Br. J. Pharmac. (1969), **37**, 650–658.

Effects of cyclic 3′,5′-AMP and other adenine nucleotides on the melanophores of the lizard (*Anolis carolinensis*)

MAC E. HADLEY AND JOEL M. GOLDMAN

Department of Biological Sciences and Division of Pharmacology, College of Pharmacy, University of Arizona, Tucson, Arizona 85721

1. Cyclic 3′,5′-AMP has been reported to darken skins of the frog, *Rana pipiens*. This suggests that cyclic 3′,5′-AMP may mediate the action of MSH on amphibian chromatophores. Since MSH also darkens skins of the lizard, *Anolis carolinensis*, we investigated the effects of cyclic 3′,5′-AMP and other nucleotides on *Anolis* melanophores to determine whether cyclic 3′,5′-AMP may be the intracellular mediator of hormone action on melanophores of another vertebrate class.

2. Cyclic 3′,5′-AMP, itself, causes a rapid melanin granule aggregation within melanophores of *Anolis*. This response is, however, somewhat nonspecific in that both 5′-ATP and 5′-ADP also lighten the skins by aggregating the melanin granules. Another nucleotide, 5′-AMP, darkens the skins by dispersing melanin granules. Cyclic 2′,3′-AMP does not darken or lighten *Anolis* skins.

3. Dibutyryl cyclic 3′,5′-AMP, which is considered to be better able to penetrate membranes and resist degradation by a specific phosphodiesterase, maximally darkens *Anolis* skins, as does MSH. This darkening by the potent dibutyryl cyclic 3′,5′-AMP suggests that cyclic 3′,5′-AMP may be the intracellular mediator of melanin granule dispersion within *Anolis* melanophores leading to skin darkening.

4. Other evidence supporting the first-messenger–second-messenger hypothesis for melanophore regulation is discussed.

5. The differences in responses of *Anolis* melanophores to adenine nucleotides may relate to the ability of these agents to penetrate melanophore membranes ; thus, the nucleotides could exert their effects either intracellularly or extracellularly on the plasma membrane.

Adenosine 3′,5′-monophosphate (cyclic 3′,5′-AMP) disperses melanin granules within melanophores of the frog, *Rana pipiens* (Bitensky & Burstein, 1965 ; Novales & Davis, 1967), similar to the dispersion induced by melanophore-stimulating hormone (melanocyte-stimulating hormone, MSH). It has been suggested (Novales & Davis, 1967) that MSH acting as " first messenger " (Sutherland, Øye & Butcher, 1965) may stimulate an increase in the intracellular level of cyclic 3′,5′-AMP, the " second messenger " (Sutherland *et al.*, 1965), which is then more directly responsible for initiating melanin granule dispersion. As in most other studies (Robison,

Butcher & Sutherland, 1967), the concentrations of the nucleotide necessary to disperse melanin granules is quite high (10^{-2}M). Lower concentrations cause little, if any, darkening of skins (Tercafs, 1966 ; Novales & Davis, 1967).

The effects of cyclic 3′,5′-AMP on the melanophore responses of members of other vertebrate classes have not been studied. The melanophores of the lizard, *Anolis carolinensis,* are about as sensitive to MSH as are those of *Rana pipiens* (personal observations). We therefore studied the effects of cyclic 3′,5′-AMP on the melanophores of this species of another vertebrate class to determine whether the results obtained on amphibian melanophores were of more general significance. The results presented here stand in contrast to those reported earlier for the frog, *Rana pipiens* (Bitensky & Burstein, 1965 ; Novales & Davis, 1967).

Methods

The lizards, *Anolis carolinensis,* used in these studies were obtained from the Snake Farm, Laplace, Louisiana. The frogs, *Rana pipiens,* were obtained from the Lemberger Company, Oshkosh, Wisconsin. Animals were killed by decapitation followed by spinal pithing. Because of their smaller size, only the back skins of the lizards could be used, whereas the leg skins of the frogs were used, the back skins being somewhat too thick. The colour changes which occur with back skins from lizards and leg skins from frogs are similar in that darkening results from melanin granule dispersion within dermal melanophores and lightening results from melanin granule aggregation within melanophores. In previous publications (Hadley & Bagnara, 1969 ; Hadley & Goldman, 1969) we have demonstrated that frog leg skins and lizard back skins respond in a similar manner. Skins were removed from the animals and then rinsed in Ringer solution and allowed to remain in Ringer solution for about 2 hr before use. During this time the skins usually become quite light in colour due to the gradual aggregation of melanin granules within the dermal melanophores.

The methods employed followed closely those described for the frog skin bioassay for melanophore-stimulating hormone (Shizume, Lerner & Fitzpatrick, 1954 ; Wright & Lerner, 1960). This assay involves the measurement of light reflectance from the outer (epidermal) surface of skins immersed in Ringer solution. The movement of melanin granules within melanophores brings about a change in reflectance. The assay is quite sensitive, being able to detect the presence of MSH at a 10^{-11}M concentration (Lerner, 1959). Skins are mounted on metal rings and held in place by outer plastic rings and then placed in 30 ml. beakers containing 10 ml. of Ringer solution (Novales & Davis, 1967). A Photovolt photoelectric reflection meter (Photovolt Corporation, New York, N.Y.) was used to measure the reflectance from each skin. An initial reflectance value was obtained for each skin, and these skins were then arranged so that the total average reflectance value for each group of skins was approximately the same. Each experimental group consisted of six or eight skins. The initial average reflectance value for each experimental group was given a value of 100%. Succeeding average values are compared with the initial value and recorded as a percent of this initial value of 100% ; for example, a value of 120% represents a 20% increase in reflectance (skin lightening).

Ringer solution containing the nucleotides (10^{-2}M) were substituted for the Ringer solution bathing the skins, and after a period of time the reflectance was

measured. Catecholamines and MSH were added in 0·1 ml. amounts to the 10 ml. of solution bathing the skins to give the final concentrations stated in the experiments. All agents were dissolved immediately before the experiment. All solutions used were at a pH of 7·4.

The porcine beta MSH used in these experiments was obtained from Dr. Aaron B. Lerner. The MSH was lyophilized with lactose as described by Novales, Novales, Zinner & Stoner (1962). The following nucleotides were obtained from Sigma Chemical Company: cyclic 3′,5′-AMP, cyclic 2′,3′-AMP, 5′-AMP, 5′-ADP and 5′-ATP. Schwarz BioResearch Co. was the source of ^{6}N-2′-O-dibutyryl cyclic 3′,5′-AMP. Both l-isoprenaline bitartrate and l-noradrenaline bitartrate were obtained from the Winthrop Laboratories. Statistical comparisons of mean values were made using Student's *t* test.

Results

We first compared the effects of cyclic 3′,5′-AMP and 5′-AMP on the melanophores of *Anolis carolinensis* (Fig. 1). Cyclic 3′,5′-AMP lightened the skins but 5′-AMP darkened them. Isoprenaline added to the skins at 120 min resulted in darkening of all skins, including those lightened by cyclic 3′,5′-AMP.

In other experiments, we have found that none of the skins from fifty-five lizards darkened in response to cyclic 3′-5′-AMP. The degree to which skins will lighten in response to agents such as cyclic 3′,5′-AMP depends on the degree to which skins have already become lightened in Ringer solution before the initial photometric reading. Skin coloration is related to the intracellular location of melanin granules within dermal melanophores. Some skins, for some as yet unexplained

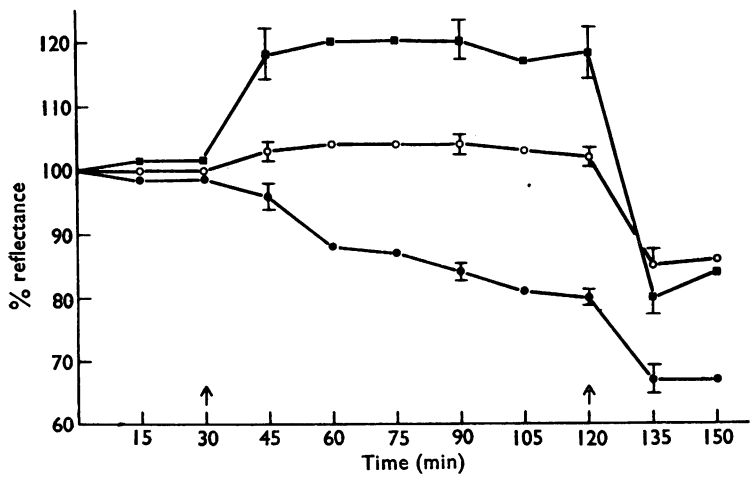

FIG. 1. After an initial base photometric reading, three groups of eight *Anolis carolinensis* skins each were preincubated in Ringer solution for 30 min. Cyclic 3′,5′-AMP (10^{-2}M) was then added (arrow) to one group of skins (■) and 5′-AMP (10^{-2}M) was added to another group (●). One Ringer group (○) was allowed to remain as a control. At 120 min isoprenaline (10^{-5}M) was added (arrow) to all groups of skins. Each point on the graph is the mean value for the reflectance measurements from the eight skins in the group. Vertical lines represent the standard error of the mean.

reason, never become as green as other skins. Melanin granules are more dispersed within melanophores of darker skins, whereas they are more nearly aggregated to a perinuclear position within melanophores of lighter skins.

The lightening of lizard skins exposed to cyclic 3′,5′-AMP was unexpected in light of the published data on the frog, *Rana pipiens*. We have used cyclic 3′,5′-AMP from a number of different lot numbers (Sigma Chemical Co.) and have

TABLE 1. *Comparative melanophore responses of* Anolis carolinensis *and* Rana pipiens *skins to cyclic 3′,5′-AMP*

Number of animals	Treatment	% change in reflectance* ±S.E.M.
Sixteen *Anolis carolinensis*	1×10^{-2}M	
	Cyclic 3′,5′-AMP	+3±1·12
	Ringer control	+3±0·80
Eight *Rana pipiens*	1×10^{-2}M	
	Cyclic 3′,5′-AMP	−10±1·17†
	Ringer control	+2±1·20

* Values are means±S.E. Results represent the greatest change in reflectance in response to cyclic 3′,5′-AMP within 60 min after addition of the nucleotide. A plus sign (+) indicates an increase in reflectance (skin lightening), and a minus sign (−) indicates a decrease in reflectance (skin darkening).

† Significantly different from control group ($P<0·001$).

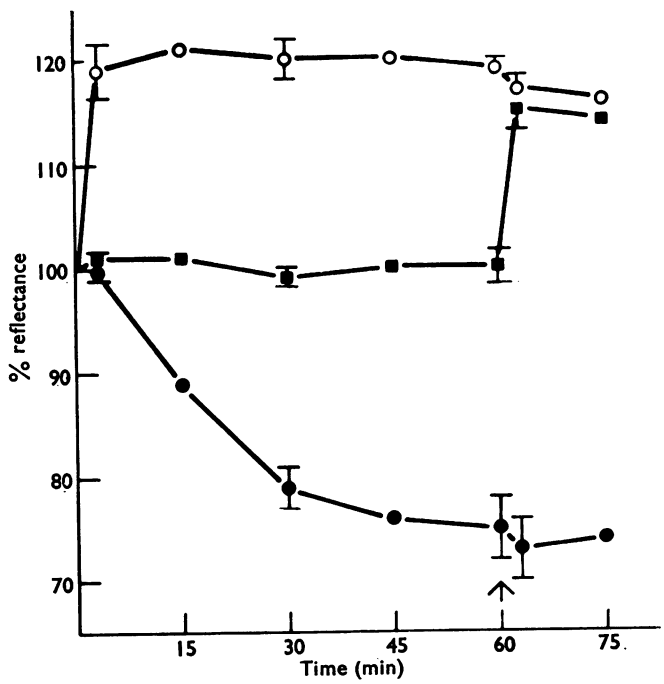

FIG. 2. Adenosine 5′-triphosphate, ATP (○), and adenosine 5′-monophosphate, AMP (●), were each added (10^{-2}M) to a group of eight *Anolis* skins. Another group of eight skins (■) remained as a Ringer control. At 60 min (arrow) adenosine 5′-diphosphate, ADP, was added to the Ringer control group (■). Each point represents the mean value of the reflectance measurements for the eight skins in the group. Standard error of the mean is indicated by vertical lines.

consistently obtained lightening. The same cyclic 3′,5′-AMP that failed to darken skins of *A. carolinensis* darkened the skins of *R. pipiens* used in the same experiment (Table 1). In this experiment *Anolis* skins did not lighten as in previous experiments, because they were already maximally light in colour. The addition of MSH to the skins of both *A. carolinensis* and *R. pipiens* treated with cyclic 3′,5′-AMP caused a rapid and profound darkening of these skins. Such darkening of skins in response to MSH, in both species, after preincubation of skins in the high concentrations of cyclic 3′,5′-AMP used in the experiment, demonstrates that the use of such concentrations of the nucleotide is not damaging to the skins.

The effects of other adenine nucleotides on *Anolis* skins were then examined (Fig. 2). Skins were incubated in either 5′-adenosine triphosphate (ATP), 5′-adenosine diphosphate (ADP), or as in the first experiment, 5′-adenosine monophosphate (AMP). Both ADP and ATP lightened the skins; AMP, in contrast, darkened the skins. In some experiments, however, the skins failed to lighten with ATP and ADP, possibly because the skins were already maximally light in colour. Cyclic 2′,3′-AMP did not, however, significantly lighten or darken the skins of *Anolis*.

The dibutyryl derivative of cyclic 3′,5′-AMP has been reported to be more effective in some systems than the parent nucleotide and, in fact, it caused a maximal darkening of *Anolis* skins equal to that caused by MSH (Fig. 3). Again, cyclic 3′,5′-AMP lightened the skins. In a second experiment, utilizing the same solutions

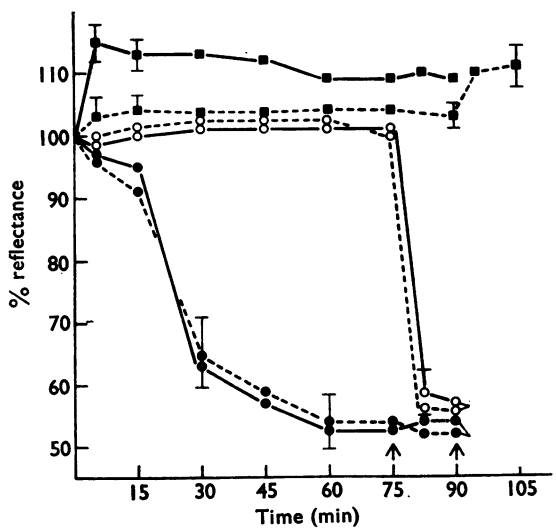

FIG. 3. Cyclic 3′,5′-AMP (■——■) and dibutyryl cyclic 3′,5′-AMP (●——●) were each added (10^{-2}M) to a group of six *Anolis* skins. One group of six skins remained as a Ringer control group (○——○). At 75 min (arrow), MSH (5×10^{-9} g/ml.) was added to the Ringer control group (○——○). After 90 min, this experiment was terminated and six fresh, previously untreated skins, were placed in beakers containing the same solutions of cyclic 3′,5′-AMP (■- - - -■) and dibutyryl cyclic 3′,5′-AMP (●- - - -●) used in the first part of the experiment. In addition, a fresh Ringer control group of six skins (○- - - -○) was also used. At 75 min (arrow) MSH (5×10^{-9} g/ml) was again added to the Ringer control group (○- - - -○). After 90 min (arrow), at the conclusion of the second part of the experiment, noradrenaline (NA) was added (10^{-5}M) to the second group of skins in cyclic 3′,5′-AMP (■- - - -■). Each point on the graph represents the mean value for the six skins making up that group. Vertical lines represent the standard error of the mean.

used in the last experiment, dibutyryl cyclic 3′,5′-AMP again caused a maximal darkening of the skins, but cyclic 3′,5′-AMP had only a minor lightening effect. MSH added to the control group of skins darkened them to about the same degree, thus suggesting that the darkening response of the skins to dibutyryl cyclic 3′,5′-AMP was maximal. In the second experiment, after 90 min, noradrenaline was added to the group of cyclic 3′,5′-AMP skins which had not lightened, in order to demonstrate their potential to lighten.

Discussion

According to the "first-messenger–second-messenger" hypothesis (Sutherland *et al.*, 1965), hormones (first-messengers) mediate their actions by controlling the intracellular level of the second-messenger, cyclic 3′,5′-AMP. The fact that cyclic 3′,5′-AMP can mimic the action of the first-messenger (Sutherland, Robison & Butcher, 1968) is considered strong evidence in support of this hypothesis. The demonstration (Bitensky & Burstein, 1965; Novales & Davis, 1967) that cyclic 3′,5′-AMP can mimic the action of MSH on *Rana pipiens* skin suggests, therefore, that cyclic 3′,5′-AMP may possibly play a role in the regulation of melanin granule movement within melanophores. The recent finding (Abe, Butcher, Nicholson, Baird, Liddle & Liddle, 1969) that the darkening of frog skin by MSH is correlated with an increased level of cyclic 3′,5′-AMP in the skin is good evidence for its suggested role in MSH action.

The present experiments have demonstrated that cyclic 3′,5′-AMP lightens the skins of *Anolis carolinensis* by causing melanin granule aggregation within melanophores. These results stand in decided contrast to those results reported by other workers (Bitensky & Burstein, 1965; Novales & Davis, 1967; Abe *et al.*, 1969) in their studies on the melanophore responses of the frog, *R. pipiens*. In the present experiments we have shown, however, that our preparations of cyclic 3′,5′-AMP darken the skins of *R. pipiens*. But, again, as demonstrated by Novales & Davis (1967) for *R. pipiens*, this response is usually quite small.

The perinuclear aggregation of melanin granules within *Anolis* melanophores in response to cyclic 3′,5′-AMP would appear to be somewhat non-specific, since 5′-ATP and 5′-ADP also produce this effect. The demonstration that 5′-AMP darkened the skins of *Anolis* and that 5′-ATP lightened them confirms results obtained by Horowitz (1958). We have been unable to darken skins of *R. pipiens* with 5′-AMP, a result previously reported by Novales and Davis (1967). We are unable to account for this melanin granule dispersion in *Anolis* melanophores in response to 5′-AMP. Horowitz (1958) suggested that the darkening response of *A. carolinensis* skins to 5′-AMP was an effect similar to that produced by methylxanthines, agents (for example, caffeine and theophylline) which, as in *R. pipiens* (Wright & Lerner, 1960; Hadley & Bagnara, 1969), disperse melanin granules within *Anolis* melanophores (Hadley & Goldman, 1969).

The demonstration that the responses of *Anolis* melanophores to cyclic 3′,5′-AMP and to 5′-AMP are opposite and that cyclic 2′,3′-AMP does not aggregate or disperse melanin granules indicates that these responses are not of a pathological nature owing to the high concentrations of the nucleotides used. In addition, *Anolis* skins preincubated in any of the nucleotides used in this study still respond maximally to either MSH or to isoprenaline.

In a previous report (Goldman & Hadley, 1969) we demonstrated that melanin granule aggregation within *Anolis* melanophores in response to sympathomimetic stimulation was regulated through alpha-adrenoceptors, whereas melanin granule dispersion in response to such stimulation was mediated through beta-adrenoceptors. Isoprenaline, a rather specific beta-agonist, dispersed melanin granules in the present as well as in previous experiments (Goldman & Hadley, 1969). Stimulation of beta-adrenoceptors of some other tissues is believed to increase the intracellular levels of cyclic 3′,5′-AMP (Robison *et al.*, 1967). It is interesting that in the present experiments the stimulation of beta-adrenoceptors leading to the theoretical increase in cyclic 3′,5′-AMP darkened the skins, but that, in contrast, the addition of the supposed second-messenger, cyclic 3′,5′-AMP, actually lightened the skins. Also, Turtle & Kipnis (1967) have suggested that alpha-adrenoceptor stimulation decreases intracellular levels of cyclic 3′,5′-AMP. Similarly, Abe *et al.* (1969) have suggested that alpha-adrenoreceptor stimulation prevents the formation of cyclic 3′,5′-AMP in frog skin in response to MSH. Nevertheless, in our experiments both alpha-adrenoceptor stimulation (Goldman & Hadley, 1969) and the addition of cyclic 3′,5′-AMP, as demonstrated in the present experiments, caused melanin granule aggregation. Further, the addition of the first-messenger, MSH, which at least in the frog, *R. pipiens*, is considered (Novales & Davis, 1967 ; Abe *et al.*, 1969) responsible for increasing intracellular levels of cyclic 3′,5′-AMP, darkened the same *Anolis* skins that had been lightened by cyclic 3′,5′-AMP.

We consider the demonstration that dibutyryl cyclic 3′,5′-AMP caused maximal darkening of *Anolis* skins as evidence for the role of cyclic 3′,5′-AMP in the regulation of melanin granule movement within melanophores. In addition, data obtained using methylxanthines (cyclic 3′,5′-AMP phosphodiesterase inhibitors) also indicates that cyclic 3′,5′-AMP is involved in melanin granule dispersion (Hadley & Goldman, 1969). However, the demonstration that 5′-AMP also darkened the skins and that cyclic 3′,5′-AMP actually caused melanin granule aggregation within melanophores should provide a note of caution for such an early interpretation.

It has been suggested that the ability of the dibutyryl derivative of cyclic 3′,5′-AMP to mimic hormone action better than cyclic 3′,5′-AMP itself is because the dibutyryl derivative is able to cross cell membranes more easily and that, once within the cells, is less susceptible to degradation by cyclic 3′,5′-AMP phosphodiesterase (Posternak, Sutherland & Henion, 1962). The rapid, maximal, and prolonged darkening of *Anolis* skins in response to dibutyryl cyclic 3′,5′-AMP supports such a suggestion. In addition, the failure of cyclic 3′,5′-AMP to maintain its ability to aggregate melanin granules when used again in a second experiment could be interpreted as indicating that it had been rapidly degraded.

Unlike muscle, melanophores represent a dispersed tissue which comprises only a small percentage of the skin. It is difficult, therefore, to determine clearly whether the effects of either hormonal or pharmacological agents are directly on the melanophores or if they act indirectly on surrounding tissue. This also makes it difficult to relate possible biochemical events to physiological responses. The responses of *Anolis* skins to the adenine nucleotides used in the present experiments are quite specific but qualitatively very different. It is presently impossible to determine whether these agents directly affect melanophores, and, if so, whether these effects are intracellularly mediated or are membrane effects. Falk & Gerard (1954) found that externally applied 5′-ATP activated the membrane of the isolated

sartorius muscle of *Rana pipiens,* with resulting twitching. It is possible, therefore, that the effects of the nucleotides on *Anolis* skins are similar to what Falk & Gerard (1954) found using muscle: that is, that the nucleotides are acting on the plasma membrane rather than intracellularly.

The work of Bartelstone, Nasmyth & Telford (1967) demonstrating that smooth muscle contraction in response to catecholamines may be mediated by cyclic 3′,5′-AMP has been criticized by Sutherland *et al.* (1968). Actually, the response of *Anolis* melanophores to cyclic 3′,5′-AMP is somewhat similar to the data of Bartelstone *et al.* (1967), in that both muscle contraction in the rat aortic strip and melanin granule aggregation within *Anolis* melanophores are regulated by alpha-adrenoceptors and, in addition, cyclic 3′,5′-AMP mimics the alpha-adrenoceptor effect in both cases. Our data differ significantly from that of Bartelstone *et al.* (1967) in that melanin granule aggregation in response to catecholamine stimulation is antagonized (Hadley & Goldman, 1969) rather than potentiated by methylxanthines as reported by these workers.

In conclusion, it has been suggested that since MSH darkens frog skins and cyclic 3′,5′-AMP also darkens frog skins, the action of MSH is mediated through increases in intracellular levels of cyclic 3′,5′-AMP. Our data on *Anolis* suggest that such a simple interpretation may be premature. Other workers should also be aware of the possibility that cyclic 3′,5′-AMP may have effects opposite to those expected.

This study was supported in part by PHS Research Grant No. 2-F2-AM-32, 622-02 from the National Institute of Arthritis and Metabolic Diseases, GB-8347 from the National Science Foundation, and a National Institutes of Health Institutional Grant to the University of Arizona.

REFERENCES

ABE, K., BUTCHER, R. W., NICHOLSON, W. E., BAIRD, C. E., LIDDLE, R. A. & LIDDLE, G. W. (1969). Adenosine 3′,5′-monophosphate (cyclic AMP) as the mediator of the actions of melanocyte stimulating hormone (MSH) and norepinephrine on the frog skin. *Endocrinology*, **84**, 362–368.

BARTELSTONE, H. J., NASMYTH, P. A. & TELFORD, J. M. (1967). The significance of adenosine cyclic 3′,5′-monophosphate for the contraction of smooth muscle. *J. Physiol., Lond.*, **188**, 159–176.

BITENSKY, M. W. & BURSTEIN, S. R. (1965). Effects of cyclic adenosine monophosphate and melanocyte-stimulating hormone on frog skin *in vitro*. *Nature, Lond.*, **208**, 1282–1284.

FALK, G. & GERARD, R. W. (1954). Effect of micro-injection of salts and ATP on the membrane potential and mechanical response of muscle. *J. cell comp. Physiol.*, **43**, 393–403.

GOLDMAN, J. M. & HADLEY, M. E. (1969). *In vitro* demonstration of adrenergic receptors controlling melanophore responses of the lizard, *Anolis carolinensis*. *J. Pharmac. exp. Ther.*, **166**, 1–7.

HADLEY, M. E. & BAGNARA, J. T. (1969). Integrated nature of chromatophore responses in the *in vitro* frog skin bioassay. *Endocrinology*, **84**, 69–82.

HADLEY, M. E. & GOLDMAN, J. M. (1969). Physiological color changes in reptiles. *Am. Zool.*, **9**, 489–504.

HOROWITZ, S. B. (1958). The energy requirements of melanin granule aggregation and dispersion in the melanophores of *Anolis carolinensis*. *J. cell comp. Physiol.*, **51**, 341–357.

LERNER, A. B. (1959). Mechanism of hormone action. *Nature, Lond.*, **184**, 674–677.

NOVALES, R. R. & DAVIS, W. J. (1967). Melanin-dispersing effect of adenosine 3′,5′-monophosphate on amphibian melanophores. *Endocrinology*, **81**, 283–290.

NOVALES, R. R., NOVALES, B. J., ZINNER, S. H. & STONER, J. A. (1962). The effects of sodium, chloride, and calcium concentration on the response of melanophores to melanocyte-stimulating hormone (MSH). *Gen. comp. Endocr.*, **2**, 286–295.

POSTERNAK, TH., SUTHERLAND, E. W. & HENION, W. F. (1962). Derivatives of cyclic 3′,5′-adenosine monophosphate. *Biochem. biophys. Acta*, **65**, 558–560.

ROBISON, G. A., BUTCHER, R. W. & SUTHERLAND, E. W. (1967). Adenyl cyclase as an adrenergic receptor. *Ann. N.Y. Acad. Sci.*, **139**, 703–723.

SHIZUME, K., LERNER, A. B. & FITZPATRICK, T. B. (1954). *In vitro* bioassay for the melanocyte stimulating hormone. *Endocrinology*, **54**, 553–560.

SUTHERLAND, E. W., ØYE, I. & BUTCHER, R. W. (1965). The action of epinephrine and the role of the adenyl cyclase system in hormone action. *Recent Prog. Horm. Res.*, **21**, 623–642.

SUTHERLAND, E. W., ROBISON, G. A. & BUTCHER, R. W. (1968). Some aspects of the biological role of adenosine 3′,5′-monophosphate (cyclic AMP). *Circulation*, **37**, 279–306.

TERCAFS, R. R. (1966). Chromatophores and permeability characteristics of frog skin. *Comp. Biochem. Physiol.*, **17**, 937–951.

TURTLE, J. R. & KIPNIS, D. M. (1967). An adrenergic receptor mechanism for the control of cyclic 3′,5′-adenosine monophosphate synthesis in tissues. *Biochem. biophys. Res. Commun.*, **28**, 797–802.

WRIGHT, M. R. & LERNER, A. B. (1960). On the movement of pigment granules in frog melanocytes. *Endocrinology*, **66**, 599–609.

(*Received August* 7, 1969)