tables[14] applied to this data showed that the responses of the pseudo-operated and operated animals to the control trials were essentially identical ($X^2 = 0.0006$, $p > 0.1$). Thus, destroying the integrity of BARTH's organ apparently did not grossly affect the level of activity in these animals under the conditions of the experiment. When the tones were presented, only 53% (16/30) of the operated animals responded, but 76% (19/25) of the pseudo-operated animals responded. The McNemar test was used to assess the significance of this difference[14]. Table II shows that there was no difference in the behavior of the operated animals between the control and sound trials ($X^2 = 0.235$, $p > 0.1$). The pseudo-operated animals responded significantly more often in the sound trials than in the control trials ($X^2 = 4.923$, $p < 0.05$). This means that the operated animals seem to have failed to react to the stimulus, while the pseudo-operated animals did react to it.

Table II. Data for the McNemar statistic

I. Operated animals ($N = 30$)

	control trials	
Sound trials	*R*	*NR*
NR	7	7
R	6	10

II. Pseudo-operated animals ($N = 25$)

	control trials	
Sound trials	*R*	*NR*
NR	2	4
R	8	11

R number responding; *NR* number not responding.

This result and the electrophysiological evidence are both consistent with the view that BARTH's organ is the only receptor in *Ocypode* sensitive to high frequency vibrational and acoustic stimuli. As such, it is analogous to the subgenual organs of insects[15–17], but is possibly more amenable to neurophysiological study than the latter. Although much less studied in this regard than insects, many decapod crustaceans communicate with acoustic signals[4–7, 11]. BARTH's organ probably is the receptor used for detection of these signals, at least for those with frequencies above 1 kHz, and thus may be crucial for intraspecific communication among crustaceans.

Zusammenfassung. Nachweis des Barth'schen Myochordotonal-Organs bei der Krabbe *Ocypode*, als sensorieller Apparat für hochfrequente, akustische Signale.

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[14] S. SIEGEL, *Nonparametric Statistics for the Behavioral Sciences* (McGraw-Hill, New York 1956), p. 63–67, 107–109.
[15] V. G. DETHIER, *Insect Physiology*, (Ed. K. D. ROEDER, Wiley, New York 1953), p. 523.
[16] H. AUTRUM, *Acoustic Behavior of Animals* (Ed. R.-G. BUSNEL, Elsevier, Amsterdam 1963), p. 412.
[17] J. SCHWARTZKOPF, *Physiology of Insecta* (Ed. M. ROCKSTEIN, Academic Press, New York 1964), vol. 1, p. 509.
[18] This work was supported by AEC Contract No. AT-(29-2)-226, and NSF Grant No. GB-20766x to M. SALMON, whom I thank, along with P. R. BURGESS for critical review of the manuscript. This is a contribution of the Eniwetok Marine Biological Laboratory.

Microsomal Na^+-Stimulated NADH- Cytochrome c Reductase: Could it be Involved in Sodium Transport?

During the last few years, there has been much discussion about the possibility of the existence of more than one sodium pump in absorbing epithelia. The most convincing evidence has been obtained with kidney proximal tubular cells[1] but research with various different epithelial cells has suggested that the dichotomy is widespread. One of the weak points of the hypothesis remains the fact that, whereas it is generally accepted that sodium-for-potassium exchange is mediated by a ($Na^+ + K^+$)-stimulated ATPase[2], no indication has been forthcoming as to the biochemical nature of the second pump. A plausible candidate for such a mechanism appeared to be afforded by the Na^+-stimulated NADH-cytochrome c reductase system of microsomal fractions, first described by SIEKEVITZ[3]. The present preliminary survey was undertaken in an attempt to discover any link between this enzyme and the sodium-pumping system of the kidney. Of the various criteria listed by SKOU[4] for the identification of an enzyme system with a pumping mechanism, we have concentrated on its cytological location, its sensitivity towards cationic

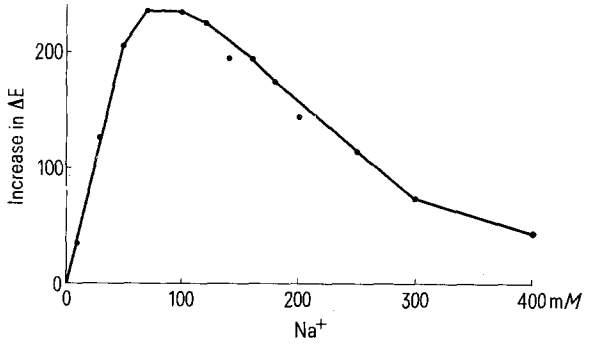

Fig. 1. Sensitivity of dog renal cortex microsomal NADH-cytc. reductase to sodium ions. Results express the increase in enzyme activity in the presence of different concentrations of sodium ions (in the form of NaCl) with reference to the basal level in the absence of sodium.

[1] G. WHITTEMBURY and F. PROVERBIO, Pflügers Arch. Eur. J. Physiol. *316*, 1 (1970).
[2] F. PROVERBIO, J. W. L. ROBINSON and G. WHITTEMBURY, Biochim. biophys. Acta *211*, 327 (1970).
[3] P. SIEKEVITZ, Fedn. Proc. *24*, 1153 (1965).
[4] J. C. SKOU, Physiol. Rev. *45*, 596 (1965).

stimulation, and its behaviour with respect to inhibitors, and we have concluded regretfully that this enzyme is probably not responsible for sodium transport across cell membranes in epithelial cells, and its physiological function remains a mystery.

Methods. Dog renal cortex, or intestinal or colonic mucosa, was homogenised as described previously[5] in a mixture of 0.3 *M* saccharose (80%) and *Tris*/HCl buffer, 0.05 *M* pH 7.2 (20%), which also contained 1 m*M* EDTA and 0.1% deoxycholate (*Tris*-salt), this latter being omitted in some experiments. The homogenate was centrifuged for 30 min at 5 000 *g*, then the residue was re-homogenised and re-centrifuged at the same speed. The residue from this centrifugation was considered to be a nuclear-mitochondrial fraction. Then the 2 supernatants were mixed and re-centrifuged for 60 min at 110,000 *g* to provide a microsomal fraction.

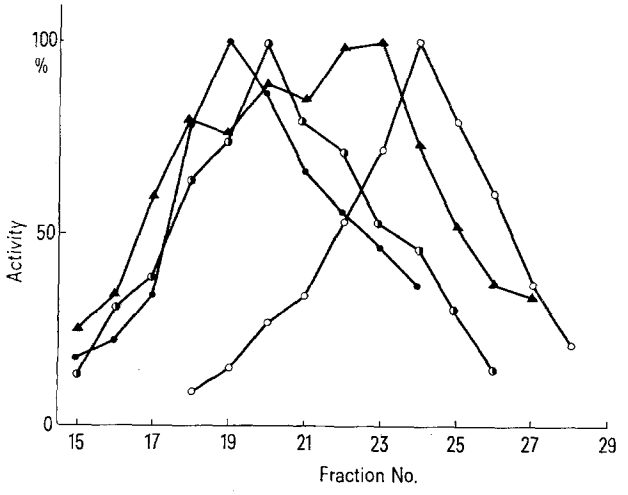

Fig. 2. Separation of activities of Na^+-K^+-ATPase (closed circles) alkaline phosphatase (open circles), Na^+-stimulated NADH-cyt. c reductase (halved circles), and Na^+-insensitive NADH-cyt. c reductase (triangles), following free-flow electrophoresis. Results are the means of 5 experiments, and each point is expressed as a percentage of the highest activity.

Most enzyme studies were performed on the crude fractions obtained in this fashion. For more exact cytological location of the Na^+-stimulated NADH-cyt. c reductase, membrane fractions were prepared from rat kidney cortex by differential centrifugation and applied to a preparative free-flow electrophoresis apparatus, as described earlier[6].

The level of NADH-cyt. c reductase was determined in a recording double-beam spectrophotometer (Perkin-Elmer/Hitachi 124) by measuring the rate of change of extinction at 550 nm. Aliquots of enzyme suspension (protein content, approx. 0.01%) were incubated at 37 °C in the presence of 1 m*M* NADH, 0.1% cytochrome c, 0.33 m*M* KCN and 10 m*M* *Tris*/HCl buffer, pH 7.8 (total volume, 0.5 ml). The NADH was omitted from the blank. Sodium ions (generally 100 m*M*) were added to appropriate samples, and the activity of the sodium-stimulated enzyme determined by the difference in activity in the presence and absence of these ions. ATPase

[5] J. W. L. ROBINSON, V. MIRKOVITCH, J. BORGEAUD and M. CAMPICHE, Biomedicine *18*, 206 (1973).

[6] H.-J. HEIDRICH, R. KINNE, E. KINNE-SAFFRAN and K. HANNIG, J. Cell Biol. *54*, 232 (1972).

Distribution of enzymes, effect of ageing and of deoxycholate in different fractions of dog renal cortex homogenates

	Na^+-K^+-ATPase	Na^+-stimulated NADH-cyt.c reductase	Na^+-independent NADH-cyt.c reductase
Total homogenate			
Without DOC (day 1)	16	46	65
Without DOC (day 3)	24	46	63
With DOC (day 1)	41	56	60
With DOC (day 3)	38	62	72
Nuclear-mitochondrial fraction			
Without DOC (day 1)	23	31	103
Without DOC (day 3)	16	31	93
With DOC (day 1)	42	0	66
With DOC (day 3)	46	0	82
Microsomal fraction			
Without DOC (day 1)	55	101	159
Without DOC (day 3)	47	109	108
With DOC (day 1)	85	120	104
With DOC (day 3)	107	81	99
Supernatant			
Without DOC (day 1)	10	7	21
Without DOC (day 3)	6	5	18
With DOC (day 1)	16	18	23
With DOC (day 3)	9	8	19

Total homogenates of dog renal cortex were prepared in saccharose/*tris*/EDTA with or without 0.1% deoxycholate. The levels of Na^+-K^+-ATPase, NADH-cyt.c reductase, and Na^+-stimulated NADH-cyt·c reductase were determined in nuclear-mitochondrial and microsomal fractions, in supernatant and in the original homogenate. The results are the means of 4 different experiments. The units of Na^+-K^+-ATPase are nmoles of phosphate liberated per min and per mg protein, while the units of the NADH-cyt.c reductases are nmoles of cyt.c oxidised per min and per mg protein (molar coefficient of extinction of cytochrome c oxidation taken as 18.5).

and alkaline phosphatase activities were determined as described previously[5,7].

Results. The basal activity of NADH-cyt. c reductase in dog renal cortex microsomes is about 100 nmoles/mg protein/min and is approximately doubled in the presence of 100 m*M* sodium ions. The stimulation of the enzyme in the presence of different NaCl concentrations is illustrated in Figure 1 where it can be observed that maximal stimulation is attained at a concentration of 75 m*M*. High concentrations of sodium become inhibitory. A similar stimulation is encountered when sodium chloride is replaced with sodium sulphate. Potassium ions have the same effect as sodium, the stimulation by choline chloride is less than that observed with sodium or potassium, but mannitol of the same tonicity has no effect. Magnesium ions, surprisingly, are inhibitory.

Studies with inhibitors, and the electrophoretic data presented below, suggest that at least 2 different enzyme systems are present in this preparation, one which is stimulated by sodium and one which is not. Rotenone only inhibits the enzyme that is insensitive to sodium. Antimycin affects neither enzyme, nor does ouabain. Ethacrynic acid (1 m*M*) had no consistent effect.

The presence of a microsomal Na^+-stimulated NADH-cyt. c reductase appears widespread, since an enzyme with essentially identical properties was located in the kidneys of dogs, rats and guinea-pigs, in the guinea-pig and dog small intestine, and in the dog colonic mucosa. Its presence in the rat liver has been described previously[3].

Various attempts at defining the cytological location of the enzyme were carried out. First, as seen in the Table, it occurs almost exclusively in the microsomal fraction, whereas the Na^+-insensitive homologue occurs both in the nuclear-mitochondrial and the microsomal fractions. Na^+-K^+-ATPase preparations have been shown to be enriched in the presence of deoxycholate and on ageing for 48 h at 0 °C in the presence of the detergent, due probably to opening of closed vesicles[5]. Deoxycholate eliminated all activity of the Na^+-dependent NADH-cyt. c reductase from the nuclear-mitochondrial fraction, though Na^+-K^+-ATPase activity was concomitantly increased in this fraction. Thus, taken together, these findings suggest that the reductase is not located within the same membrane as the Na^+-K^+-ATPase.

Sophisticated separation techniques (free-flow electrophoresis) were applied to crude membrane fractions from rat kidney cortex, and the activity of the enzyme, as well as that of other marker enzymes, was determined in the fractions eluting from the electrophoresis (Figure 2). It was found that the Na^+-stimulated NADH-cyt. c reductase did not follow either the elution pattern of Na^+-K^+-ATPase or that of alkaline phosphatase. Secondly, there was a notable difference in elution pattern of the Na^+-stimulated activity from that of the Na^+-insensitive NADH-cyt. c reductase.

Discussion. The results disclosed in the present survey would appear to preclude the possibility that the Na^+-stimulated NADH-cyt. c reductase activity in microsomal fractions might be involved in membrane sodium transport, and to render unnecessary in the present context a more profound study of the enzyme. Thus it does not seem to be located in either of the plasma membranes of the proximal tubular cell, its stimulation by sodium is not specific for that ion, and it is not affected by ethacrynic acid, which is apparently a specific inhibitor of the ouabain-insensitive sodium pump in this tissue[1,2]. Thus the enzyme fails to fulfil three of the vital requirements for identification with sodium-pumping activity.

The biochemical role of this enzyme (which was discussed previously by SIEKEVITZ[3]) has not been clarified by the present investigations. It is known that the microsomal cytochrome reductases are extremely complex[8-11]. However, it seems that at least the Na^+-stimulated fraction is not bound to either plasma membrane. The possibility remains that it is identical with the rotenone-insensitive, antimycin-insensitive NADH-cyt. c reductase of the golgi apparatus described by FLEISCHER at al.[12] who unfortunately did not test the ion sensitivity of their enzyme[13].

Résumé. En présence de sodium, l'activité totale de la NADH-cytochrome c réductase rénale était doublée; le potassium induit le même effet stimulateur. Cette enzyme sensible aux cations n'est inhibée ni par la roténone ni par l'antimycine. Elle était également insensible à l'ouabaïne et à l'acide éthacrynique. Des expériences destinées à révéler sa localisation cytologique n'ont que partiellement réussi: l'enzyme ne semble pas être associée a la membrane de la bordure en brosse, ni à la membrane péritubulaire. Ces résultats suggèrent qu'elle n'est pas impliquée dans les mécanismes des pompes à sodium.

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21 January 1974.

[7] R. KINNE, J.-E. SCHMITZ and E. KINNE-SAFFRAN, Pflügers Arch. Eur. J. Physiol. *329*, 191 (1971).
[8] H.-U. SCHULZE, H. GALLENKAMP and HJ. STAUDINGER, Hoppe-Seyler's Z. physiol. Chem. *351*, 809 (1970).
[9] T. HARA and S. MINAKAMI, J. Biochem. *69*, 317 (1971).
[10] K. MIHARA and R. SATO, J. Biochem. *71*, 725 (1972).
[11] K. STAROŃ and Z. KANIUGA, Hoppe-Seyler's Z. physiol. Chem. *353*, 14 (1972).
[12] B. FLEISCHER, S. FLEISCHER and H. OZAWA, J. Cell Biol. *43*, 59 (1969).
[13] This work was supported by the Fonds National Suisse and the Deutsche Forschungsgemeinschaft. We are grateful to Mlles H. ASPER and D. METTRAUX in Lausanne and Frau R. PLATE in Frankfurt for their excellent technical assistance.

Young and Old Rats. ATP, Alkaline Phosphatase, Cholesterol and Protein Levels in the Blood; DNA and RNA Contents of the Liver. Regulation by an Aqueous Thymus Extract

Various authors have attributed to the thymus an important role in the physiology of development[1]. In addition, since the involution of the thymus also entails a general decline of immunocompetence, and different authors[2-4] have suggested that aging could depend on an immunological disorder, PANTALOURIS[5] has postulated the hypothesis of a direct correlation between the thymus and senescence.