of the skeletal muscle strongly increased in the same exercised mice[9]. The lack of the hydrolytic response in cardiac muscle after exercise may indicate that mouse cardiac muscle has an inherent protective mechanism against overstrain. Such a mechanism can be induced in skeletal muscle by endurance training[18,19]. Species variation also influences the susceptibility of myocytes to cell damage[20]. Acid proteolytic capacity increases in cardiac muscle during ageing[21], starvation[22] or insulin deprivation[23].

In addition to lysosomal proteolytic enzymes there are also neutral and alkaline proteases[24] and protease inhibitors[25] in cardiac muscle. The activities of these nonlysosomal proteases have been found to increase very considerably during ageing[26], starvation[22,26], and some other conditions causing cardiac atrophy[26]. In mouse cardiac muscle the activities of alkaline protease, neutral autolysis and trypsin inhibitor were unaffected by strenuous exercise (table) as also in skeletal muscle[9]. Problems associated with inhibitors may, however, disturb the analysis of these nonlysosomal proteases[25].

In spite of some ultrastructurally similar alterations in reversibly damaged rat cardiac and skeletal muscle fibres[5-7] there seems to be no distinct enzymatic response to strenuous exercise in mouse cardiac muscle. This may be due to different responses between the lysosomal systems of cardiac and skeletal muscles or to species differences in the properties of the cardiac muscle.

1 Supported by grants (Nos 8840/78/77 and 8208/78/78) from the Ministry of Education and the Academy of Finland.

2 R.B. Jennings, Acta med. scand. *587*, suppl., 83 (1976).
3 R.B. Jennings and C.E. Ganote, Circulation Res. *38*, suppl., 80 (1976).
4 S. Patterson and L. Klenerman, J. Bone Joint Surg. *61B*, 178 (1979).
5 R. Paniagua, L. Ceballos and J.J. Vazquez, An. Anat. *26*, 325 (1977).
6 E.W. Banister, R.J. Tomanek and N. Cvorkov, Am. J. Physiol. *220*, 1935 (1971).
7 P.D. Gollnick and D.W. King, Am. J. Physiol. *216*, 1502 (1969).
8 V. Vihko, J. Rantamäki and A. Salminen, Histochemistry *57*, 237 (1978).
9 A. Salminen and V. Vihko, unpublished observations.
10 N.N. Kipshidze, in: Prevention of Ischemic Heart Disease, p. 67. Ed. W. Raab. Charles C. Thomas, Springfield 1966.
11 A.U. Arstila, P. Hirsimäki and B.F. Trump, Beitr. path. Anat. *152*, 211 (1974).
12 V. Vihko, A. Salminen and J. Rantamäki, Pflügers Arch. *378*, 99 (1978).
13 A.D. Shannon and F.C. Courtice, Austr. J. exp. Biol. Med. Sci. *52*, 157 (1974).
14 R.J.T. Pennington, in: Proteinases in Mammalian Cells and Tissues, p. 515. Ed. A.J. Barrett. Elsevier, Amsterdam 1977.
15 T. Noguchi, E. Miyazawa and M. Kametaka, Agr. Biol. Chem. *38*, 253 (1974).
16 W.T. Stauber, A.-M. Hedge and B.A. Schottelius, Life Sci. *18*, 1441 (1976).
17 A. Okitani, Y. Otsuka, M. Sugitani and M. Fujimaki, Agr. Biol. Chem. *38*, 573 (1974).
18 V. Vihko, A. Salminen and J. Rantamäki, J. appl. Physiol. *47*, 43 (1979).
19 B. Highman and P.D. Altland, Am. J. Physiol. *205*, 162 (1963).
20 D.J. Hearse, S.M. Humphrey, D. Feuvray and J. DeLeiris, J. molec. cell. Cardiol. *8*, 759 (1976).
21 K. Wildenthal, R.S. Decker, A.R. Poole and J.T. Dingle, J. molec. cell. Cardiol. *9*, 859 (1977).
22 A.L.N. Smith, Cytobios *18*, 111 (1977).
23 K. Wildenthal, Circulation Res. *39*, 441 (1976).
24 A.L. Smith and J.W.C. Bird, J. molec. cell. Cardiol. *7*, 39 (1975).
25 L. Waxman and E.G. Krebs, J. biol. Chem. *253*, 5888 (1978).
26 W.S.T. Griffin and K. Wildenthal, J. molec. cell. Cardiol. *10*, 669 (1978).

Alpha- and beta-adrenergic receptors in rat myocardium membranes after prolonged ethanol inhalation[1]

D. Sabourault, Françoise Bauché, Y. Giudicelli, J. Nordmann and R. Nordmann

Laboratoire de Biochimie de la Faculté de Médecine de Paris-Ouest et Groupe de Recherches (U 72) de l'INSERM, 45 rue des Saints-Pères, F-75006 Paris (France), 3 July 1980

Summary. After 3 weeks of continuous ethanol intoxication by inhalation, the maximal number and affinity of the α- and β-receptors of rat heart were unchanged. These data indicate that adrenergic receptor disturbances are not involved in the mechanism of chronic ethanol-induced triglyceride deposition in the heart.

Tremor, agitation, tachycardia and hypertension are characteristic features frequently observed after the discontinuation of long-term heavy ethanol consumption[2,3]. Similar symptoms also occur in hyperadrenergic states such as thyrotoxicosis[4], and propranolol, a β-adrenergic blocker, has been successfully used to control some of the clinical signs of ethanol withdrawal such as hypertension and tremor[5]. This led to the suggestion that the peripheral manifestations of increased adrenergic activity in ethanol withdrawal may be the result of increased β-adrenergic sensitivity[6].

In a recent study, Banerjee et al.[7] reported that the β-receptor density in rat brain membranes decreased after chronic ethanol-treatment and conversely increased during the withdrawal state. In addition, the same authors presented some partial data suggesting that the same alterations may affect the cardiac β-receptors as well[7]. The latter data, however, concerned experiments in which a single concentration of labeled ligand was used, thus allowing no conclusion about possible alterations in the maximal number and/or affinity of the measured binding sites. Therefore, and because no attempt was made by these authors[7] to investigate the cardiac α-receptors, we have studied both the α- and the β-receptors in heart membranes of rats submitted to a 3-week ethanol inhalation treatment[8].

Material and methods. Male Wistar rats weighing 300–350 g were exposed for 21 days to ethanol vapor according to Le Bourhis[8]. Food and water intake and total body weight were monitored daily. The ethanol concentration in the air was gradually raised from 15 mg/l on day 1 to 20 mg/l on day 21, resulting in blood ethanol concentrations of about 70 mg/100 ml on day 21. Animals were then sacrificed, and the hearts were quickly excised, rinsed and weighed.

Hearts were minced in cold buffer (0.25 M sucrose, 5 mM Tris/HCl, 1 mM $MgCl_2$, pH 7.4) and homogenized in a Potter-Elvejhem homogenizer. The homogenate was filtered through 1 layer of cheese-cloth and centrifuged at $1000 \times g$ for 10 min at 4 °C. The pellets were discarded and

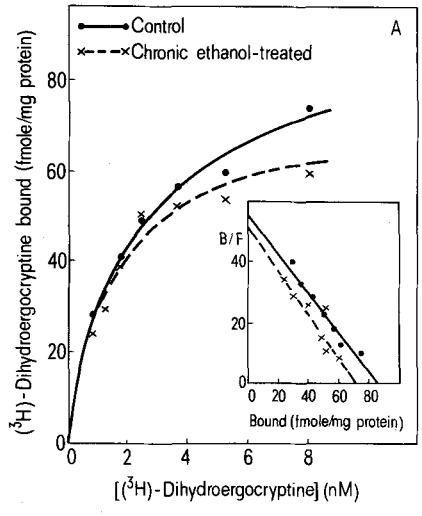
the supernatant was centrifuged at 40,000 × g for 10 min. The resulting pellet was washed twice in cold incubation buffer (50 mM Tris/HCl, 10 mM $MgCl_2$, pH 7.5) and was finally resuspended at 3–5 mg of protein/ml in incubation buffer. This suspension was used in the binding assays. The yields of final membrane preparation per g of initial heart wet weight from control and from ethanol-treated animals were similar (about 5 mg of membrane protein/g initial wet weight).

α- and β-receptors were studied according to the procedures described by Williams and Lefkowitz[9] using (^{3}H)-dihydroergocryptine (DHEC) and (−)-(^{3}H)-dihydroalprenolol (DHA) as ligands respectively. Nonspecific binding, determined in the presence of either 10 µM phentolamine (α-receptors) or 10 µM (±)-propranolol (β-receptors), was similar in membranes from control and ethanol-treated rats [35–40% of total binding at 3 nM (^{3}H)-DHEC and 12–15% of total binding at 3 nM (^{3}H)-DHA respectively]. The origin of the chemicals used was as by Giudicelli and Pecquery[10] and Pecquery et al.[11].

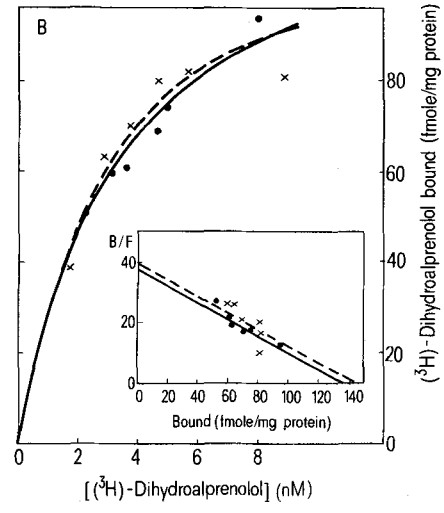
Results and discussion. The figure shows the results of a typical experiment in which the concentration-dependence of the specific binding of (^{3}H)-DHEC and (^{3}H)-DHA is measured in heart membranes from control and chronic ethanol-treated rats. As can be seen, no obvious difference was found between (^{3}H)-DHA binding to the β-receptors in the control and the ethanol-treated group. Concerning the α-receptors, however, binding of (^{3}H)-DHEC at high concentrations was apparently lower in heart membranes from the ethanol-treated animals.

In order to have a better insight into the effect of chronic ethanol inhalation on the properties of cardiac α- and β-receptors, the results of 3 separate experiments were analyzed according to Scatchard[12] (table). Chronic ethanol inhalation induced a non-significant reduction of the maximal number of α- and β-receptors and did not alter their binding affinities.

The lack of any modification of the number of cardiac β-receptors found in the present work contrasts with the significant decrease (−43%) observed by Banerjee et al.[7]. There are at least 2 explanations for these discrepant results. Firstly, as already pointed out above, the assumption made by these authors that the number of cardiac β-receptors is markedly decreased by chronic ethanol administration was based on binding data obtained at a single (^{3}H)-DHA concentration. Considering the fact that (^{3}H)-DHA binding to heart membranes follows the law of mass action, the only way to assess the total number of β-receptors is to perform saturation binding experiments[13]. Secondly, the chronic ethanol treatment used by Banerjee et al.[7] was different from ours and consisted of giving ethanol in an homogenized liquid diet[14] for 60 days. From



A Specific binding of (^{3}H)-DHEC to rat myocardial membranes as a function of increasing concentrations of (^{3}H)-DHEC. Inset: Scatchard plot of (^{3}H)-DHEC binding. The ratio (B/F) of bound (^{3}H)-DHEC (fmole/mg protein) to free (^{3}H)-DHEC (nM) is plotted as a function of the (^{3}H)-DHEC bound per mg protein. Each value is the mean of triplicate determinations.



B Specific binding of (^{3}H)-DHA to rat myocardial membranes as a function of increasing concentrations of (^{3}H)-DHA. Inset: Scatchard plot of (^{3}H)-DHA binding. Each value is the mean of triplicate determinations. Experimental conditions are described in the legend to the table.

Influence of chronic ethanol inhalation on the α- and β-adrenergic receptor density and affinity of rat heart membranes

	α-Adrenergic receptors		β-Adrenergic receptors	
	Maximal number (fmole/mg protein)	Dissociation constant, K_D (nM)	Maximal number (fmole/mg protein)	Dissociation constant, K_D (nM)
Control	102 ± 19	3.2 ± 1.3	164 ± 26	3.4 ± 0.9
Chronic ethanol-treated	80 ± 16	2.3 ± 1.3	142 ± 9	3.4 ± 0.8
	$p > 0.2$	$p > 0.2$	$p > 0.2$	$p > 0.2$

Rats were subjected to a 3-week ethanol inhalation treatment and the maximal number and affinity of α- and β-receptors were determined as described in the text. Ventricular membranes were incubated at 25 °C with different concentrations (1–8 nM) of either (^{3}H)-DHEC or (^{3}H)-DHA for 20 min or 18 min respectively. Each value represents the mean ± SD of the data from 3 separate experiments, each performed on pooled membranes from 10 to 12 hearts, with specific binding assessed in triplicate in each experiment as described in the text.

recent studies[15], it is clear that the method used for ethanol administration plays an important role in studies such as these. In fact, while Banerjee et al.[7] reported marked modifications in the density of β-receptors in the brain during both ethanol-treatment and ethanol-withdrawal, Hunt et al.[15] failed to observe any significant modification of the concentration of these receptors in most brain areas of rats rendered dependent after a 4 day oral administration of ethanol. In this connection, it is interesting to note that brain benzodiazepin receptors have also been reported to be unaffected in rats subjected to an ethanol treatment similar to that used here[16].

From this study, we can conclude that the chronic ethanol inhalation treatment presently used does not alter the density and affinity of cardiac α- and β-receptors. Moreover, since hydrolysis of cardiac triglycerides depends on a catecholamine-sensitive lipase[17], this study also indicates that the triglyceride accumulation that we have recently found in rat heart after the same ethanol inhalation treatment[18] does not result from an ethanol-induced alteration of the cardiac β-receptors.

1 Acknowledgments. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale. We wish to thank Mr M. Clément for skilled technical assistance in performing chronic ethanol inhalation treatments.

2 M. Victor, Post-grad. med. J. *47*, 68 (1970).
3 E.M. Sellers and H. Kalant, New Engl. J. Med. *294*, 757 (1976).
4 C.D. Marsden, T.M. Gilmette, R.G. Mc Allister, D.A.L. Owen and T.N. Miller, Acta endocr. *57*, 353 (1968).
5 D.H. Zilm, E.M. Sellers, S.M. Mac Leod and N.C. Degani, Ann. int. Med. *83*, 234 (1974).
6 E.M. Sellers, N.C. Degani, D.H. Zilm and S.M. Mac Leod, Lancet *1*, 94 (1976).
7 S.P. Banerjee, V.K. Sharma and J.M. Khanna, Nature *276*, 407 (1978).
8 B. Le Bourhis C.r. Soc. Biol. *169*, 898 (1975).
9 R.S. Williams and R.J. Lefkowitz, Circulation Res. *43*, 721 (1978).
10 Y. Giudicelli and R. Pecquery, Eur. J. Biochem. *90*, 413 (1978).
11 R. Pecquery, L. Malagrida and Y. Giudicelli, FEBS Lett. *98*, 241 (1979).
12 D. Scatchard, Ann. N.Y. Acad. Sci. USA *51*, 660 (1949).
13 L.T. Williams and R.J. Lefkowitz, in: Receptor Binding Studies in Adrenergic Pharmacology, p. 27. Raven Press, New York 1978.
14 J.M. Khanna, H. Kalant and G. Bustos, Can. J. Physiol. Pharmac. *45*, 777 (1967).
15 W.A. Hunt, T.K. Harden and R.G. Thurman, Drug Alcohol Depend. *4*, 327 (1979).
16 M. Karobath, J. Rogers and F.E. Bloom, Neuropharmacology *19*, 125 (1980).
17 D.R. Christian, G.S. Kilsheimer, G. Pettett, R. Paradise and J. Ashmore, in: Advances in Enzyme Regulation vol. 7, p. 71. Ed. G. Weber, Pergamon Press. Oxford 1969.
18 R. Nordmann, B. Le Bourhis, M. Clément, G. Jean and G. Aufrère, in: Proceeding 25th International Institute on the Prevention and Treatment of Alcoholism, p. 296. ICAA Publication, Lausanne 1979.

Urease inhibition by hydroxamic acids[1]

H.A. Akers

Department of Chemistry, Lamar University, Beaumont (Texas 77710, USA), 12 May 1980

Summary. Ureases from jack beans and *Rhodototorula pilimanae* were observed to be inhibited by primary hydroxamic acids but were not inhibited by acyclic-secondary (N-alkyl) hydroxamic acids.

During nutritional studies with the yeast *Rhodotorula pilimanae* it was observed that urea would serve as a nitrogen source in cultures deprived of iron. During iron deprivation *R. pilimanae* and related yeasts produce millimolar quantities of their iron transport agent, the hydroxamic acid rhodotorulic acid[2-4]. Urease inhibition and the retardation of microbial growth on urea by hydroxamic acids are well known[5,6], yet paradoxically *R. pilimanae* thrives (table 1) in low-iron cultures with urea as the sole nitrogen source in spite of the presence of millimolar concentrations of rhodotorulic acid. To resolve this problem the effect of a number of hydroxamic acids on *R. pilimanae* and jack bean ureases was determined.

Ureases were assayed spectrophotometrically and the inhibition by hydroxamic acids determined[7]. N-Methylhydroxylamine was obtained from Aldrich Chemical Co. while N-butylhydroxylamine was prepared by reducing the corresponding oxime[8]. Rhodotorulic acid was isolated from low iron *R. pilimanae* (ATCC 26423) cultures[2], glycine hydroxamic acid, jack bean powder, and avidin were from Sigma Chemical Co., while acetohydroxamic acid, propionohydroxamic acid, N-methylacetohydroxamic acid, and N-butylacetohydroxamic acid were prepared by acylation of the hydroxylamines[9]. Hydroxamic acid concentrations were determined spectrophotometrically with 5 mM $Fe(ClO_4)_3$ in 0.1 M $HClO_4$ using typical hydroxamic acid extinction coefficients[2,10]. For cultures of *R. pilimanae* grown with urea, 1.7 g/l was substituted for the ammonium contained in the normal culture medium[2]. Urea solutions were filter sterilized. *R. pilimanae* extracts were prepared from cells grown on urea containing medium for 2 days from a 1% inoculum by grinding with alumina, extracting with 0.02 M potassium phosphate 0.002 M EDTA, pH 6.5, and centrifuging at 14,000 × g for 20 min. Avidin and hydroxamic acid solutions were prepared with the same buffer (pH readjusted to 6.5).

Both jack bean and *R. pilimanae* ureases were found to be inhibited (table 2) by primary (unsubstituted nitrogen) hydroxamic acids while secondary (n-substituted) hydrox-

Table 1. The effect of nitrogen source and iron on the formation of rhodotorulic acid and cell yield in *Rhodotorula pilimanae* cultures[a]

N source[b]	Fe[c]	Rhodotorulic acid concentration (mM)	cell yield[d]
Urea	−	3.23	7.8
Urea	+	0.02	9.6
Ammonium	−	3.83	8.2
Ammonium	+	0.01	10.1

[a] Determinations were made after 3 days of growth at 23 °C from a 1% inoculum. The data represents an average of 3 separate determinations. [b] Cultures were prepared with 0.8 g of nitrogen per liter as urea or ammonium acetate. [c] A negative (−) sign indicated no iron salts were added to the cultures while a plus (+) indicated 5 mg of iron as ferric citrate was added per liter of culture. [d] Cells were washed twice with 1% saline and the resulting pellets were dried to a constant weight at 90 °C.