However, before a causal relationship between the increase in hypothalamic somatostatin and the decrease in pituitary GH can be suggested with certainty, the release of somatostatin from the hypothalmus will have to be measured.

Plasma levels of GH in old female rats have been reported to be decreased[13] or unchanged[16] when compared to young female rats. The reason for this difference may reside in the fact that neither study accounted for the pulsatile release of GH into the plasma, by measuring GH levels in the same animal over time, as was done in aged male rats[8]. A decrease in plasma levels of GH in old CE female rats is suggested by the growth stasis observed in these old animals[17] which preliminary findings suggest can be reversed by the administration of exogenous GH (Forman, Sonntag and Meites, unpublished observation).

Sonntag et al.[18] have reported that various centrally acting drugs increase GH to a greater extent in young as compared to old male rats. In addition, these investigators reported that systemic administration of growth hormone releasing factor (hp-GRF) increased plasma levels of GH more in young than in old animals[19]. By contrast, passive immunization with antiserum to somatostatin increased GH levels to the same or greater extent in old than in young animals[18]. Moreover, when pituitaries from young and old rats were exposed to hp-GRF in vitro, the release of GH from these pituitaries was essentially the same[19]. These data appear to indicate that the decreased release of GH characterizing aged male rats may be attributed to the observed increase in hypothalamic somatostatin[8].

Similar to the aged male rat, centrally acting drugs were observed to increase plasma levels of GH to a greater extent in young than in aged CE female rats (Forman, Sonntag and Meites unpublished observation). The decreased ability of old CE female rats as compared to young female rats to increase plasma levels of GH may partially be attributed to the decrease in pituitary GH levels, and the increase in hypothalamic somatostatin levels observed in these old animals in the present study.

Thus in the present study, hypothalamic somatostatin was increased and pituitary GH decreased, in old CE as compared to young female rats. Furthermore, these age-related alterations in the hypothalamic-pituitary unit in the old CE female rat, appear to be similar to those changes observed in aged male rats.

1 This work was supported by NIH Research grants AG-00416 (J.M.) from the National Institute on Aging, AM-04784 (J.M.) from the NIAMDD, and NIA postdoctoral fellowships AG-05208 (L.J.F.) and AG-05147 (W.E.S.).

2 Neuroendocrine Research Laboratory, Department of Physiology, Michigan State University, East Lansing, MI 48824.

3 Tannenbaum, G.S., and Martin, J.B., Endocrinology *98* (1976) 562.
4 Eden, S., Albertsson-Wikland, K., and Isaksson, O., Acta endocr. *88* (1978) 676.
5 Eden, S., Endocrinology *105* (1979) 555.
6 Martin, J.B., Physiologist *22* (1979) 23.
7 Kastings, N.W., Martin, J.B., and Arnold, M.A., Endocrinology *109* (1981) 1739.
8 Sonntag, W.E., Steger, R.W., Forman, L.J., and Meites, J., Endocrinology *107* (1980) 1875.
9 Jansson, J.-O., Ekberg, S., Isaksson, O.G.P., and Eden, S., Endocrinology *114* (1984) 1287.
10 Huang, H.H., and Meites, J. Neuroendocr.*17* (1975) 289.
11 Lu, J.K.H., Damassa, D.A., Gilman, D.P., Judd, H.L., and Sawyer, C.H., Biol. Reprod. *23* (1980) 345.
12 Bruni, J.F., Huang, H.H., Marshall, S., and Meites, J., Biol. Reprod. *17* (1977) 309.
13 Dickerman, E., Dickerman, S., and Meites, J., Excerpta med. int. Congr. Ser. *244* (1971) 252.
14 Lowry, D.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
15 Arimura, A., Smith, W.D., and Schally, A.V., Endocrinology *98* (1976) 540.
16 Florini, J.R., Harned, J.A., Rickman, R.A., and Weiss, J.P., Mech. Aging Dev. *15* (1981) 165.
17 Greenspan, F.S., Li, C.H., Simpson, M.E., and Evans, H.M., in: Hormone Assay. p. 274. Ed. C.W. Emmons. 1950.
18 Sonntag, W.E., Forman, L.J., Miki, N., Steger, R.W., Ramos, T., Arimura, A., and Meites, J., Neuroendocrinology *33* (1981) 73.
19 Sonntag, W.E., Hylka, V.W., and Meites, J., Endocrinology *113* (1983) 2305.

0014-4754/85/050653-02$1.50 + 0.20/0

An *Anolis* skin melanophore assay suitable for photoaffinity labeling studies with α-MSH[1]

A.N. Eberle and J. Girard

Laboratory of Endocrinology, Department of Research, University Hospital and University Children's Hospital, CH-4031 Basel (Switzerland), 28 May 1984

Summary. Visual determination of MSH-induced pigment migration in melanophores of small pieces of *Anolis carolinensis* skin is standardized by first measuring photoelectrometrically the change in reflection/transmission of the whole dorsal skin in response to different hormone concentrations. This method allows the rapid and precise recording of time-response curves after photoaffinity labeling of MSH receptors or of dose-response curves of large series of synthetic compounds.
Key words. α-MSH; bioassay; *Anolis carolinensis;* melanophores; photoaffinity labeling.

In vitro bioassays for the measurement of melanocyte-stimulating hormones (MSHs) are based on the ability of these peptides to induce pigment dispersion in the melanophores of amphibian *(Rana, Xenopus)* or reptilian *(Anolis)* skin. The biological response is usually quantitated photoelectrometrically *(→Rana pipiens)* by measuring the reflectance change of the isolated skin[2-4] or microscopically *(→Xenopus laevis)* by visual comparison of the degree of pigment dispersion with that of the Hogben-Slome index[5,6]. This latter method is very rapid and although subjective, is precise as demonstrated recently with a new assay using tail-fin pieces of *Xenopus* tadpoles[7].

Pigment dispersion in melanophores of *Anolis carolinensis* skin cannot easily be observed under the microscope. The skin has, however, the advantage that it changes color from bright green to dark brown in response to increasing hormone concentrations. This color change forms the basis for the quantal *Anolis* skin assay[8,9] in which the degree of pigment dispersion in small skin pieces is assessed visually by comparison with a standard series. The method is particularly useful for titrating the lowest hormone concentration that produces a response, but it is not suitable for constructing dose-response curves. So far the latter could only be obtained with the reflectometric assay[10,11] or with the rate assay[12] which measures the time for the generation of the response. Since neither method is suitable for recording time-response curves of large series of skin pieces, we have developed an assay in which the entire dorsal skin of each

animal is calibrated reflectometrically before cutting into small pieces for rapid visual determination. This approach has made it possible to perform photoaffinity labeling studies with the *Anolis* system and to record time-response curves with good precision.

Methods. Adult male *Anolis carolinensis* were obtained from De Natuurvriend, Utrecht, Holland. They were kept in a terrarium at 20–25 °C in a normal day/night cycle and fed with larvae of the wax moth *(Galleria mellonella)*. Water contained 4 g calcium lactate and 0.25 mg vitamin D_3 per liter and was supplied daily by sprinkling over the vegetation in the terrarium.

After decapitation and mid-ventral incision, the whole skin of the torso was removed and washed for 40 min by floating on Krebs-Ringer solution (8.3 g NaCl, 0.33 g KCl, 0.12 g $CaCl_2$, 0.21 g $MgCl_2 \cdot 6H_2O$, 0.10 g $NaHCO_3$, 0.01 g BSA per liter of distilled water[13]). The skin was then transferred into a 10 × 80 mm petridish containing 20 ml of Krebs-Ringer buffer, and the reflectance/transmission of the skin was determined in a dark room with a Photovolt reflectometer model 670 whose probe 670-Y was arranged as shown in figure 1. The instrument was set to 100 scale units before each measurement, using the same type of petridish filled with 20 ml of buffer. Green *Anolis* skins usually exhibited about 100 ± 5 scale units. For a routine log dose-response curve the skin was incubated in 20 ml buffer containing either 0.1, 0.5, 2.0 or 10 ng/ml of α-MSH and the change in reflectance/transmission was measured after 15 min. The skin was washed with buffer between the different incubations with hormone until the pigment was fully aggregated again (15–40 min). For a complete log dose-response curve, five additional α-MSH concentrations were tested (0.025, 0.05, 0.2, 1.0 and 5 ng/ml). 10 ng/ml α-MSH produced a maximal reflectance/transmission change of 50–60 scale units. This value was defined as 100% reflectance change and the other values were normalized accordingly.

The skin was cut into 50–60 pieces of about 2.5 × 2.5 mm. 6 × 2 pieces were used as reference in a standard dilution of 2, 1, 0.5, 0.25, 0.125 and 0.063 ng α-MSH/ml buffer arranged in a 24-well Costar plate. Serial dilutions of the test substances containing 2–3 skin pieces each were placed alongside the standard series. Skin darkening was assessed visually against a black background after a 15-min incubation using a macroscopic melanophore index (MI) of 1–5 that was similar to the microscopic Hogben-Slome index[5,7] and corresponded with 0 to ~85% reflectance change (see fig. 2; higher reflectance changes could not be unequivocally assessed by eye).

For photoaffinity labeling studies of MSH receptors, the whole skin or small skin pieces were preincubated in a solution containing 20 ng/ml of photoreactive p-azidophenylalanine[13]-α-MSH[14] for 5 min and irradiated with light of 310–550 nm for 5 min in an Oriel apparatus (for details see Eberle[15]). This procedure was repeated once with fresh hormone solution. The skins were then washed in buffer (with 4 buffer changes) and the time course of the melanosome aggregation was followed reflectometrically or visually by comparison with a standard series.

p-Azidophenylalanine[13]-α-MSH[14] was synthesized in our laboratory by a classical solution approach. α-MSH was kindly provided by Ciba-Geigy, Basel.

Results and discussion. Calibration of the whole dorsal skin of *Anolis carolinensis* with photoelectrometric determination of reflectance and transmission changes produces a log dose-response curve which is linear from 0.06–2 ng/ml of α-MSH, i.e. in the range that contains the minimal and maximal dose of

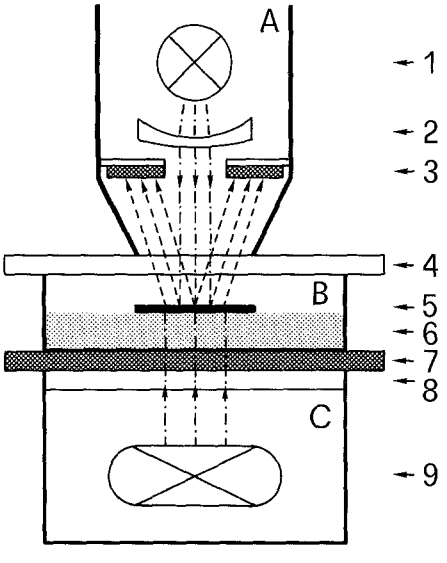

Figure 1. Apparatus for a mixed reflection/transmission measurement of the melanophore response of *Anolis* skin in vitro. A: Photovolt Reflectometer Probe 670-Y consisting of lamp (1), lens (2) and photodiode (3). B: Petridish containing a glass plate (4), the skin (5) floating on the hormone solution (6). C: Transluminator consisting of a red filter (7), an opaque glass plate (8) and the light source (40 Watts, 9).

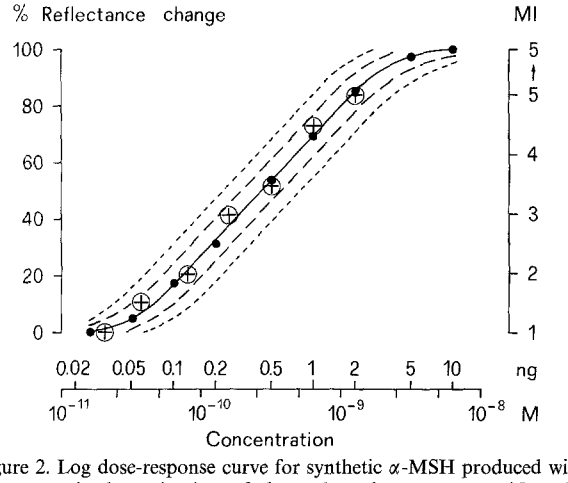

Figure 2. Log dose-response curve for synthetic α-MSH produced with reflectometric determination of the melanophore response (●—●; mean of 6 assays) and standard points for visual estimation (⊕). 95% confidence limits for visual determination with (– – –) or without (– – – –) reflectometric calibration.

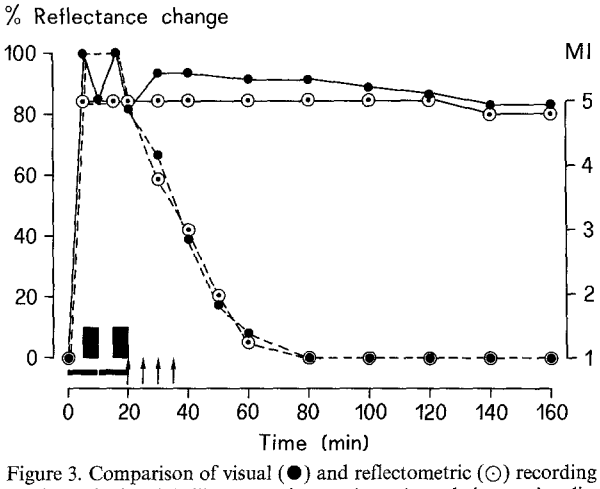

Figure 3. Comparison of visual (●) and reflectometric (⊙) recording in a photoaffinity labelling experiment (——) and in nonirradiated controls (– – –). Each point represents the mean of 2–6 determinations. ——: incubation with hormone; ▮: UV-irradiation; ↑: buffer change.

the peptide for visual estimation. The linear part covers about 75% of the total reflectance/transmission change and corresponds to a hormone concentration range of 1–30. The composite line of six individual reflectometric log dose-response curves for α-MSH is shown in figure 2. A statistical analysis of the lines showed that the mean precision (λ)[16] of the reflectometric assay was 0.11 which corresponds well with the values obtained with other melanophore assays, such as the method using tail-fin pieces of *Xenopus* larvae[7], or the frog skin assay[4]. Visual estimation of skin darkening is less precise than reflectometric determination since not more than seven intermediate steps between minimal and maximal response can be distinguished. It is particularly unfavorable to use a set of noncalibrated skin pieces from different animals because this would introduce a significant error (fig. 2). If on the other hand the skin pieces for standard and unknowns originate from the same (calibrated) dorsal skin, the 95% confidence interval is not larger than that for the microscopic *Xenopus* assay. The precision of the assay reaches 0.2 which is sufficiently accurate for time-response curves in photoaffinity labeling studies.

UV-irradiation of *Anolis* skin in the presence of photoreactive p-azidophenylalanine[13]-α-MSH produces irreversible pigment dispersion[15]. Visual and reflectometric recording of this long-lasting stimulation elicits an almost identical time-response curve, except for a slight difference during the irradiation phase (fig. 3): as melanophores are UV-sensitive, there is a tendency for their pigment to aggregate slightly during exposure to UV-light. In most cases this can only be observed reflectometrically; moreover it is always reversible and does not affect the further course of the time-response curve. Visual and reflectometric recording of the nonirradiated controls hardly differ either (fig. 3) thus demonstrating the usefulness and validity of the visual method using calibrated skin pieces.

1 Acknowledgments. We wish to thank Ms V. Jäggin, Ms C. Schulthess and Ms G. van Hees for excellent technical assistance, Dr. R. Andreatta, Ciba-Geigy AG, Basel, for his generous gift of α-MSH and Prof. A. Pletscher for his continuous interest. This work was supported by the Swiss National Science Foundation.

2 Shizume, K., Lerner, A.B., and Fitzpatrick, T.B., Endocrinology *54* (1954) 553.
3 Geschwind, I.I., and Huseby, R.A., Endocrinology *79* (1966) 97.
4 Eberle, A.N., and Schwyzer, R., Helv. chim. Acta *58* (1975) 1528.
5 Hogben, L.T., and Slome D., Proc. R. Soc., B *108* (1931) 10.
6 Landgrebe, F.W., and Waring, H., in: Methods in hormone research, vol. 2, p. 517. Ed. R.I. Dorfman. Academic Press, New York 1962.
7 De Graan, P.N.E., Molenaar, R., and van de Veerdonk, F.C.G., Molec. cell. Endocr. *32* (1983) 271.
8 Burgers, A.C.J., Ann. N.Y. Acad. Sci. *100* (1963) 669.
9 Tilders, F.J.H., van Delft, A.M.L., and Smelik, P.G., J. Endocr. *66* (1975) 165.
10 Hadley, M.E., and Goldman, J.M., Am. Zool. *9* (1969) 489.
11 Björklund, A., Meurling, P., Nilsson, G., and Nobin, A., J. Endocr. *53* (1972) 161.
12 Carter, R.J., and Shuster, S., J. invest. Derm. *71* (1978) 229.
13 Waring, H., Color change mechanisms of cold-blooded vertebrates. Academic Press, New York 1963.
14 Eberle, A.N., de Graan, P.N.E., and Hübscher, W., Helv. chim. Acta *64* (1981) 2645.
15 Eberle, A.N., J. Receptor Res. *4* (1984), in press.
16 Bliss, C.I., and White, C., in: The Vitamins, p. 21. Eds P. György and W.N. Pearson. Academic Press, New York 1967.

0014-4754/85/050654-03$1.50 + 0.20/0

Malignant hyperthermia: Molecular defects in membrane permeability

K.S. Cheah and A.M. Cheah

Agricultural and Food Research Council, Food Research Institute, Langford, Bristol BS18 7DY (England), 20 February 1984

Summary. Malignant hyperthermia (MH), a genetically inherited disorder of skeletal muscle, is due to molecular defect in membrane permeability. The alteration in membrane permeability is suggested to be due to enhanced phospholipase A_2 activity which is responsible for the increased level in sarcoplasmic Ca^{2+}. The excess Ca^{2+} is responsible for muscle hyper-rigidity and enhanced rate of glycolysis, resulting in a rapid rate of lactic acid production and a low pH in MH muscle.
Key words. Malignant hyperthermia; membrane permeability; sarcoplasmic Ca^{2+}; phospholipase A_2 activity; calmodulin.

Malignant hyperthermia, a genetically inherited disorder, affects primarily the skeletal muscle of humans and pigs. The syndrome can be induced in apparently healthy humans[1–8] and in stress-susceptible pigs[9–16] by halothane[1–16], a fluorinated hydrocarbon anesthetic, and suxamethonium[3, 17–23], and particularly in pigs, by either environmental or physiological stress triggered by changes in temperature and excitement[10, 23–25]. Once initiated, the classical symptoms of the syndrome are characterized by gross muscular rigidity, rapid rise in body temperature, hyperventilation, severe metabolic acidosis and elevated levels of serum metabolites[26–28]. The body temperature may increase at a rate of 1 °C per 5 min[7], and the metabolic rate can exceed a 17-fold increase over normal[25], if uncontrolled, death occurs[11, 24, 29]. In humans, malignant hyperthermia can occasionally occur without any sign of muscle rigidity[2, 7], with 70% of malignant hyperthermia patients showing skeletal muscle rigidity[2]. The occurrence of anesthetic deaths in apparently healthy patients is about 1 in 15,000 anesthesia[2]. In healthy pigs, the incidence of malignant hyperthermia is dependent on the breed and in highly stress-susceptible breeds, it can be as high as 88%[30]. Malignant hyperthermia susceptible pigs are responsible for a substantial economic loss in the pig industry through transportation deaths[23, 24] and in the production of a commercially undesirable pale, soft and exudative meat[10, 23, 25, 31]. The latter condition is principally due to the denaturation of sarcoplasmic and myofibrillar proteins[32–34] caused by a combination of low muscle pH due to rapid glycolysis and high temperature immediately after death[11, 31, 33, 35]. The syndrome in humans and pigs[36] shows striking similarities, and it is generally assumed that the human and porcine malignant hyperthermia are identical. Thus, genetically selected pigs are frequently used as experimental models for the investigation of human malignant hyperthermia, particularly on the biochemical and physiological aspects of the syndrome.

In humans, the genetic inheritance appears to be autosomal dominant[1, 37–40] with reduced penetrance although multifactorial