstitution experiment. Figure 2, left, shows the results for the same-species membrane crossover experiments. For each species, the original detergent-activated maximal molecular activity is shown followed by the molecular activity after the first detergent treatment. The next set of values shows the molecular activity of each preparation after the addition of the heat-inactivated membrane from the same species. The next values show the molecular activity after the second detergent treatment, and the final values show the molecular activities recovered after buffer dilution in the presence of original heat-inactivated membrane added in the first reconstitution. The same pattern is shown in Fig. 2, right, but the values shown here are for molecular activities reconstituted with heat-inactivated membrane from the other species.

For both cattle and crocodile membrane preparations, the presence of original heat-inactivated membrane in the first and second reconstitution returned the molecular activity of the sodium pump to their original levels. Cattle sodium pumps recovered 102 and 102% of their original activity using cattle heat-inactivated membrane in the first and second reconstitution, respectively. Crocodile sodium pumps recovered 90 and 108% of their original activity using crocodile membrane on the first and second reconstitution, respectively. The first and second reconstitutions showed no statistical differences between one another or with the original activity (P values ranged from 0.98 to 0.11).

The presence of heat-inactivated membrane from the other species in the crossover experiments resulted in sodium pump molecular activity that was influenced by the source of the added membrane. When crocodile membrane was initially added to delipidated cattle membrane, a significant (P < 0.005) decrease (40%) occurred compared with the original molecular activity of the cattle sodium pumps. A second delipidation and subsequent reconstitution of cattle sodium pumps with crocodile membrane lipid produced a further significant (P < 0.003) decrease (47%) in molecular activity. The combined effect of the double reconstitution where crocodile membrane lipid exchanged with cattle membrane lipid resulted in cattle sodium pump molecular activity decreasing from an average 3,245 ± 347 ATP/min to 1,953 ± 207 and then 1,031 ± 62 ATP/min with each subsequent reconstitution, an overall reduction of 68% (P < 0.001).

In contrast, when cattle membrane lipid was initially added to delipidated crocodile membrane, a significant (P < 0.01) increase (25%) in the molecular activity of the crocodile sodium pumps occurred. A second delipidation and reconstitution in the presence of cattle membrane lipid produced a further significant (P < 0.01) increase (63%) in sodium pump activity.

Fig. 2. Molecular activities of cattle and crocodile sodium pumps after species membrane crossover experiments using kidney microsomal membrane preparations and the double membrane-lipid reconstitution technique (as detailed in MATERIALS AND METHODS). Graphs show effects on the molecular activity of sodium pumps from cattle and crocodile membranes having undergone 2 consecutive delipidations and reconstitutions using heat-inactivated membranes from both the original and other species sources. Top left: cattle sodium pumps reconstituted with heat-inactivated cattle membrane. Top right: cattle sodium pumps reconstituted with heat-inactivated crocodile membrane. Bottom left: crocodile sodium pumps treated with heat-inactivated cattle membrane. Bottom right: crocodile sodium pumps reconstituted with heat-inactivated crocodile membrane. Top left: cattle sodium pumps reconstituted with heat-inactivated crocodile membrane. Bottom right: crocodile sodium pumps reconstituted with heat-inactivated cattle membrane. In the first reconstitution, 8–10 preparations were used, and in the second reconstitution, 2–5 preparations were used. Values are means ± SE.
molecular activity. The combined effect of the double reconstitution, where cattle membrane lipids exchanged with crocodile membrane lipids, was for the crocodile sodium pumps to increase their molecular activity from 729 ± 35 ATP/min to 908 ± 45 and 1,476 ± 173 ATP/min over the two reconstitutions, an overall 103% increase (P < 0.001) as shown in Fig. 2.

Table 2 shows the fatty acid composition of phospholipids of kidney microsomal membranes of cattle and crocodile. Although statistically different (on a percentage basis), the two species had similar levels of saturates (34–31%), monounsaturates (24–22%), and polyunsaturates (41–46%; cattle-crocodile, respectively). The differences between the cattle and crocodile membranes included the types of saturates present, with palmitic acid (16:0) dominant in the membrane phospholipids of crocodile and stearic acid (18:0) dominant in the membrane phospholipids of crocodiles. For monounsaturated fatty acids, there were no major significant differences in either the types or percentage present in the membrane phospholipids of the two species, apart from slightly more 18:1 n-9 in the cattle membranes.

Within the polyunsaturates, the two membrane preparations differed considerably. Cattle membrane phospholipids had significantly higher levels of n-3 fatty acids compared with the crocodile phospholipids. The cattle had 11.2% of its fatty acids as long-chain n-3 polyunsaturates with 4-fold higher linolenic acid (18:3 n-3), 4.5-fold higher eicosapentaenoic acid (EPA; 20:5 n-3), 6-fold higher docosapentaenoic acid (DPA; 22:5 n-3), and 3.5-fold higher levels of DHA (22:6 n-3) compared with crocodile membranes. The crocodile kidney membrane n-3 polyunsaturates included only 0.7% DHA, 0.4% DPA, 1.1% EPA, together with other minor n-3 polyunsaturates contributing to 2.9% of their total fatty acids. In compensation the crocodile membrane had increased levels of n-6 fatty acids with 93% more linoleic (18:2 n-6), 24% more arachidonic acid (20:4 n-6), and 8.5-fold higher docosatetraenoic acid (22:4 n-6). The crocodiles had 43% of their total membrane fatty acids as n-6 polyunsaturates, whereas the cattle had only 29%.

The level of phospholipid per milligram of membrane protein was significantly higher (2.4 ×, P < 0.05) in the cattle compared with the crocodile (Table 2). Similarly the level of cholesterol was significantly higher (2.8 ×, P < 0.05) in the cattle membrane compared with the crocodile. Consequently, the average cholesterol:phospholipid ratios were similar at 0.34 for cattle and 0.31 for crocodile membrane, indicating that approximately one cholesterol molecule was present for every three phospholipid molecules in the microsomal membrane of both species.

**DISCUSSION**

The aim of this work was to experimentally examine the influence of natural membrane lipids on the activity of a common membrane protein, the sodium pump, as a test of the membrane pacemaker theory of metabolism. These experiments were conducted using kidney microsomal membranes from two disparate species, cattle and crocodile. The kidney microsomes from these two species displayed large differences in both the molecular activity of their sodium pumps and in their lipid composition. The molecular activity of sodium pumps from the cattle was 4- to 5-fold faster than those from the crocodile (~3,400 vs. ~800 ATP/min) at the same temperature (37°C). The major difference in membrane lipid composition was among the polyunsaturated fatty acids with cattle membranes having more as n-3 fatty acids (11.2% in cattle vs. 2.9% in crocodile), whereas crocodiles had predominantly n-6 fatty acids as their membrane polyunsaturates (43% in crocodile vs. 29% in cattle).

To test the ability of membrane lipids to influence the activity of the sodium pump, the pumps were first mildly delipidated and then reconstituted with membrane from either the original source or from the other species. The emphasis was on lipid exchange with the reconstitution procedure carried out twice in succession. Reconstitution that involved membrane from original species showed that, for both cattle and crocodile, the molecular activity of sodium pumps could be fully restored after both an initial and second delipidation. However, when crocodile pumps were delipidated and reconstituted with cattle membrane lipid, the molecular activity of crocodile

**Table 2. Phospholipid fatty acid profiles of cattle and crocodile kidney microsomal membrane**

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Composition, % of total fatty acids</th>
<th>Cattle (n = 4)</th>
<th>Crocodile (n = 5)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>19.1 ± 0.3</td>
<td>11.4 ± 0.2</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>C17:0</td>
<td>1.0 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>C18:0</td>
<td>14.1 ± 0.3</td>
<td>18.9 ± 0.1</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Σ Saturates</td>
<td>34.2 ± 0.4</td>
<td>30.6 ± 0.6</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Monounsaturates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1 n-7</td>
<td>0.9 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>C17:1 n-7</td>
<td>1.0 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>C18:1 n-7</td>
<td>2.0 ± 0.1</td>
<td>3.2 ± 0.2</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>C18:1 n-9</td>
<td>20.8 ± 0.3</td>
<td>18.3 ± 0.2</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Σ Monounsaturates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.7 ± 0.6</td>
<td>22.8 ± 0.2</td>
<td>0.012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-9 Polyunsaturates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:3 n-9</td>
<td>0.8 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>n-6 Polyunsaturates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>9.2 ± 0.2</td>
<td>17.8 ± 0.1</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>C20:3 n-6</td>
<td>1.8 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>C20:4 n-6</td>
<td>17.8 ± 0.2</td>
<td>22.0 ± 0.3</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>C22:4 n-6</td>
<td>0.2 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>n-3 Polyunsaturates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:3 n-3</td>
<td>0.8 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>C20:3 n-3</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>C20:5 n-3</td>
<td>4.9 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>C22:5 n-3</td>
<td>2.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>C22:6 n-3</td>
<td>2.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Σ Polyunsaturates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41.2 ± 0.5</td>
<td>46.6 ± 0.3</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indexes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σn-9</td>
<td>21.6 ± 0.3</td>
<td>19.0 ± 0.2</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Σn-7</td>
<td>3.9 ± 0.4</td>
<td>4.5 ± 0.3</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Σn-6</td>
<td>29.2 ± 0.3</td>
<td>43.0 ± 0.2</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Σn-3</td>
<td>11.2 ± 0.4</td>
<td>2.9 ± 0.1</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>n-6n-3</td>
<td>2.6 ± 0.1</td>
<td>15.0 ± 0.2</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Unsaturation index</td>
<td>179 ± 2</td>
<td>174 ± 1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Average chain length</td>
<td>18.3 ± 0.1</td>
<td>18.3 ± 0.1</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

Lipid parameters (n = 4)  

|                   |                           |
|                   | (n = 4)                   |
| Cholesterol, μg/mg protein | 149.8 ± 4.0              |
| Phospholipid, μg/mg protein | 862 ± 58                  |
| Cholesterol/phospholipid  | 0.34 ± 0.01               |

Values are means ± SE. Fatty acids < 0.5% of total fatty acids in both species were not included. Unsaturation index is the number of double bonds per 100 acyl chains. Σ indicates the sum for all fatty acids within designated group. NS indicates a nonsignificant difference between cattle and crocodile at the P < 0.05 level.
sodium pumps increased with each subsequent delipidation-reconstitution cycle, leading to a doubling of the original molecular activity. Conversely, each time cattle sodium pumps were reconstituted with crocodile membrane, molecular activity decreased resulting in a total 70% decrease compared with their original molecular activity.

The strength of this experimental approach is the emphasis on lipid exchange and the significant stepwise changes after each cycle of delipidation and reconstitution. On technical grounds it is easy to envisage how a decrease in molecular activity might occur due to the failure of the reconstitution procedure, but it is difficult to explain how an increase in molecular activity could occur. One possibility is that the presence of the heat-inactivated membrane differentially "pulled down" more active membrane out of solution after delipidation (compared with the buffer controls used to determine protein yield in the final pellet). A separate series of experiments, however, showed that there was no difference in the amount of radiolabeled membrane pelleted between buffer and membrane treatments (19, 55). Furthermore, the number of sodium pumps per milligram of membrane protein determined by [3H]ouabain binding was unaffected by the two mild detergent treatments for both crocodile and cow membrane. These results indicate that the use of buffer controls to determine protein yield and sodium pump concentration was not artifically contributing to changes in molecular activity. A weakness of the experimental approach used in these experiments is the inability to determine the actual composition of the membrane of the pumps after reconstitution. This inability to determine the membrane lipid composition surrounding the sodium pumps in the original membrane is due to the dominant presence (4:1) in the final pellet of the added heat-inactivated membrane.

The idea of using heat-inactivated microsomal membrane originates with the work of Ottolenghi (41) and the period of the mid-1970s when much of the original work examining the interactions between membrane lipids and proteins was performed (34, 35, 41, 44, 54). In the case of the sodium pump, previous work has shown that molecular activity could either be fully recovered (41) or additional activity obtained after detergent treatment and repurification with lipid (54). Ottolenghi (41) used heat-inactivated ox brain (gray matter) as a lipid source to reconstitute sodium pumps purified from the salt gland of the dogfish. In these experiments, the ox brain extract was heat inactivated and used to reconstitute Na+-K+-ATPase after pump purification. The activity of the purified Na+-K+-ATPase was ~0.2% of the original activity, and after the addition of the heat-inactivated ox brain lipid extract, it returned to ~100% of original activity. The phospholipid acyl composition of dogfish salt gland (10) and ox brain (11) is quite similar, with ~15% DHA, 28–24% n-3s, 12–15% n-6s, and ~33% saturates for dogfish and ox lipids, respectively. The reason for the similarity is likely that cold-water fish such as dogfish show increased levels of polyunsaturation, particularly DHA, in their membrane phospholipids to compensate for the effects of low temperature (22, 23). In such species the molecular activity of the sodium pump is often high and similar to those of mammals (when measured at the same temperature) with which they share a high level of membrane polyunsaturation, particularly of the n-3 type (18).

Previous work examining the effects of phospholipids upon delipidated Na+-K+-ATPase has found phosphatidylserine (PS) to be most effective at restoring ATPase activity (53). It is of interest that during dietary n-3 deficiency, PS is one of the few phospholipids to show a significant decrease in content with the affected PS species commonly being 18:0:22:6 n-3 (40). When used to reconstitute sodium pump activity, PS has also been shown to be able to double the original activity (54). This extra activity was assumed to be due to an increase in sodium pump concentration relative to the protein content resulting from the differential solubilization of the proteins during the detergent treatment (54). However, actual pump concentration was not measured in the study, and increased molecular activity is a possible alternative explanation for this reported effect.

Only a few studies have directly investigated the effect of exchanging natural membrane phospholipids on the activity of membrane-bound proteins. When the mitochondrial enzyme succinic dehydrogenase was delipidated and reconstituted with mitochondrial lipid from warm (25°C)- and cold (5°C)-acclimated goldfish, maximum activity was obtained using lipid extracted from the cold-acclimated fish (21). As previously mentioned, cold acclimation results in an increased level of polyunsaturation of membrane lipids. Use of phospholipids to reconstitute activity in the same study found PS resulted in the highest activity and that the degree of unsaturation seemed to be the predominant influence. In a similar study, comparing trout, cold acclimation increased sodium pump activity in erythrocyte by 60% across all temperatures measured. However, the concentration of sodium pumps was found not to have changed with acclimation, and therefore the increase in sodium pump activity was due to changes in the molecular activity of the pumps (43). Similar changes have been found in a study substituting membrane lipids between marine fish species adapted to different pressures (20). Membrane lipid from species adapted to high pressure are more polyunsaturated and resulted in an increased Na+-K+-ATPase activity over a range of pressures when exchanged into the membranes of low-pressure-adapted species.

Changes in the molecular activity of the sodium pump might be suggested to be due to effects other than changes in membrane lipid composition. The large α-catalytic unit of the sodium pump occurs as four known isoforms, with the dominant type being α1. Different isoforms, and/or their lipid sensitivities, could explain differences in molecular activity between cattle and crocodile sodium pumps. There have been reports of small differences in molecular activity for the different isoforms of the sodium pump (39). However, different isoforms are unlikely to explain the molecular activity differences in the present study because the kidneys of most vertebrates uniformly express the α1- or α1-like isoform (49), suggesting the catalytic unit of the sodium pump of the cattle and crocodile kidney are likely to be very similar. A number of external factors, including various hormones and cofactors, are known to affect the activity of the sodium pump (50). However, external factors are unlikely to be active in the semipurified membrane preparations used here, as the heat inactivation used will likely denature most proteins in the added membrane. There also exists the potential for changes in the lipid environment of the membrane to influence interactions between the subunits of the sodium pump, particularly the γ-
and α-subunits. At present, however, it is not known if crocodile pumps possess γ-subunits.

The measurement of sodium pump activity at 37°C for both cattle and crocodile was chosen to allow for direct comparison between the two species. Previous experiments examining the thermal dependence of sodium pump activity has shown it to be resilient over the temperature range of 25–37°C, even for ectotherms with a low preferred body temperature (see Table 2 in Ref. 18). The crocodile species used in the present study has a high preferred body temperature at 32–33°C (35), with 37°C within the range of environmental temperatures these animals might normally experience, and suggests that the sodium pump of the crocodile would not be adversely affected by measurement at 37°C.

The ability of polyunsaturates to influence the molecular activity of the sodium pump was recently examined (52) in a broad comparison across three organs (heart, kidney, and brain) from five mammalian and eight avian species, where molecular activity varied 18-fold and membrane DHA levels varied 80-fold. This study found a highly significant (P < 0.001) positive correlation between membrane DHA content and sodium pump molecular activity. The differences in membrane composition and molecular activity between the two species measured in the present study show a similar relationship. Membrane cholesterol (1, 8, 9) and unsaturation (37, 48) are also known to affect the activity of membrane proteins, including the sodium pump. Although the cholesterol:phospholipid molar ratios of the two membrane preparations used here were similar, the actual cholesterol concentration (relative to protein) was higher in the cattle membrane. The effect of cholesterol at higher concentrations is to reduce activity, but in these experiments the cattle membrane with its higher cholesterol concentration did not exhibit such an effect. This might suggest that the cholesterol:phospholipid molar ratio might be important. With a value of ~0.3 in both species, this ratio suggests a predominance of plasma membrane and/or golgi membranes in our microsomal preparations as other potential membrane contributors tend to have much lower cholesterol:phospholipid ratios (0.03–0.05, Ref. 42).

A major difference in the membrane phospholipids of the two species used here was in their n-6:n-3 ratios at 2.6:1 for cattle and 15:1 for crocodile. These ratios are interesting in light of the changes in essential fatty acid consumption that has occurred during recent human history. The ancestral n-6:n-3 dietary ratio of humans is believed to have been in the order of 1:1; in Western diets the ratio is now 16:1, which is clearly dominated by n-6 polyunsaturates (46). Whether these dietary changes are affecting membrane phospholipid ratios and subsequently having an effect such as slowing down the molecular activity of membrane proteins in the age of increasing human obesity is an interesting speculation.

In conclusion, the experiments conducted in this study provide a test of the membrane pacemaker theory of metabolism (27, 28). The results both support this theory and reinforce earlier findings from rat and toad membrane crossover experiments (19). Specifically, we show that changes in polyunsaturates of cattle and crocodile membranes modify the molecular activity of the sodium pump. The results support the idea that the lipid composition of membranes significantly influences the molecular activity of membrane proteins.

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Present address of B. J. Wu: Centre for Thrombosis and Vascular Research, School of Medical Science, Univ. of New South Wales, Sydney, New South Wales 2052, Australia (E-mail: bj.wu@unsw.edu.au).

Present address of L. H. Storlien: Disease Area Scientific Leader, Obesity, AstraZeneca, R&D, Molndal S-431 83, Sweden (E-mail: Leonard.Storlien@astrazeneca.com).

GRANTS

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REFERENCES


