amount of the enzyme. Incubation mixture (310 µl) contained: enzyme, 100 µl; water 100 µl; 1 *M Tris*-HCl buffer, pH 6.0 10 µl; 40 m*M* ascorbic acid in the *Tris* buffer, 30 µl; 40 m*M* sodium fumarate, 30 µl; 6 m*M* pargyline, 10 µl; 1 m*M* $CuSO_4$, 10 µl; 30 m*M* tyramine, 10 µl; and catalase (5 mg crystals in 2 ml of water), 10 µl. Incubation was carried out at 37 °C for 20 min. After the incubation the following reaction mixtures were added: 1 *M Tris*-HCl buffer, pH 8.6, 80 µl; 0.5 m*M* EDTA, 20 µl; phenylethanolamine-N-methyltransferase partially purified from bovine adrenal medulla by the method of CONNETT and KIRSHNER[13] (0.17 nmoles/min/mg protein, 12 mg/ml), 10 µl; S-adenosylmethionine-(methyl-C^{14}), 10 µl (0.1 µCi, 1.8 nmoles). The incubation was continued further for 30 min at 37 °C. The radioactive N-methyl-octopamine formed was extracted into toluene-isoamyl alcohol (3:2, v/v) and counted. Boiled enzyme (95°C, 5 min) was used for the blank incubation. Octopamine, 0.4 nmole, was added into a reaction mixture as an internal standard.

The results are shown in the Table. Serum dopamine-β-hydroxylase activities of male or female SH rats were slightly lower than those of normotensive male or female Wistar rats, but the differences were not statistically significant. The enzyme activities of female rats of normotensive Wistar or SH strains were slightly lower than those of male rats of normotensive Wistar or SH strains, but the differences were not statistically significant. The results suggest that the increase in dopamine-β-hydroxylase in adrenal glands of SH rats[7] does not cause the elevation of the level of the serum enzyme and that the rate of secretion of the enzyme from the peripheral sympathetic nerves of SH rats may not be different from that of normotensive Wistar rats[14].

Serum dopamine-β-hydroxylase activity of normotensive wistar rats and spontaneously hypertensive rats

Enzyme activity	Normotensive wistar rats	Spontaneously hypertensive rats
Male (numbers)	(5)	(9)
*p*moles/min/ml serum±S.E.M.	76 ±17	52 ±12
*p*moles/min/mg protein±S.E.M.	1.7± 0.3	1.1± 0.3
Female (numbers)	(5)	(8)
*p*moles/min/ml serum±S.E.M.	58 ±22	47 ± 9
*p*moles/min/mg protein±S.E.M.	1.0± 0.4	1.0± 0.2

Zusammenfassung. Es wird gezeigt, dass die Dopamin-β-Hydroxylase-Aktivität im Serum bei spontan hypertonischen Ratten im Vergleich zu normotonen Kontrollen nicht verändert ist.

T. NAGATSU, T. KATO, H. KUZUYA, H. UMEZAWA[15], M. MATSUZAKI[15] and T. TAKEUCHI[15]

Department of Biochemistry, School of Dentistry, Aichi-Gakuin University, 2-11 Suemori-dori, Chikusa-ku, Nagoya 464 (Japan); and Institute of Microbial Chemistry, Shinagawa-ku, Tokyo (Japan), 14 January 1972.

1 K. OKAMOTO and K. AOKI, Jap. Circulation J. *27*, 282 (1963).
2 H. UMEZAWA, T. TAKEUCHI, H. IINUMA, K. SUZUKI, M. ITO, M. MATSUZAKI, T. NAGATSU and O. TANABE, J. Antibiotics *23*, 514 (1970).
3 M. OHNO, M. OKAMOTO, N. KAWABE, H. UMEZAWA, T. TAKEUCHI, H. IINUMA and S. TAKAHASHI, J. Am. chem. Soc. *93*, 1285 (1971).
4 T. NAGATSU, I. NAGATSU, H. UMEZAWA and T. TAKEUCHI, Biochem. Pharmac. *20*, 2505 (1971).
5 H. HIDAKA, T. NAGATSU, K. TAKEYA, T. TAKEUCHI, H. SUDA, K. KOJIRI, M. MATSUZAKI and H. UMEZAWA, J. Antibiotics *22*, 228 (1966).
6 T. NAGATSU, H. HIDAKA, H. KUZUYA, K. TAKEYA, H. UMEZAWA, T. TAKEUCHI and H. SUDA, Biochem. Pharmac. *19*, 35 (1970).
7 I. NAGATSU, T. NAGATSU, K. MIZUTANI, H. UMEZAWA, M. MATSUZAKI and T. TAKEUCHI, Nature, Lond. *230*, 381 (1971).
8 I. NAGATSU, T. NAGATSU, K. MIZUTANI, H. UMEZAWA, M. MATSUZAKI and T. TAKEUCHI, Experientia *27*, 1013 (1971).
9 J. TARVER, B. BERKOWITZ and S. SPECTOR, Nature, Lond. *231*, 252 (1971).
10 Y. YAMORI, W. LOVENBERG and A. SJOERDSMA, Science *170*, 544 (1970).
11 R. WEINSHILBOUM and J. AXELROD, Circulation Res. *28*, 307 (1971).
12 M. GOLDSTEIN, L. S. FREEDMAN and M. BONNAY, Experientia *27*, 632 (1971).
13 R. J. CONNETT and N. KIRSHNER, J. biol. Chem. *245*, 329 (1970).
14 We thank Dr. K. OKAMOTO, School of Medicine, University of Kyoto, for spontaneously hypertensive rats and Dr. J. AXELROD, National Institute of Mental Health, Bethesda, for his personal information on the assay of serum dopamine-β-hydroxylase. We also wish to acknowledge the assistance of Miss Y. SUDO with the preparation of phenylethanolamine-N-methyltransferase and the technical assistance of Miss Y. NISHIKAWA and Miss Y. SHIBAHARA.
15 Institute of Microbial Chemistry, Shinagawa-ku, Tokyo (Japan.)

The Affinity of Mitochondria for Ca^{++}

It has been shown that the transport of Ca^{++} and the phosphorylation of ADP in mitochondria are alternative processes[1]. It is thus reasonable to suggest that the former may be a means of regulating the concentration of Ca^{++} in the cytosol, and affecting a number of Ca^{++} dependent reactions. Among these, the contraction and relaxation of heart is particularly important. Indeed, whereas the role of the sarcoplasmic reticulum (SR) in the contraction and relaxation of fast skeletal muscle seems well established[2-4], several lines of evidence point to its inefficiency in cardiac muscle, and indicate that mitochondria could play a role in the process. In heart, SR is poorly developed, and has a very limited capability for Ca^{++} transport[5].

Mitochondria, on the other hand, are very abundant, and are capable of efficient Ca^{++} transport both in vitro[6-8] and in vivo[9]. An important factor in assessing their possible role in the relaxation and contraction of heart is their affinity for Ca^{++}. Since contraction begins when the free Ca^{++} concentration in the sarcoplasm approaches $10^{-7}M$, mitochondria must be able to take up Ca^{++} with reasonable efficiency at concentrations not far removed from this level. Most measurements of the mitochondrial K_m for Ca^{++}, based on the shift of the redox state of cytochrome b, or on the rate of O_2 consumption, have, however, yielded figures around $5 \times 10^{-5}M$[10], clearly inconsistent with a role in heart relaxation. However, since the uptake of Ca^{++} by mitochondria is

very rapid, at very low Ca^{++} concentrations the changes in the parameters considered are very difficult to evaluate. In this study, mitochondria have been treated with Ca-EGTA (ethylene glycol-*bis* aminoethyl tetra-acetic acid) buffers, and thus with stabilized, low free Ca^{++} concentrations[11]. It has thus been possible to establish an apparent K_m for Ca^{++} close to $10^{-6}M$.

Methods. Rat liver mitochandria were prepared with a 0,25 *M* sucrose procedure, rabbit heart mitochondria as indicated by SORDAHL and SCHWARTZ[12], and pigeon heart mitochondria according to CHANCE and HAGIHARA[13]. Energy-linked uptake of Ca^{++} was studied in the medium specified in the legends to the Figures. The concentration of mitochondrial protein, in a final volume of 2 ml, at room temperature, was 6 mg. The uptake of Ca^{++} was indicated by the shift of the redox state of cytochrome b in a dual wavelength spectophotometer, at 430 minus 410 nm. The Ca^{++} EGTA buffers were prepared as indicated by PORTZEHL et al. [11]. The protein was measured with a biuret reaction.

Results and discussion. When succinate is added to a suspension of rat liver mitochondria in the presence of rotenone, a reduction of cytochromes occurs, which is maintained while the substrate is present. The new steady state of cytochrome b depends on the rate at which electrons arrive and depart from the cytochrome, and it is also under the control of the second coupling site, which slows down the flow of electrons between cytochromes b and c. When the effect of the second coupling site is decreased by the addition of ADP, uncouplers, or Ca^{++}, an oxidation of cytochrome b and a reduction of cytochrome c can be observed. In Figure 1, a reduction of cytochrome b in the Soret region was induced by succinate in about 20 sec. At this point, the Ca-EGTA buffer was added (final free Ca^{++} concentration of 1 μM, and a rapid reoxidation of cytochrome b followed.

In Figure 2, the logarithm of the molar concentration of calcium added to rat liver mitochondria is plotted against the Ca^{++} induced absorbance change of cytochrome b. The concentration which induced half maximal oxidation of cytochrome b was between 2 and 3 μM. This figure is several times lower than those previously obtained 1. from measurements of the oxygen consumption, 2. with the murexide technique, or 3. with the cytochrome b technique based on additions of Ca^{++} without EGTA. Rabbit heart mitochondria behaved very similarly: half-maximal effects were obtained at about the same concentrations of Ca^{++} as in liver mitochondria.

It is thus clear that mitochondria transport Ca^{++} optimally at the concentrations where relaxation occurs. With pigeon heart mitochondria, half maximal stimulation was obtained with 20 μM Ca^{++}. This difference could be the consequence of some non-specific damage to the Ca^{++} transporting system, since pigeon heart mitochondria were prepared with the proteinase method; evidence has been provided[14] that the proteinase could damage the Ca^{++} transporting system.

A calculation can now be made of the total amount of Ca^{++} that must be removed from troponin (the receptor protein for Ca^{++} in muscle[15]), during relaxation. Cardiac troponin binds about 20 nMoles of Ca^{++} per mg[15,16] and there are about 3 mg of troponin per g of muscle in heart. A total of about 60 nMoles of Ca^{++} must therefore be removed from troponin as the heart relaxes after each contraction[16]. Calculations of LANGER[17] have shown that relaxation lasts not less than 200 msec; we have calculated the initial velocity of Ca^{++} uptake by heart mitochondria (i.e. the rate of uptake during the first 10 sec) from media containing free Ca^{++} concentrations increasing from 10^{-6} to $10^{-4}M$, and have extrapolated values of at least 1–1.5

[1] C. S. ROSSI and A. L. LEHNINGER, J. biol. Chem. *239*, 397 (1969).
[2] S. EBASHI, Progr. theor. Physics, Suppl. *17*, 35 (1961).
[3] W. HASSELBACH and M. MAKINOSE, Biochem. Z. *333*, 518 (1961).
[4] A. WEBER, R. HERZ and I. REISS, J. gen. Physiol. *46*, 679 (1963).
[5] J. GERGELY, D. PRAGAY, A. F. SCHOLZ, J. C. SEIDEL, F. A. SRETER and M. M. THOMPSON, *Molecular Biology of Muscle Contraction*, BBA Library, (Elsevier, Amsterdam 1965), vol. 9, p. 145.
[6] G. P. BRIERLEY, E. MURER, E. BACHMANN and D. E. GREEN, Arch. Biochem. Biophys. *105*, 85 (1969).
[7] M. C. O. FEHMERS, Ph. D. Dissertation, Univ. of Amsterdam (1968).
[8] N. HAUGAARD, E. S. HAUGAARD, N. H. LEE and R. S. HORN, Fedn Proc. *28*, 1657 (1969).
[9] P. PATRIARCA and E. CARAFOLI, J. Cell Physiol. *72*, 29 (1968).
[10] B. CHANCE, *Energy-linked Functions of Mitochondria* (Ed. B. CHANCE; Academic Press, New York 1963), p. 253.
[11] H. PORTZEHL, P. C. CALDWELL and J. C. RÜEGG., Biochim. biophys. Acta *79*, 581 (1964).
[12] L. SORDAHL and A. SCHWARTZ, Molec. Pharmac. *3*, 509 (1967).
[13] B. CHANCE and B. HAGIHARA, in Vth *Int. Congress of Biochem.*, Moscow 1961, (Ed. A. N. M. SISSAKIAN, Pergamon Press, New York 1963), vol. 5, p. 3.
[14] C. H. CHEN, E. CARAFOLI and A. L. LEHNINGER, in preparation.
[15] S. EBASHI, A. KODAMA and F. EBASHI, J. Biochem. *64*, 465 (1968).
[16] S. HARIGAYA and A. SCHWARTZ, Circulation Res. *25*, 781 (1969).
[17] G. A. LANGER, Physiol. Rev., *48*, 708 (1968).

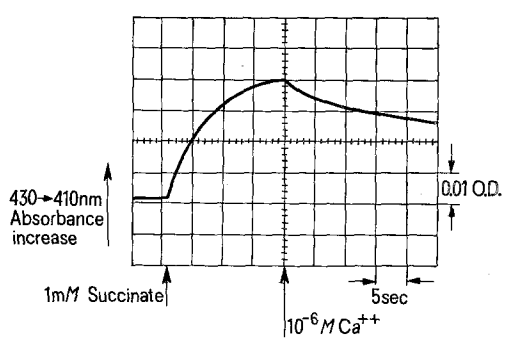

Fig. 1. Kinetics of cytochrome b changes in rat liver mitochondria on addition of Ca^{++}. Cytochrome b was measured in the double-beam spectrophotometer at 430–410 nm. The incubation mixture contained: 0.3 *M* mannitol-sucrose, 0.005 *M* *Tris*-HCl pH 7.4, 1 μM rotenone, 3 mg protein per ml. After succinate addition, 3 m*M* Ca-EGTA (0.87:1) complex was added to give a final free Ca^{++} concentration of $10^{-6}M$.

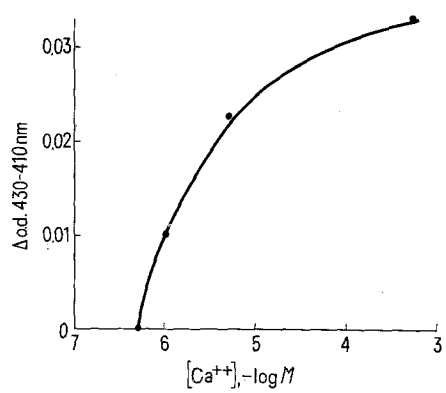

Fig. 2. Plot of cytochrome b changes in rat liver mitochondria as a function of Ca^{++} concentrations. Conditions as in Figure 1. The ratio Ca^{++}: EGTA varied according to PORTZEHL et al.[11] to give the final concentrations indicated.

nMoles of Ca^{++} bound per mg of mitochondrial protein per 200 msec, at $5 \times 10^{-7} M$ Ca^{++}. Estimates of the total mitochondrial content of cardiac muscle[9] indicate values of 30–40 mg of protein. As many as 60 nMoles of Ca^{++} could thus be removed by mitochondria from troponin in 200 msec at room temperature, and this limit would certainly be higher at 37 °C. The rate of Ca^{++} uptake by mitochondria is thus adequate to explain relaxation.

Riassunto. E' stata studiata l'affinità dei mitocondri di fegato di ratto e di cuore di coniglio per il Ca^{++}, nel sistema dipendente da energia. Si sono usati tamponi EGTA-Ca^{++} per mantenere la concentrazione del Ca^{++} costante durante l'esperimento. Si è dimostrato che la K_m apparente per il Ca^{++} non è lontana da $10^{-6} M$.

E. CARAFOLI[18,19] A. AZZI[20]

Johnson Research Foundation, University of Pennsylvania School of Medicine, Philadelphia (Pennsylvania, USA), 4 January 1972.

[18] We are indebted to Dr. B. CHANCE for many stimulating discussions and for facilitating the progress of the work with the generous hospitality in his Institute.

[19] Present address: Institute of General Pathology, University of Modena, Via Campi 287, I-41100 Modena (Italy).

[20] Present address: Institute of General Pathology, University of Padova, Italy.

Muscle Afferent Outflow During Ethanol Intoxication

KUCERA and SMITH[1] have reported that the addition of ethanol into a Krebs' solution bath surrounding the rat caudal muscle in situ caused a concentration-dependent increase in the activity of afferent nerves from sensory endings in the muscle spindles and tendon organs. The authors suggested that alterations in muscle afferent outflow might be involved in the motor incoordination of ethanol intoxication.

The aim of the present investigation was to examine whether disturbances in the limb muscle spindle afferent function are encountered in vivo following systemic administration of an intoxicating dose of ethanol.

Materials and methods. Male Wistar rats, 250–300 g body weight, were anesthetized by a commercial (Ciba) mixture of allobarbital (8 mg/100 g), urethane (32 mg/100 g) and monoethyl urea (32 mg/100 g) injected i.p. After cannulation of the trachea, a laminectomy exposed the spinal cord from segment L_3 to S_1. A pool containing the exposed spinal cord was filled with mineral oil at 36°C, using the technique described by GLADDEN and KIDD[2]. A hind limb was denervated except for the nerves to the gastrocnemius-soleus complex; the muscles could be stretched to various degrees by weights hung from the toes. The dorsal root L_5 was teased until afferent discharges were obtained from a single muscle spindle stretch receptor. Only afferents exhibiting a sustained discharge in response to loading the Achilles tendon were considered.

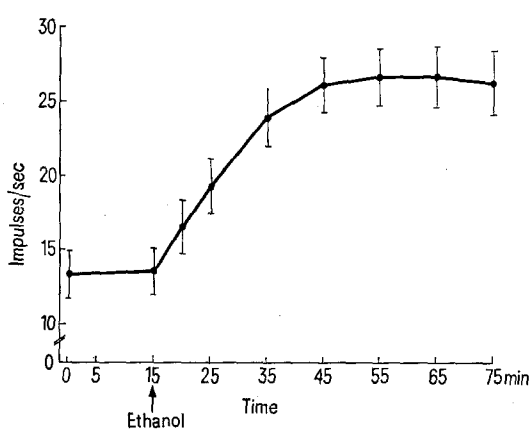

Fig. 1. The effect of i.p. injection of ethanol (2.5 g/kg) on the frequency of firing of 15 muscle spindle afferents. Ordinate: mean frequency of discharge in impulses/sec; brackets encompass ± 1 standard error of mean. Abscissa: time in min.

The afferent activity was measured as described by KUCERA and SMITH[1]. The mean carotid artery pressure was recorded throughout all experiments, using a Statham pressure transducer connected to a polygraph.

Ethanol (Gold Shield absolute alcohol) was given by i.p. injection as a 15% (w/v) solution in isotonic saline in volumes required to provide a dose of 2.5 g/kg body wt. In 3 control experiments, the animals were injected with 1.7 ml/100 g body wt. of a 0.9% sodium chloride solution.

In a parallel study, 15 rats anesthetized as described above were used to determine the rate of appearance of ethanol in blood after the i.p. administration. A cannula was inserted into the central end of the femoral vein and heparin (500 IU/kg) was given. Venous blood samples (0.3 ml) were collected 10, 30, and 60 min after the i.p. injection of ethanol (2.5 g/kg). The plasma samples were analyzed for ethanol content using a Perkin Elmer Model F-11 gas chromatograph fitted with a hydrogen flame ionization detector and a 7% Hallcomid on Porapak Q column.

Results and discussion. The effects of ethanol were studied using 15 muscle spindle afferent fibers from acutely de-efferented muscles. Routinely, the muscle was stretched to obtain an initial discharge rate of 5–20 imp/sec. The i.p. injection of ethanol provoked an acceleration in the activity of afferent nerves originating in muscle spindles; a decrease in the discharge rate was never observed. A noticeable rise in the rate of firing appeared typically as soon as 5 min after the ethanol injection; the firing rate approximately doubled within 60 min. The acceleration was most pronounced within the first 30 min; the response then leveled off and the sensory activity remained fairly constant for the next 30 min. However, the effects of ethanol were not followed longer than 60 min. Figure 1 illustrates the mean time course for excitation by ethanol of 15 spindle afferents; Figure 2 presents oscillo-

[1] J. KUCERA and C. M. SMITH, J. Pharmac. exp. Ther. *179*, 301 (1971).

[2] M. H. GLADDEN and G. L. KIDD, J. appl. Physiol. *26*, 501 (1969).