adherent medullary tissue. By inspection, less than 10% of the weight of the central vein pieces was made up of medullary tissue. Therefore, unless there is a gradient of renin-like enzyme in the medulla, such that most of the medullary enzyme is adjacent to the central vein, it would appear that central vein itself contains relatively large quantities of renin-like activity.

These data show that the bovine adrenal gland contains an enzyme that is similar to renin in all respects tested. The enzyme does not pass through dialysis membranes and does not have an absolute requirement for those metals chelated by EDTA and dimercaprol[7-9]. The enzyme is stable for several hours at pH 2.6, 4°C. It reacts with rabbit renin substrate to form a product capable of causing a transient rise of mean arterial blood pressure. No product is formed in the absence of substrate, and no product is formed if either the enzyme or substrate is heated in a boiling water bath before preparing the reaction mixture. The reaction product is stable in boiling water but is inactivated by trypsin and chymotrypsin. The adrenal renin-like enzyme is distinguished from pseudorenin in that the adrenal enzyme is reactive with native renin substrate in the presence of other plasma components[11].

The apparent wide distribution in bovine adrenal gland of an enzyme capable of releasing angiotensin is consistent with the concept that the intraglandular release of angiotensin could regulate some adrenal secretions. As shown by LARAGH, et al.[3] and FELDBERG and LEWIS[4], angiotensin II, in low concentrations, stimulates the release of medullary catecholamines and aldosterone. Higher concentrations stimulate the secretion of corticosterone[12]. Although renin-like enzymes occur in several other tissues, in no other organ or gland are there more clearly demonstrated effects of angiotensin. However, there is, as yet, no evidence that the adrenal renin-like enzyme has access to and reacts with renin substrate in vivo.

Résumé. La glande surrénale bovine contient un enzyme qui réagit avec l'angiotensinogène pour former de l'angiotensine. L'enzyme est distribué dans toute la glande surrénale, en plus grande quantité dans la médullosurrénale et dans la veine centrale. Vu les effects bien connus de l'angiotensine sur la sécrétion de l'aldostérone et sur les catécholamines de la médullosurrénale, il est possible que l'enzyme «rénine-semblable» influence les fonctions spécifiques de la glande surrénale.

J. W. RYAN

University of Miami, Department of Medicine, Biscayne Annex, P.O. Box 875, Miami (Florida 33152, USA), 28. August 1972.

[11] L. T. SKEGGS, K. E. LENTZ, J. R. KAHN, F. E. DORER and M. LEVINE, Circulation Res. *25*, 451 (1969).
[12] F. C. BARTTER, A. G. T. CASPER, C. S. DELEA and J. D. H. SLATER, Metabolism *10*, 1006 (1961).
[13] Supported in part by a grant (No. HL 14360) from the U.S. Public Health Service.

The Reaction of Ca^{++} with the Inner and Outer Membrane of Mitochondria

In addition to the energy-linked transport of Ca^{++}[1], and to the metabolism independent Ca^{++} binding[2], another type of Ca^{++} binding has been described in isolated mitochondria[3,4]. In this reaction, small amounts of Ca^{++} are bound with very high affinity to specific sites in the mitochondrial membranes. Scatchard plots of this type of binding have led REYNAFARJE and LEHNINGER[3] to postulate carrier molecules specific for Ca^{++}, a conclusion also reached by MELA and CHANCE[5,6]. The role of the high affinity binding in the active translocation of Ca^{++} has been discussed[3,7-9], and the two processes go parallel in some mitochondrial and submitochondrial preparations[3]. It was thought that information on this problem could be obtained from a study of the distribution of the Ca^{++} binding sites between the inner (IM) and the outer (OM) mitochondrial membrane. Indeed, the latter does not participate in the energy-linked transport of Ca^{++}; if the high affinity sites play any role in the active translocation, they should be absent from the OM.

Materials and methods. Mitochondria were prepared from rat livers by the standard sucrose procedure of SCHNEIDER[10]. The 2 membranes were separated according to SCHNAITMAN and GREENAWALT[11]. Malic dehydrogenase and monoamine oxidase were determined as described by SCHNAITMAN et al.[12], but the temperature of the monoamine oxidase medium was 21°C instead of 37°C. Cytochrome oxidase was determined polarographically with a Clark electrode, in a medium containing 0.04 *M* phosphate buffer, pH 7.4, 0.0004 *M* $AlCl_3$, 0.015 *M* Na-ascorbate, 0.0001 *M* cytochrome C, 1 mg lubrol, and 0.05–2 mg of enzyme protein. Volume, 2 ml, temperature, 25°C. Respiratory control by ADP or by

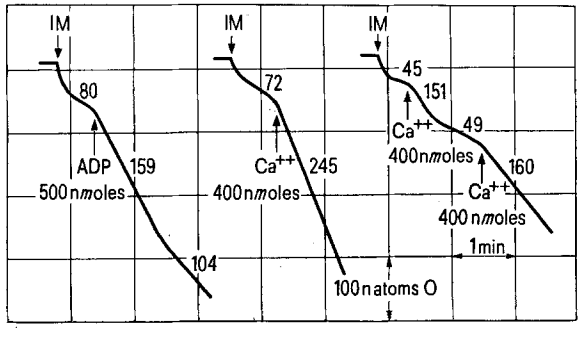

Fig. 1. Stimulation of the respiration of the inner membrane plus matrix fraction by ADP and by Ca^{++}. Technical details are described in Methods. Temperature 25°C. Final volume 2 ml. 1st and 2nd trace from the left: inorganic phosphate present. 3rd trace from the left: inorganic phosphate absent.

[1] A. L. LEHNINGER, E. CARAFOLI and C. S. ROSSI, Adv. Enzymol. *29*, 259 (1967).
[2] C. ROSSI, A. AZZI and G. F. AZZONE, J. biol. Chem. *242*, 951 (1967).
[3] B. REYNAFARJE and A. L. LEHNINGER, J. biol. Chem. *244*, 584 (1969).
[4] E. CARAFOLI and A. L. LEHNINGER, Biochem. J. *122*, 681 (1971).
[5] L. MELA, Arch. Biochem. Biophys. *123*, 286 (1968).
[6] L. MELA and B. CHANCE, Biochem. biophys. Res. Commun. *35*, 556 (1969).
[7] A. L. LEHNINGER and E. CARAFOLI, in *Biochemistry of the Phagocytic Process* (Ed. J. SCHULZ; North Holland, Amsterdam 1969), p. 9.
[8] E. CARAFOLI, Biochem. J. *116*, 2 (1969).
[9] E. CARAFOLI and C. S. ROSSI, Adv. Cytopharmac. *1*, 209 (1971).

Ca^{++} was followed polarographically in the medium described by SCHNAITMAN and GREENAWALT[12], without EDTA and without $MgCl_2$. Succinate was the respiratory substrate. $^{45}CaCl_2$ uptake was determined with Millipore filtration. High and low affinity Ca^{++} bindings were measured as described by REYNAFARJE and LEHNINGER[3], in a total volume of 1 ml.

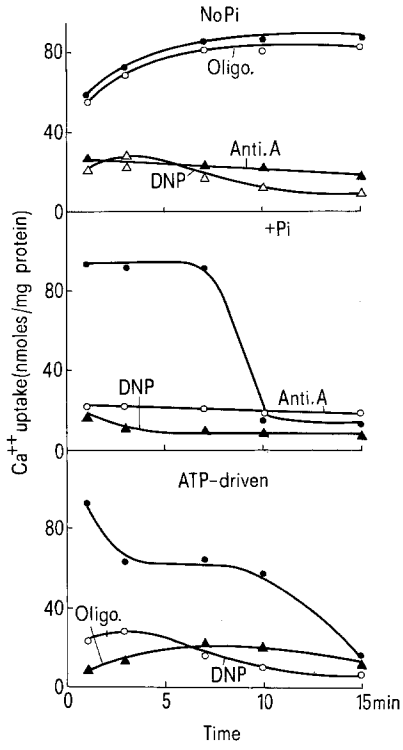

Fig. 2. Respiration driven and ATP driven Ca^{++} uptake by the inner membrane plus matrix fraction. Technical details in Methods. Concentrations: DNP, 0.0001 M; antimycin A, 0.2 µg per mg of protein; enzyme protein, 20 mg. Temperature 25 °C. In the upper and middle graph the uptake was energized by respiration, in the lower by ATP. In the ATP-driven system, no respiratory substrate was added. 0.0005 M ATP and 0.2 µg antimicyn A per mg of enzyme protein were present.

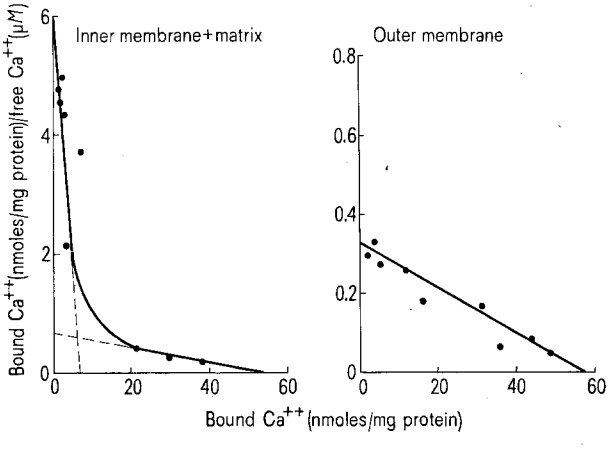

Fig. 3. Scatchard plots of high and low affinity Ca^{++} binding by the inner and outer membranes of mitochondria. 1 ml total volume, containing 2.5 mg enzyme protein. 1 min incubation, at 0 °C. Other technical details in Methods.

Results and discussion. The specific activities of cytochrome oxidase, malic dehydrogenase, and monoamine oxidase indicated a good separation of the 2 membranes. In the electron microscope, the IM fraction consisted of closed sacs containing a dense matrix material, while most of the OM fraction consisted of empty vesicles and non-vesicular fragments. After negative staining, both profiles of these non-vesicular fragments appeared smooth, ruling out the IM as their possible source.

As already shown by SCHNAITMAN and GREENAWALT[11], the respiration of the IM (plus matrix) fraction was increased reversibly by ADP. Figure 1 shows that respiratory stimulation was induced also by Ca^{++}. The stimulation was reversible in the absence of inorganic phosphate, and irreversible in its presence. The presence of energy-linked transport of Ca^{++} in the IM fraction was confirmed directly by the Millipore filtration experiment shown in Figure 2. The uptake could be supported by either respiration or ATP, and the sensitivity to inhibitors was as expected. In this respect, the IM (plus matrix) fraction differs from the purified IM vesicles in which PEDERSEN and COTY[13] could not find ATP-supported uptake of Ca^{2+}. As expected from the results obtained in intact mitochondria[14], Ca^{++} was maintained in the IM from shorter times in the presence of inorganic phosphate. No active transport of Ca^{++} was found on the other hand in the OM fraction.

Scatchard plots of high and low affinity Ca^{++} binding were biphasic in the IM, and rectilinear in the outer (Figure 3). Thus, both membranes possessed the low-affinity Ca^{++} binding sites (50–60 per mg of protein in both membranes), but the affinity of these sites for Ca^{++} was slighthly lower in the OM (K_d 100–150 µM versus 50–80 µM in the IM). The high-affinity sites, on the other hand, are present only in the IM. Their number (4–7 mg protein) and their affinity for Ca^{++} (K_d, 1 µM) is about the same as in intact mitochondria. The presence of the high affinity sites in the inner mitochondrial membrane, across which the energy-linked transport of Ca^{++} takes place, and their absence from the outer membrane, which has no active transport capabilities, provide additional evidence for their involvement in the energy-linked transport of Ca^{++}[15].

Riassunto. È stata studiata la distribuzione del trasporto attivo del Ca^{++} e dei siti ad alta e bassa affinità per il Ca^{++} tra la membrana esterna e interna dei mitocondri di fegato. La membrana interna trasporta attivamente il Ca^{++}, la membrana esterna non è in grado di farlo. Ambedue le membrane posseggono i siti a bassa affinità, mentre i siti ad alta affinità si trovano solo sulla membrana interna.

E. CARAFOLI and P. GAZZOTTI

Istituto di Patalogia Generale dell' Università, Via Campi 287, I-41100 Modena (Italy),
20 September 1972.

[10] W. C. SCHNEIDER, in *Manometric Techniques* (Eds. W. W. UMBREIT, R. BURRIS and J. E. STAUFFER; Burgess, Minneapolis 1956), p. 188.
[11] C. SCHNAITMAN and J. W. GREENAWALT, J. Cell Biol. *38*, 158 (1968).
[12] C. SCHNAITMAN, V. S. ERWIN and J. W. GREENAWALT, J. Cell Biol. *32*, 719 (1967).
[13] P. L. PEDERSEN and W. L. COTY, J. biol. Chem. *247*, 3107 (1972).
[14] C. S. ROSSI and A. L. LEHNINGER, J. biol. Chem. *239*, 3971 (1964).
[15] Acknowledgments. The research was supported by the National Research Council of Italy.