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THE BIOCHEMISTRY OF MELANOTROPIC AGENTS[1]

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I. INTRODUCTION

Control of skin pigmentation entered the realm of pituitary endocrinology in 1916 when Allen (2) and Smith (110) independently found that hypophysectomized tadpoles become light in color and finally turn a transparent silvery gray. Later it was shown that such animals darken rapidly after injection with extracts of bovine pituitary intermediate lobe or merely on swimming in solutions containing this material (4). The active principle has been termed melanocyte-stimulating hormone, MSH, melanotropin, intermedin, chromatophore-stimulating hormone, and chromotropin. In conformity with general biochemical usage the names melanocyte-stimulating hormone, melanotropin, or MSH will be used interchangeably in this review of the biochemistry of melanotropic agents.

II. BIOASSAY

The action of a variety of agents in darkening pigment cells of the skin of frogs, fish, or lizards *in vitro* or *in vivo* forms the basis for quantitative bioassay.

A. Assay in vivo

MSH is assayed by a microscopic examination of melanocytes in interdigital webs after injection of active material into the dorsal lymph sac of hypophysectomized frogs (65). The amount of agent required to disperse the pigment granules can be measured quantitatively by comparison with the Hogben index (Fig. 1), which defines standard levels of dispersion. The index illustrates the appearance of a typical melanocyte as it passes from the resting state to the fully stimulated state, in which the melanin granules are completely dispersed. Light skin corresponds to Stage 1 and fully darkened skin to Stage 5. Contrary to appearances, the phenomenon is not literally one of expansion and contraction, although it is conveniently described by these terms, but rather a movement of melanin granules from about nuclei into cytoplasmic processes making them visible (83).

B. Assay in vitro

A widely employed assay for MSH *in vitro* measures photometrically the decrease in reflectance, *i.e.*, darkening, of unit areas of frog skin bathed in a buffer solution (78). Statistically, this assay is more accurate than assay *in vivo* since each measurement is the sum of the response of a large number of melanocytes; however, the assay *in vivo* more closely approximates physiological conditions.

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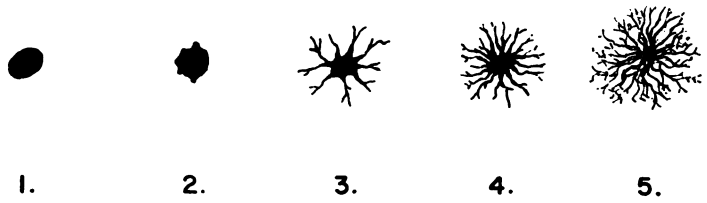


FIG. 1. The Hogben index. Frog skin melanocytes in Stage 1 contain melanin granules aggregated about the nucleus. In Stage 5 the granules are fully dispersed throughout the cellular processes. (From Snell, R. and Kulovich, S, J. invest. Derm., 1966, in press, with permission of authors and Williams & Wilkins Co., Publ.)

Burgers (10) described an assay with the isolated skin of *Anolis carolensis*. In this animal melanocytes are equally distributed over the entire dorsal area and as many as 80 usable portions can be obtained from a single skin; a frog yields only 4.

C. Immunochemical assay

It has not been possible to demonstrate the production of specific antibodies to α-MSH, but when this peptide is coupled to rabbit serum albumin (RSA) by a carbodiimide reagent, an antiserum specific for the MSH-RSA conjugate can be developed (84). This technique also has been used to form antibodies to angiotensin- and bradykinin-RSA conjugates (35). Antibodies are detected by complement fixation (122). Specificity is assessed by measuring the extent of cross reaction with other peptide-RSA complexes and by the hapten inhibition of complement fixation by free peptides, in this case, α- and β-MSH and corticotropin (ACTH). Anti-α-MSH-RSA does not cross-react with β-MSH or ACTH but can neutralize the effect of α-MSH on frog skin *in vitro*. Therefore, this technique can be usefully applied toward enhancing the specificity of assay *in vitro*.

D. Assay of lightening activity

Several agents that lighten melanocytes are known and will be discussed in Section IV. The development of a quantitative assay for the lightening phenomenon (78) led to the isolation of melatonin, a highly potent MSH inhibitor found in the pineal gland and nerve tissue (71).

Maximally darkened skin cannot be stimulated to revert completely to its original light state, possibly because of a difference in the response of dermal and epidermal melanocytes to lightening agents (85). While both types darken rapidly with MSH and lighten when MSH is washed away, only the dermal cells respond to lightening agents such as melatonin, acetylcholine, or norepinephrine. The epidermal melanocytes of frogs appear strikingly similar to mammalian pigment cells in morphology and in physiologic characteristics as well (85). For example, long-term administration of melatonin lightens neither the melanocytes of guinea pigs (115) nor epidermal melanocytes of frogs. The mode of response of frog epidermal melanocytes appears to lie between that of typical submammalian and mammalian melanocytes and is, therefore, worthy of special study.

III. BIOCHEMISTRY OF MELANOTROPIC PEPTIDES

A. Naturally occurring peptides

1. Isolation procedures. The first stages in the isolation of pituitary melanotropins are similar to those used in the purification of ACTH (14): acid extraction of defatted glands followed by adsorption of the acid extract on oxycellulose. Countercurrent distribution and zone electrophoresis of the oxycel-adsorbate provide homogeneous preparations. The original experiments yielded only very small quantities of pure material, but improved methods for the preparative isolation of melanotropic peptides, as well as other peptides, have become available. A gram of β-MSH was obtained from an oxycellulose concentrate of porcine pituitary extracts by a combination of gradient elution on carboxymethyl cellulose (CMC) and diethylaminoethyl cellulose (99); in the same series of experiments, α-MSH was separated from lysine vasopressin by countercurrent distribution. About a gram of α-MSH was also isolated from a defatted posterior pituitary preparation of bovine origin (101). In this case, α-MSH was separated easily from vasopressin by ion exchange chromatography since the bovine pressor hormone contains arginine, not lysine. It is noteworthy that large amounts of α-MSH are present in the neurohypophysis.

On an even larger preparative scale, the first stages in the fractionation of extracts from about 7 million pituitary glands have been reported (63). Chromatography on CMC followed by gel filtration on Sephadex G-25 revealed the presence of a large number of peptides, many still inhomogeneous. The purpose of these experiments is to isolate and characterize many of the peptides as well as to obtain large quantities of homogeneous α- and β-MSH for clinical studies in man. New peptides isolated in these experiments are available to other investigators for biological studies.

A generally held belief is that the pituitary gland produces several distinct peptide hormones that regulate specific metabolic processes. We know that some amino-acid sequences in α- and β-MSH and ACTH overlap. Hence, it would not be surprising if the many peptides in the anterior and intermediate lobes of the pituitary contained similar sequences. That is, growth hormone and thyroid-stimulating hormone may possess large segments identical to those found in the hormones with structures already known. In addition, all these peptides may have an effect on all cells in the body. In some cells the activity may be pronounced; in others it may be very small but not zero. The study just described may help to clarify this issue.

2. Structure of MSH peptides. The first homogeneous melanotropic hormone, α-MSH, a basic acetyl tridecapeptide amide, was described in 1955 (74); the sequential structure of α-MSH was reported 2 years later (39) followed by proof of structure by synthesis (51, 106). An α-melanotropin has also been found in the pituitary glands of frogs (11), cattle (32, 33), horses (16), and monkeys (68) (Table 1).

During this period β-MSH, a second, highly active pituitary peptide, was isolated and characterized. β-Melanotropins from hypophyses of pigs (29, 40),

TABLE 1

The structure of the naturally-occurring melanotropins[a]

ACTH (N-terminus, all species)				H-	Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val	Gly	Lys	Lys	etc.
α-MSH (Beef, pig, horse, monkey)				Ac														NH_2			
β-MSH																					
(Beef, sheep)	H-	Asp	Ser	Gly	Pro		Lys								Ser		Pro	Lys	Asp	-OH	
(Pig, sheep)	H-		Glu																	-OH	
(Horse)	H-																Ala			-OH	
(Monkey)	H-						Ala										Pro			-OH	
(Human)	H Ala Glu Lys Lys																			-OH	

[a] Abbreviations are as follows: Asp = aspartic acid; Arg = arginine; Glu = glutamic acid; Gly = glycine; His = histidine; Lys = lysine; Phe = phenylalanine; Pro = proline; Ser = serine; Trp = tryptophan; Tyr = tryosine; Val = valine. In any column, a blank space indicates that the residue is the same as that above it. A capital "H" to the left of a sequence indicates the amino terminus; an "OH" to the right is the carboxyl terminus of the peptide. Within the heavy lines are enclosed sequences that ACTH and α- and β-MSH hold in common. Shaded areas indicate the positions of variation of the β-melanotropins from porcine β-MSH.

cattle (30, 31), horses (17), monkeys (68), and man (15, 38) have been described. Pituitaries of sheep contain β-MSH of both the bovine and porcine types (28). Extracts of single ovine glands on electrophoresis can be resolved into three components with MSH activity with mobilities identical to those of standard samples of α-MSH, β-(beef)-MSH, and β-(pig)-MSH. The melanocyte-stimulating hormones of deer, lizards, and codfish have also been studied (11). The total synthesis of porcine β-MSH was described in 1963 (107).

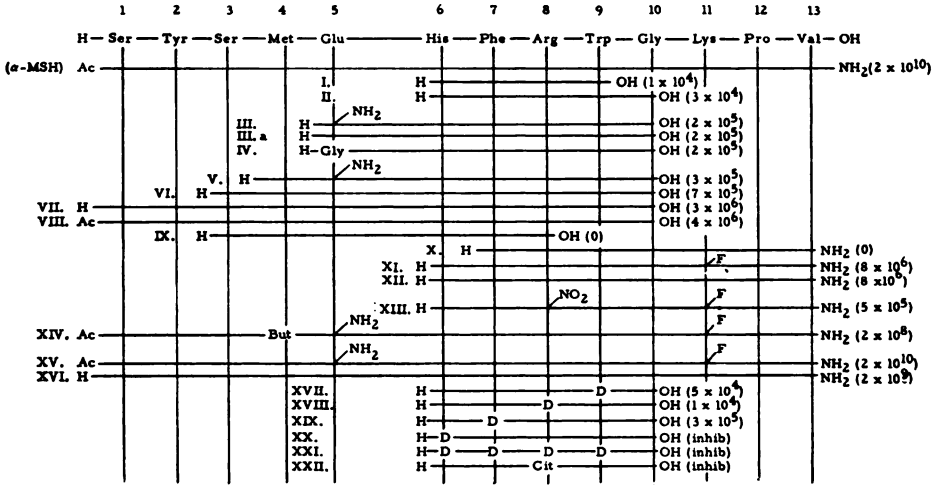
A comparison of the structures of ACTH and α- and β-melanotropins (Table 1) discloses that the first and second hormones contain identical amino terminal tridecapeptide sequences while all three share the heptapeptide sequence -Met-Glu-His-Phe-Arg-Try-Gly-. This common core is considered to be the portion of the molecule directly responsible for eliciting the melanocytic response, and its presence in the corticotropin structure accounts for the MSH activity of this peptide.

B. Synthetic peptides: analogues of α-MSH

There are many reasons for preparing peptide hormones synthetically. Only when the physical, chemical, and biological properties of a natural and a synthetic product are identical can the proposed structure of the former be considered proved. When a synthetic product exhibits the expected biologic activity, the possibility is eliminated that traces of a potent contaminant were responsible for the effect of the natural product. The question of possible contamination also arises when a natural product exhibits more than one pharmacologic effect. Furthermore, synthesis makes available peptide subunits or other desired ana-

TABLE 2

Structure-activity relationships of synthetic α-MSH analogues[a]



[a] Abbreviations are as follows (see Table 1): Ac = acetyl; But = 1-α-aminobutyric acid; F = formyl; NO_2 = nitro-*l*-arginine; Cit = *l*-citrulline; D = dextro configuration. Vertical lines indicate the amino acid residues at the top of the columns. Horizontal lines indicate the length of the MSH-analogue: each vertical line crossed by a horizontal stands for an amino acid included in the sequence. On the horizontal, secondary lines are attached to the right of those residues containing ω-blocked groups. The numbers to the right express the number of international units of melanotropic activity *in vitro* per gram of compound.

logues containing unnatural amino acids. Such analogues may be useful in correlating the chemical structure of a hormone with its function or specific activity. Finally, production of synthetic peptides can provide pure material in quantities often difficult or even impossible to obtain from natural sources. Biochemical and physiological studies of bradykinin and angiotensin are based almost entirely on synthetic hormones.

Many analogues of α-MSH have been synthesized. These experiments have been reviewed in detail (42) and only some of the highlights will be presented here (Table 2). The hypothesis that the seat of melanotropic activity is the heptapeptide core common to the melanotropins and ACTH was substantiated experimentally when this and even smaller peptides exhibited a measurable melanotropic effect (48). Peptide II, a key intermediate in the synthesis of larger MSH peptides, is one of the smallest in which melanotropic activity was found. Recently tetrapeptide I was reported to be active (92). The addition of amino acids to the amino terminus of II results in a stepwise increase in biological activity [III (56), V (57), VI (46), VII (49, 50, 108)]. Blocking the terminal amino group generally is accompanied by a further enhancement of activity; compare VIII (50) to VII and XVI (37) to XV. Peptides containing high molecular weight derivatives of the terminal amino moiety are less active than those with smaller blocking groups (52). Amidation of the γ-glutamyl carboxyl group at position 5

does not affect activity significantly (compare III and IIIa); in fact since the glutamyl moiety can be replaced by glycine (IV) without loss of activity, the free carboxyl group must be nonfunctional in the stimulation of melanocytes (104).

The importance of tryptophan at position 9 and histidine at 6 is suggested by the lack of biologic effect in peptides IX and X (47). Comparison of these values with the high activity of peptide XI provides further evidence for the important role of histidine.

The ϵ-amino functional group of lysine at position 11 can be formylated without detriment (compare XI and XII) (50) but similar peptide derivatives with larger N^{ϵ}-acyl moieties are less active (52). The diminished biological activity that accompanies N^{α}-carbobenzoxylation or N^{ϵ}-tosylation may be the result of steric hindrance of interaction between the peptide derivative and the target cell receptor site (50). Alternatively, the effect may be related to the diminished solubility or transportability of these derivatives across cellular membranes. The highly ionic guanido function of arginine at position 8 can be neutralized by nitration without complete obliteration of activity (XIII) (45); this indicates that the guanido moiety enhances activity but is not indispensable. Oxidation of methionine at position 4 to the sulfoxide causes a sharp drop in melanotropic action but if the thiomethyl group is removed entirely (XIV) (44), the product is still quite potent (compare XIV with α-MSH). Other cases are known in which analogues containing a blocked functional group are less active than those from which the functional group is totally absent (19). Amino terminal acylation enhances the melanotropic activity of the tridecapeptide, since desacetyl α-MSH (XVI) (37, 49, 108) is an order of magnitude less active than the parent hormone.

Lengthening the chain beyond the tridecapeptide size of peptides related to α-MSH results in a hundredfold diminution in melanocytic stimulatory power. The N-terminal hexadecapeptide of ACTH has about 4×10^8 units per gram (43); the tricosa peptide about 2×10^8 units per gram (53); and ACTH (a nonatriacontapeptide) exhibits 1×10^8 units per gram (67). On a molar basis these are almost equal.

A limited number of MSH-peptides containing D-amino acids have been prepared, for studying a hormonal potentiation and prolongation effect that will be described later. The melanotropic activity of these peptides indicates that the stereospecificity of the target cell is rather low. Pentapeptides with the sequence 6 to 10 of α-MSH (Table 2) containing D-phenylalanine (XIX) (103) or D-tryptophan (XVII) (127) are many times more active than the all-L-diastereomers. The D-histidyl isomer (XX) is weakly inhibitory and the all-D-derivative (XXI), strongly so, exhibiting about one millionth the activity of melatonin on a weight basis (128). It will be interesting to see whether the anti-MSH activity of D-histidyl peptides is enhanced with increasing chain length in parallel with the increase of MSH activity with chain length in the all-L-peptides. A pentapeptide containing citrulline in place of arginine (XXII) also exhibits a weak melanotropin antagonism (9).

Gros and Leygues (36) reported the isolation of Phe-Arg-Try-Gly-Lys-

TABLE 3

Activity in vitro (MSH units per gram) reported from various laboratories

Peptides (all synthetic)	Lerner *et al.*	Activity[a] Liddle *et al.*	Schally *et al.*
His-Phe-Arg-Trp-Gly	0	4×10^3	4×10^3
Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0	1×10^6	2×10^4
Form I: Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0	1×10^4	2×10^4
Phe-Arg-Trp-Gly-Ser-Pro-Pro	0	2×10^3	4×10^3

[a] For these assays, the authors are grateful to Drs. G. W. Liddle and R. Ney of Vanderbilt University and Drs. A. V. Schally and A. Kastin of the Veterans Administration Hospital, New Orleans.

Pro-Val-NH_2 from extracts of neural lobes of porcine pituitaries. This peptide, the carboxyl-terminal heptapeptide of α-MSH, showed a melanotropic activity *in vitro* of 10^6 units per gram. The synthetic derivative was similarly found to be active (88) in contrast to previous observations indicating the requirement of histidine for melanotropic activity (42). We have been unable to confirm the results of Gros and Leygues. An analogous heptapeptide derived from β-MSH, Phe-Arg-Try-Gly-Ser-Pro-Pro was found in pituitary extracts (63) and exhibited weak melanotropic activity, but the fact that the synthetic analogue was inert (129) indicates that traces of extraneous material were responsible for the melanotropic activity of the native peptide and further substantiates the initial observations relating the presence of histidine to melanotropic activity.

To correlate all of these results, we assayed the compounds in Table 3 and submitted them to other laboratories for assay. It is apparent that even qualitative agreement is difficult to attain in a study of peptides with marginal biologic activity. These "interlaboratory variations" in the assay of weakly active peptides might be caused by differences in the test animals, such as variations in their source, rate of growth, type of feeding, methods of transportation or storage, or seasonal changes in skin response. The data obviously do not provide a sound basis for determining structure-activity relationships, and until additional (and more reproducible) evidence is presented, the original observations concerning the requirement for histidine and tryptophan should be considered valid (42). It should also be noted that by more recent analyses the pentapeptide His-Phe-Arg-Try-Gly is about an order of magnitude less active than recorded in the older literature.

Interpretations regarding the requirement of functional groups for activity based on the study of subunit analogues can be qualitatively wrong for yet another reason. In studies on structure-activity relationships of angiotensin analogues, Schwyzer *et al.* (105) found that a tetrapeptide containing tyrosine was weakly active while a similar one containing phenylalanine was inert. In a larger, more active peptide, replacement of tyrosine with phenylalanine caused diminution in activity but not obliteration. While the former experiment indi-

cated an absolute requirement for the phenolic hydroxyl group, the latter showed that the hydroxyl moiety merely enhanced activity.

The study of subunit analogues is only one approach toward assessing structural requirements for biological activity. Small peptides are employed in these experiments because they are much easier to synthesize than large ones. Furthermore, many of them represent intermediates in the synthetic route to larger peptides. Although these products should be included in biologic studies, intermediates prepared for further synthesis are often not the peptides of choice for the evaluation of structure-activity relationships. Perhaps a more fruitful approach is to assay analogues equal to the native hormone in molecular size in which one amino acid at a time is varied rather than removed. Many oxytocin-vasopressin derivatives of this kind have been prepared (42) but very few in the α-MSH series (44) and none in the β-MSH. The replacement of histidine or tryptophan in acetyl tridecapeptide amide analogues might provide a more secure basis for determining the level of requirement of these key amino acids. Perhaps with improved methods of peptide synthesis such as the "solid phase" technique (86) some new, full-sized analogues of the melanotropins will become available. The amino-acid replacement approach has its limitations, however: substitution of one amino-acid residue for another can cause a sharp change in activity, but what effect would follow replacement at the same position in the chain by a second amino acid or a third? Speculations and experiments of this kind can continue endlessly.

In spite of a sizable accumulation of empirical data concerning structure-activity relationships of melanotropic peptides, a basis for the relationship between peptide structure and melanotropic function has not become evident, nor is the nature of the reaction between hormone and target cell any better understood. In view of the observed specificity requirements for hormone action, one assumes that at some level a direct interaction occurs between peptide and receptor site, perhaps in a manner analogous to the formation of an enzyme-substrate complex. For example, studies on enzyme-substrate-inhibitor interactions have been useful in determining the characteristics of the active sites of enzymes (124). Similar investigations in the peptide hormone field have not been as successful.

As mentioned above, weakly active or biologically inert peptide analogues rarely have exhibited even a minor degree of inhibition, indicating that peptide-receptor site and enzyme-substrate interactions may not be analogous phenomena. The hypothesis of a specific hormone-receptor site interaction requires the structures of the peptide and receptor site to be complementary in some way. Were binding forces primarily ionic, the charge distribution of receptor site should be opposite that of hormone. Thus, the structure of a hormone should provide some clue to the nature of its receptor site. Yet, a comparison of the structure of α- and β-MSH reveals no common relationship to account for the millionfold increase in specific activity that these two peptides exhibit relative to the core peptide whose structure they share (Table 1). At present there is little basis for speculation on the chemical nature of the receptor site. Perhaps the peptide bonds of the remainder of the molecule enhance biologic activity by binding the active

core to the receptor site; in this case the length of the chain rather than its specific amino-acid sequence would be the factor contributing to the high activity of the native hormone relative to subunit analogues. In accord with this view, the activity of peptides III and IV (Table 2) are equal and about tenfold that of II, one residue smaller in size.

A model system for the study of possible peptide hormone-receptor site interactions has been presented in which enzymatically active ribonuclease is formed from the stoichiometric but noncovalent reaction of inactive ribonuclease S-protein and S-peptide (96). The relationships between the structure of various synthetic subunit analogues of S-peptide and their enzyme-regenerating capacity with S-protein form a pattern similar to the structure-activity relationships found in the MSH series (25). A weakly active "core" was found and its intrinsic enzyme-regenerating powers could be enhanced in a steplike manner by lengthening the polypeptide chain. A mechanism of peptide hormone action based on enzyme activation is attractive because it accounts for the catalytic behavior exhibited by these highly potent physiologic agents. Although a small degree of enzyme activation can be demonstrated in glandular preparations stimulated by specific tropic hormones (97), there is no evidence that this results from direct interaction between hormone and enzyme precursor.

C. Prolongation and potentiation of MSH peptides

1. Treatment of peptides with alkali. A study of alkali-treated MSH peptides may be another approach toward clarifying the nature of the peptide-receptor site interaction. When fully darkened, MSH-treated frog skin is washed with fresh buffer, rapid lightening occurs; this indicates that the hormone is rapidly inactivated or dissociated from the skin. Lightening is not observed when skin darkened with alkali-treated MSH is washed repeatedly with fresh buffer (66) (Fig. 2) (73). A prolongation is observed also *in vivo*; the darkening action of a single dose of hormone lasts for days instead of hours as with native material.

Originally, alkali treatment of crude preparations was reported to effect potentiation as well as prolongation of activity (111). When homogeneous α- or β-MSH is treated similarly, *i.e.*, with 0.1 N sodium hydroxide for 10 minutes in a boiling water bath, prolongation ensues but not potentiation (66). The marked potentiation in crude preparations may have resulted from partial degradation of ACTH present in the crude extracts to peptides more nearly the size and activity of α-MSH. An apparent potentiation also might result from the destruction of proteolytic enzymes. MSH activity is particularly susceptible to trypsin and chymotrypsin.

2. Differences in chemical structure between native peptides and those with the prolonged effects. Speculations on the chemical changes responsible for prolongation have included the possibilities of degradation of the guanido moiety of arginine to ornithine (80) or citrulline (9). Studies with synthetic ornithyl or citrullyl peptides have not supported these theories. When Raben (94) noted a decrease in optical activity of a partially purified pituitary extract after alkali treatment, he proposed that racemization of amino-acid residues occurred within intact pep-

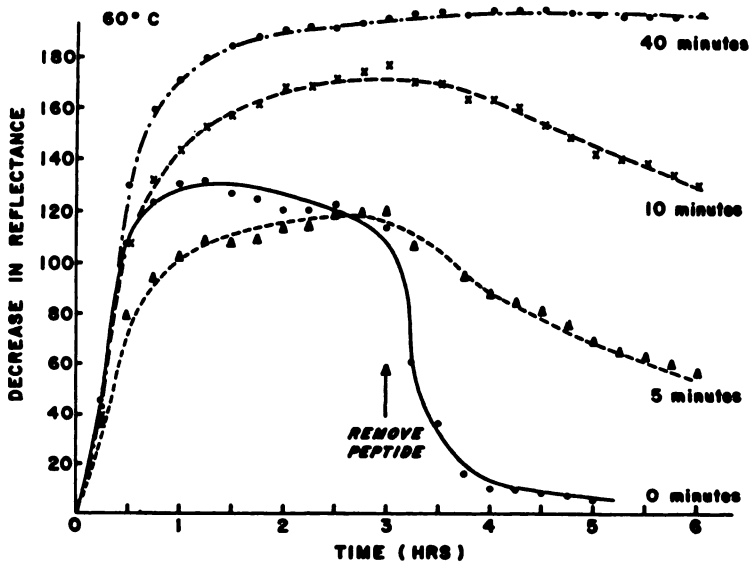


FIG. 2. Prolongation of the action of α-MSH *in vitro* as a result of preincubation of the hormone with alkali for various periods of time. Activities are shown for peptides treated for 0, 5, 10, and 40 minutes in 0.1 N sodium hydroxide at 60° C. A decrease in reflectance is directly proportional to darkening of skin, *i.e.*, melanocyte expansion. Skins were exposed to peptide for 3 hours and then washed with fresh buffer. Skin treated with native hormone reverted to its initial light state. Skin exposed to alkali-treated peptides became resistant to lightening. (From Lerner, A. B., Lande, S., Kulovich, S.: Exerpta Medica int. Congr. Series No. 83: 392–397, 1965, with permission of Exerpta Medica Foundation.)

tide chains. Synthetic L-histidyl-D-phenylalanyl-L-arginyl-L-tryptophylglycine does exhibit slight prolongation (103). In another experiment, when the same D-containing pentapeptide and the all-L-isomer were treated with alkali, the former exhibited no further increase in prolongation while the latter gained this property (5). Lee and Butter-Janusch (66) showed experimentally that incubation of pure α-MSH with alkali causes partial racemization. A quantitative evaluation of degradation of the alkali-treated product by trypsin, chymotrypsin, and various D- and L-amino acid oxidases indicated that the peptide contained fully racemized arginine, largely racemized phenylalanine, and partially racemized tyrosine, methionine, and histidine. Similar results were reported by Pickering and Li (93), who found that alkali-treated ACTH contained extensively racemized histidine, methionine, and arginine, with serine and phenylalanine racemized to a lesser degree. In this study the accompanying loss of corticotropic activity was attributed to hydrolysis of the amino-terminal seryl and tyrosyl peptide bonds.

By means of milder alkaline treatment of MSH and ACTH peptides (0.1 N sodium hydroxide, 40 minutes, 60°C), the full effect of prolongation is attained, as determined by the assay *in vitro*, but without accompanying degradation of peptide material (73). Under these conditions an enhancement in specific MSH-activity as well as prolongation is observed; this is consistent with the higher

specific activity of L-His-D-Phe-L-Arg-L-Try-Gly relative to the all L-isomer (103). With a similar mild alkaline treatment, ACTH exhibits prolonged MSH activity and retains at least one half of the corticotropic activity of the native hormone. However, no evidence could be found for prolonged action on the adrenal cortex (73). Studies are in progress to determine the extent of racemization of these alkali-treated peptides prepared under milder conditions.

Although racemized MSH exhibits resistance to enzymic degradation, prolonged hormonal action is not necessarily due to an increased half-life of the alkali-treated peptide in the tissue. Skin darkened with racemized α-MSH is resistant to the lightening action of melatonin as well as to washing with fresh buffer (62), whereas the effect of the native hormone is readily reversed by either method. Unless the response to melatonin is also mediated enzymically these observations indicate that the prolongation effect is related to a phenomenon other than simple resistance to enzyme attack. Perhaps because of configurational changes in the peptide it is bound more strongly to the receptor site. It is important that α-MSH be made with D-amino acids in one or two places. If the site of racemization can be pinpointed, it may be possible to produce well-characterized peptides more active than the natural hormone.

3. Prolongation unrelated to alkaline treatment. A comparison of the melanotropic activities of corticotropin A_1 and N^{α}-acetyl corticotropin A_1 1 hour after administration *in vivo* shows them to be equipotent (120); 6 hours after administration the activity or potency ratio of acetylated to free peptide is 10. Similarly, β-MSH is 100 times more active than corticotropin A_1 after 1 hour *in vivo* but only 33 times more active after 6 hours. In another experiment *in vivo*, the durations of activity of native α-corticotropin and alkali-treated, partially purified porcine MSH were almost identical (64). The inherent prolonged melanotropic action of native α-corticotropin can be diminished by esterification of the hormone with methanolic HCl (79); this abridgement can be reversed by mild saponification of the esterified hormone. The chemical basis for these effects *in vivo* is unknown; it may involve changes in susceptibility to enzymic degradation.

D. Intermediary metabolism of the melanotropins

Although the mechanism of MSH biosynthesis in the pituitary is unknown, a study of the synthesis of ACTH in hypophyseal tissue has been reported (1). In view of the structural similarities of ACTH and α-MSH, it is probable that their biosyntheses will be *via* similar if not intimately associated routes.

It appears that secretion of pituitary tropic hormones is mediated by polypeptide "releasing factors" produced by the hypothalamus (95). Evidence for an MSH-releasing factor was reported by Taleisnik and Orias (118), who showed that intravenous injection of extracts of rat stalk median eminence decreased MSH levels in rat pituitary. Conversely, Schally et al. (100) have found evidence for the existence of an inhibitor of MSH release in hypothalamic tissue.

The fate of circulating MSH is only partially known. In spite of the susceptibility to proteolysis of the melanotropic hormones, 4 to 6 % of the activity introduced by intramuscular injection into man can be recovered in the urine (76). Whether this represents degraded and hence less active material or a small per-

centage of the fully active hormone is not known. The liver was shown by Shizume and Irie (109) to be an important MSH-inactivating site.

IV. AGENTS OTHER THAN MELANOTROPIC PEPTIDES THAT AFFECT MELANOCYTES

A. Organic reagents

Caffeine has been the most widely studied melanin-dispersing agent other than MSH (126). *In vitro* 5×10^{-6} M solutions elicit complete melanocyte expansion. Theophylline, another xanthine, is also quite active (13). MSH is the most potent melanotropin, however, as skin responds well to 2×10^{-11} M solutions.

Several inhibitors of melanotropic action are known: the dispersing action of MSH or caffeine *in vitro* is reversed by melatonin (1×10^{-11} M) (71), epinephrine (2×10^{-5} M) (121), and norepinephrine (1×10^{-6} M) (125). Acetylcholine (1×10^{-6} M), a more specific reagent, inhibits or reverses the effect of MSH but not that of caffeine *in vitro* (125). Skins of only one third of frogs tested respond to acetylcholine (87); the difference between acetylcholine-sensitive and -resistant frogs is not known. Another lightening agent, hydrocortisone (1×10^{-3} M), inhibits the effect of MSH in *Rana pipiens* (125) but not in *Hyla arborea* (13).

B. Influence of cations

The kind and concentration of cations in the bath medium strongly affect the melanocytic response *in vitro*. In an isotonic buffer lacking sodium, *Rana pipiens* skin is completely resistant to the melanin-dispersing action of MSH (90). The same phenomenon is observed in hypotonic sodium-Ringer's buffer (125). The effect of MSH in *Rana* skin is apparently mediated in some way by sodium ion. Novales (90) believes that MSH acts by stimulating sodium transport into the melanocyte and suggests that MSH may act to increase the sodium permeability of the cell membrane, inhibit an intracellular sodium extrusion pump, or affect a sodium-sensitive intracellular enzyme system. At present there is no experimental basis for a choice among these or other possibilities.

The dispersing action of caffeine on frog skin *in vitro* is independent of sodium ion concentration (89, 125). Since the action of caffeine can be inhibited or reversed by melatonin or epinephrine (125), it is apparent that both dispersion and aggregation are functions independent of sodium ion. Under physiologic conditions, the expansion of melanocytes may involve a series of stages in which the action of MSH, with accompanying sodium uptake, precedes any movement of melanin granules. Similarly, the aggregation of melanin granules, which is also independent of sodium concentration, may be complete before sodium extrusion occurs. Acetylcholine, an inhibitor of MSH but not of caffeine, may operate at the same level as MSH in the overall reaction. A rough scheme of these experimental observations is illustrated in Figure 3.

The qualitative requirement for ions varies in different test animals. In *Hyla arborea* skin, calcium rather than sodium ion is required for stimulation by MSH (14, 24, 119). Dikstein and Sulman (13) concluded that, in *Hyla*, melanin dispersion is accompanied by calcium uptake and aggregation by its release.

The melanocytic response to stimulation includes a change in membrane polar-

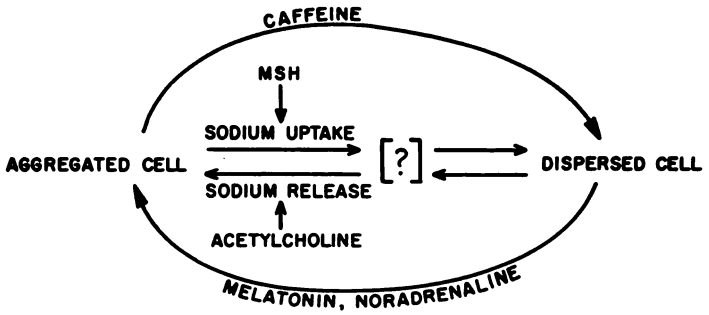


FIG. 3. A possible relationship of the factors which stimulate or inhibit melanin dispersion *in vitro*.

ity as well as ion transport. In this respect, the response of melanocytes resembles that of nerves. In expanded fish scale melanocytes the charge on the centrosphere is negative relative to cell processes, whereas in contracting or contracted cells this polarity is reversed (59). Embryologically, melanocytes are derived from the neural crest. Melanin-aggregating agents such as norepinephrine and acetylcholine are better known as chemical transmitters of nerve stimulation. Serotonin is another one of a large group of reagents active in both systems (91). β-MSH has a stimulatory effect on certain nerve tracts (61) and is present in neural tissue. The MSH-inhibitor melatonin is also found in nerve cells (72).

Caffeine, well known for promoting alertness by some kind of action on the central nervous system, also stimulates contraction of skeletal muscle. Calcium-deficient muscle refractory to stimulation still responds to caffeine (7); this is analogous to the behavior of sodium-deficient melanocytes. The action of caffeine on different types of cell appears to be associated with effects on enzymes controlling cyclic 3′,5′-adenosylmonophosphate (AMP) levels (11a). Many types of target cells respond to hormonal stimulation by formation of increased quantities of intracellular cyclic AMP (41); frog skin melanocytes are no exception. A correlation between extent of melanocyte dispersion and increase in cyclic AMP in frog skin *in vitro* has been shown (8). Although cyclic AMP activates certain enzymes, its role in the skin-darkening response remains to be established. The melanocyte may be a slow-motion model of stimulation phenomena in general, often too rapid for convenient observation or study (13).

C. Energy requirements for the melanocytic response

The response of *Rana pipiens* skin to MSH or caffeine is completely inhibited in the absence of oxygen (77); this indicates that aerobic metabolism must continue for expansion to occur and that melanocyte dispersion requires energy. Aggregation of cells proceeds normally in the absence of oxygen. When skin is repeatedly caused to lighten and darken, it becomes unresponsive to further stimulation. This "fatigued" state can be relieved by adenosine triphosphate (ATP), a weak dispersing agent. Whether ATP will replace oxygen in melanocyte expansion is not known.

Dinitrophenol, an uncoupler of oxidative phosphorylation, inhibits melano-

cyte dispersion *in vitro* in *Rana pipiens* skin (77). These experiments support the above observations and indicate that dispersion of melanin granules requires energy and that ATP formed *via* oxidative phosphorylation is the energy source for the cellular response. In *Anolis* skin, the opposite effects are seen: dinitrophenol is a darkening agent and ATP stimulates lightening (54). In this species, aggregation of granules apparently consumes more energy than dispersion. The same appears true in *Hyla arborea*, in which the rate of aggregation is temperature-dependent, but dispersion continues independently of this parameter (13). It will be interesting to see if the formation of cyclic AMP is related to any of these processes.

V. PROPOSED INTRACELLULAR CHANGES DIRECTLY RELATED TO THE MOVEMENT OF MELANIN GRANULES

A. Protoplasmic sol-gel transformation and streaming

Although it is generally agreed that ion transport accompanies the melanocytic response under physiologic conditions, the fact that melanocytes respond to caffeine in the absence of sodium indicates that other changes in the cell are more directly involved in the movement of melanin granules. Marsland (82) showed that gel-to-sol transformation occurs in the protoplasm of expanding melanocytes. He found that cells exposed to high hydrostatic pressures expand spontaneously, and that at higher temperatures higher pressures were required. Gels classified as type III colloids behave in this manner (26). Marsland also found that melanin granules of dispersed cells can be displaced by centrifugation at 70,000 $\times$ g while particles in lightened or aggregated cells do not move at 125,000 $\times$ g (82). These data are consistent with the view that in the dispersed melanocyte the protoplasm is in a sol-state, but gelled in the contracted cell.

It has been proposed that melanin granules are attached to a network of protein fibrils located on cytoplasmic structures radiating from the center of the cell and that MSH-induced sodium uptake causes solation and streaming of protoplasm toward the cell center; melanin granules then disperse outwards to fill the void left by the inward flow of protoplasm (77).

B. The action of contractile fibers

Falk and Rhodin (21) showed that fibrils were present in the zone between the inner and outer membraneous linings of the sacs containing melanin in *Lebistes reticulatus* melanocytes. They suggested that the repeated expansion and contraction of these fibrils was the driving force that moved melanin granules. Earlier studies showed similar findings (117). Novales (91) has observed that in cell culture embryonic melanocytes literally expand and contract when stimulated by darkening and lightening agents respectively.

C. Intracellular electrophoresis

Experiments described by Kinosita (58, 59, 106) indicate that the movement of melanin granules in fish scale melanocytes is the result of electrophoretic migration in a potential field formed between the centrosphere and cellular pro-

cesses. In a study of the electrical potential changes in single melanocytes, it was found that the centrosphere has the higher negative potential in dispersed cells whereas the cellular processes have the higher potential in aggregated cells. When an external electric field is introduced (6, 58, 59), melanin granules migrate toward the anode and therefore must be negatively charged. Interestingly, the movement of melanin granules in dissected processes is similar to that observed in whole cells. When cut processes are exposed to aggregating agents, melanin granules retain a capacity for orderly movement; they migrate toward, and finally clump at the cut, proximal end. This suggests that aggregation is not the response to forces deriving from the centrosphere, but rather is the result of repulsion of melanin granules away from the cell membrane. Kinosita also found that granules in a light cell are resistant to electrophoretic migration in an externally applied field, while those in a darkened cell respond readily to electrophoresis; this indicates that protoplasm in the expanded cell is in the sol form and in the contracted cell it is gelled.

D. Effect of colchicine on the melanophore

Colchicine appears to inhibit the formation of ordered structures. It disrupts the formation of mitotic spindles and thereby inhibits mitosis (55). It also disorganizes sarcoplast ribbons formed from striated muscle in tissue culture (34) and affects ciliary structure and function similarly (123). Colchicine-treated frog skin darkens normally but does not respond to lightening agents (81); this suggests that the aggregation of melanin granules not only is accompanied by protoplasmic gelation but is dependent upon the formation of this ordered state.

E. Conclusions

Many of the experiments just described were carried out in different systems, and it is usually not possible or wise to extrapolate results from one species to another. Kinosita's experiments (59) suggest that reversed pole electrophoresis causes aggregation while those of Malawista (81) indicate that aggregation depends on gelation. However, if reversed-pole electrophoresis is responsible for melanin aggregation, why should maintenance of the sol-state interfere with lightening? A combination of the electrophoresis and colchicine experiments might be of interest. It is possible that the mechanism of melanocyte dispersion includes protoplasmic solation *and* electrophoretic migration of granules toward the cell membrane, while aggregation is the reverse. A wave of gelation moving inward from the cell membrane could also help to displace the granules toward the nucleus.

VI. SIGNIFICANCE OF MELANOCYTIC ACTION

A. Pigmentation changes

1. Submammalian species. The functional significance of rapid color change in lower vertebrate and invertebrate types involves protective coloration and possibly thermoregulation. The former is related to active and passive phenomena such as camouflage and color displays associated with rage, fear, attraction

of mates, *etc.* These effects may be under hormonal or direct neural control, or both, depending on the species.

Although the melanocyte is the most widely studied chromophore, many other types of pigment-carrying cell are known, *e.g.*, cells containing red, yellow, white, blue, green, and brown pigments; Fingerman has prepared an extensive review of the comparative aspects of chromatophores [see (23)]. A number of MSH peptides have been tested to compare the structural requirements of peptides in stimulating melanocytes and guanophores (5). The relationships between peptide structure and chromotropic activity were identical and included a prolonged response of both cell types to alkali-treated peptides. However, the effect of peptides on the two types of cells is opposite: MSH stimulates the expansion of melanocytes but the contraction of guanophores. Guanophores are unresponsive toward any known melanin-aggregating agents.

2. Mammalian species. The functional significance of the melanocyte-stimulating hormones in mammalian species is not clear. The skin of man has been darkened over a period of days as the result of prolonged administration of large quantities of purified α- and β-MSH or ACTH, the latter in adrenalectomized subjects (75). Some of these peptides may be involved in hyperpigmentation associated with pituitary hyperfunction (70), but the role of the MSH peptides in normal physiology is still unknown. It is possible that both the presence of MSH and the capacity of mammalian melanocytes to respond to this hormone are vestigial traits and that other as yet unknown factors control skin pigmentation in higher animals.

Snell found that α-MSH (114) and β-MSH (112) increase the size and complexity of the dendritic processes of epidermal melanocytes as well as the amount of intra- and extracellular melanin in the guinea pig. Melanocytes in the sexual skin of female guinea pigs are more sensitive to estrogen than to MSH peptides (116). In the intact animal ACTH decreases intra- and intercellular melanin presumably indirectly *via* the cortisol inhibition of melanogenesis (113).

B. The relationship between intracellular melanin distribution and melanocyte metabolism

The melanotropins may stimulate melanocyte metabolism as well as the dispersion of melanin granules. An increase in total melanin content occurs after prolonged administration of MSH (27). Although no increase in cellular tyrosinase activity was found (77), there was evidence for an increase in "phenolase complex" action (60). Similarities in size, RNA content, and enzymatic activities of mitochondria and of amelanotic melanoma granules led duBay *et al.* (18) to suggest that mitochondria might be the precursors of melanin granules. Perhaps all tropic hormones affect the metabolic activity of their target cells *via* an induced rearrangement of metabolically active subcellular organelles, similar to the movement of melanin granules in melanocytes stimulated with MSH (69).

C. Extra-melanocytic activities of the melanotropins

A number of extra-melanocytic activities of MSH peptides have been reported, including the stimulation of an aqueous flare response (an increase in the concen-

tration of protein in aqueous humour resulting from leakage of protein through the blood-aqueous barrier of the eye) (20), thyrotropinlike activity (12), influence on neural transmission (61), induction of a stretching and yawning reflex in mammals after intracranial injection (22), and release of free fatty acids into serum (98). The structural requirements for lipolytic activity closely parallel those for melanocyte stimulation. A possible relationship between α-MSH and a corticotropin-releasing factor is worthy of mention (102) as well as the possibility that α-MSH is an intermediate in the biosynthesis of ACTH. Perhaps the availability of larger quantities of the pure melanotropins will help in determining the physiologic or pharmacologic significance of these activities.

REFERENCES

1. Adiga, P. R., Uemura, I. and Winnick, T.: Biosynthesis of ACTH and protein in slices of bovine anterior pituitary tissue. Biochemistry. N. Y. 4: 246–253, 1965.
2. Allen, B. M.: The results of extirpation of the anterior lobe of the hypophysis and of the thyroid of *Rana pipiens* larvae. Science 44: 755–758, 1916.
3. Astwood, E. B., Raben, M. S., Payne, R. W. and Grady, A. B.: Purification of corticotropin with oxycellulose. J. Am. chem. Soc. 73: 2969–2970, 1951.
4. Atwell, W. J.: On the nature of the pigmentation changes following hypophysectomy in the frog larva. Science 49: 48–50, 1919.
5. Bagnara, J. T.: Stimulation of melanophores and guanophores by MSH peptides. Gen. comp. Endocr. 4: 290–294, 1964.
6. Beauvallet, M. and Veil, C.: Action combinée du courant electrique et de divers poisons sur les melanophores du poisson. C. R. Seanc. Soc. Biol. 110: 698–700, 1932.
7. Bianchi, C. P.: Action of contracture-producing drugs on calcium transfer in striated muscle. J. gen. Physiol 45: 591A, 1962.
8. Bitensky, M. W. and Burstein, S. R.: Effects of cyclic AMP and MSH on frog skin *in vitro*. Nature, Lond. 206: 1283–1284, 1965.
9. Bodansky, M., Ondetti, M. A., Rubin, B., Piala, J. J., Fried, J., Sheehan, J. T. and Berkhimer, C. A.: Biologically active citrulline peptides. Nature, Lond. 194: 483–483, 1962.
10. Burgers, A. C. J.: Occurrence of three electrophoretic components with melanocyte stimulating activity in extracts of single pituitary glands from ungulates. Endocrinology 68: 698–703, 1961.
11. Burgers, A. C. J.: MSH's in vertebrates. Ann. N. Y. Acad. Sci. 100: 669–677, 1963.

11a. Butcher, R. W. and Sutherland, E. W.: Adenosine-3,5′-phosphate in biological materials. J. biol. Chem. 237: 1244–1250, 1962.

12. Cehovic, G.: Action des hormones mélanophoretiques sur la function thyroidienne chez le Cobaye. C. R. hebd. Seac, Acad. Sci., Paris 254: 1872–1874, 1962.
13. Dikstein, S. and Sulman, F. G.: Mechanism of melanophore dispersion. Biochem. Pharmac. 13: 819–826, 1964.
14. Dikstein, S., Weller, C. P. and Sulman, S. G.: Effect of calcium ions on melanophore dispersal. Nature Lond. 200: 1106, 1963.
15. Dixon, B. F.: Chromatographic isolations of pig and human MSH's. Biochim. biophys. Acta 37: 38–41, 1960.
16. Dixon, J. S. and Li, C. H.: Isolation and structure of α-MSH from horse pituitary glands. J. Am. chem. Soc. 82: 4568–4572, 1960.
17. Dixon, J. S. and Li, C. H.: The isolation and structure of β-MSH from horse pituitary glands. Gen. comp. Endoc. 1: 161–169, 1961.
18. du Buy, H. G., Showacre, J. L. and Hesselbach, M. L.: Enzyme and other similarities of melanoma granules and mitochondria. Ann. N. Y. Acad. Sci. 100: 569–583, 1963.
19. du Vigneaud, V., Winestock, G., Murti, V. V. S., Hope, D. B. and Kimbrough, R. D.: Synthesis of desamino oxytocin, a highly potent analogue of oxytocin. J. biol. Chem. 235: PC 64–66, 1960.
20. Dyster-Aas, K. and Krakau, C. E. T.: Increased permeability of the blood-aqueous humor barrier in the rabbit's eye provoked by melanocyte stimulating peptides. Endocrinology 74: 255–265, 1964.
21. Falk, S. and Rhodin, J.: Mechanism of pigment migration within teleost melanophores. *In* Proc. Stockholm Conf., pp. 213–215, Academic Press, Inc., New York, 1957.
22. Ferrari, W., Gessa, G. L. and Vargui, L.: Behavioral effects induced by intracisternally injected ACTH and MSH. Ann. N. Y. Acad. Sci. 104: 330–343, 1963.
23. Fingerman, M.: Chromatophores. Physiol. Rev. 45: 296–339, 1965.
24. Fingerman, M., Mitsuhara, M. and Oguru, C.: Effects of osmotic pressure and cations on the response of melanophores of the Fiddler Crab, *Uca pugnax*, to the melanin-dispersing principle from the sinus gland. Gen. comp. Endoc.: 3: 496–504, 1963.
25. Finn, F. M. and Hofmann, K.: Studies on polypeptides. 33. Enzymatic properties of partially synthetic ribonucleases, J. Am. chem. Soc. 87: 645–651, 1965.
26. Freundlich, H.: Some recent work on gels. J. phys. Chem. 41: 901–910, 1937.
27. Frieden, E. H. and Bozer, J. M.: Effect of administration of intermedin upon melanin content of the skin of *Rana pipiens*. Proc. Soc. exp. Biol. Med. 77: 35–37, 1951.

28. Grschwind, I. I.: *In:* Comparative Endocrinology, ed. by A. Gorbman, pp. 421–443, Wiley, New York, 1959.
29. Grschwind, I. I., Li, C. H. and Barnafi, L.: Isolation and structure of MSH from porcine pituitary glands. J. Am. chem. Soc. 78: 4494–4495, 1956.
30. Grschwind, I. I., Li, C. H. and Barnafi, L.: The isolation and structure of an MSH from bovine pituitary glands. J. Am. chem. Soc. 79: 1003, 1957.
31. Grschwind, I. I., Li, C. H. and Barnafi, L.: The isolation, characterization and amino acid sequence of an MSH from bovine pituitary glands. J. Am. chem. Soc. 79: 6394–6401, 1957.
32. Grschwind, I. I., Li, C. H. and Barnafi, L.: The structure of β-MSH. J. Am. chem. Soc. 79: 620–625, 1957.
33. Grschwind, I. I., Li, C. H. and Barnafi, L.: The isolation and structure of an MSH from bovine pituitary glands. J. Am. chem. Soc. 79: 1003–1004, 1957.
34. Godman, G. C.: Effect of colchicine on straited muscle in tissue culture. Exp. Cell Res. 8: 488–499, 1955.
35. Goodfriend, T. L., Levine, L. and Fasman, G. D.: Antibodies to bradykinin and angiotensin: a use of carbodiimide in immunology. Science 144: 1344–1346, 1964.
36. Gros, C. and Leygues, C.: Étude de peptides isolés a partir de la posthypophyse de porc. Bull. Soc. Chem. France 1964: 2840–2842.
37. Guttman, S. and Boissonnas, R. A.: Influence of the structure of the N-terminal extremity of α-MSH on the MSH activity of this hormone. Experientia 17: 265–266, 1961.
38. Harris, J. I.: Structure of a melanocyte-stimulating hormone from the human pituitary gland. Science 134: 167–168, 1959.
39. Harris, J. I. and Lerner, A. B.: Amino acid sequence of the α-melanocyte-stimulating hormone. Nature, Lond. 179: 1346–1347, 1957.
40. Harris, J. I. and Roos, P.: Amino-acid sequence of a melanophore stimulating hormone. Nature, Lond. 178: 90, 1956.
41. Haynes, R. C., Sutherland, E. W. and Rall, T. W.: The role of cyclic AMP in hormone action. Recent Progr. Horm. Res. 16: 121–138, 1960.
42. Hofmann, K. and Katsoyannis, P. G.: The Proteins I, ed. by H. Neurath, 2nd ed., pp. 54–188, Academic Press, Inc., New York, 1963.
43. Hofmann, K., Liu, T., Yanaihara, N., Yanaihara, C. and Humes, J. L.: Studies on polypeptides. 22. High ACTH activity in the rat and in man of a synthetic eicosapeptide amide. J. Am. chem. Soc. 84: 1054–1056, 1962.
44. Hofmann, K., Rosenthaler, J., Wells, R. D. and Yajima, H. Polypeptides. 28. Elimination of the methionine residue as an essential function unit for *in vivo* ACTH activity. J. Am. chem. Soc. 86: 4991–4999, 1964.
45. Hofmann, K., Stutz, E., Spuhler, G., Yajima, H. and Schwartz, E. T.: Studies on polypeptides. 16. Preparation of N^{ϵ}-formyl-L-lysine and its application to the synthesis of peptides. J. Am. chem. Soc. 82: 3727–3732, 1960.
46. Hofmann, K., Thompson, T. A. and Schwartz, E. T.: Studies on polypeptides. 11. Preparation of an octapeptide possessing MSH activity. J. Am. chem. Soc. 79: 6087–6088, 1957.
47. Hofmann, K., Thompson, T. A., Woolner, M. E., Spuhler, G., Yajima, H., Cipera, J. D. and Schwartz, E. T.: Studies on polypeptides. 15. Observations on the relationship between structure and melanocyte-expanding activity of synthetic peptides. J. Am. chem. Soc. 82: 3721–3726, 1960.
48. Hofmann, K., Wolner, M. E., Spuhler, G. and Schwartz, E. T.: Studies on polypeptides. 10. The synthesis of a pentapeptide corresponding to an amino acid sequence present in corticotropin and in the MSH's. J. Am. chem. Soc. 80: 1486–1489, 1958.
49. Hofmann, K. and Yajima, H.: Studies on polypeptides. 20. Synthesis and corticotropic activity of the amide of the N-terminal tridecapeptide sequence of ACTH. J. Am. chem. Soc. 83: 2289–2293, 1961.
50. Hofmann, K. and Yajima, H.: Synthetic pituitary hormones. Recent Progr. Horm. Res. 18: 41–83, 1962.
51. Hofmann, K., Yajima, H. and Schwartz, E. T.: Studies on polypeptides. XIII. The synthesis of a derivative of α-MSH possessing a high degree of melanocyte expanding activity *in vitro*. Biochim. biophys. Acta 36: 252–253, 1959.
52. Hofmann, K., Yajima, H. and Schwartz, E. T.: Studies on polypeptides. 17. The synthesis of 3 acyltridecapeptide amides possessing a high level of melanocyte-expanding activity *in vitro*. J. Am. chem. Soc. 82: 3732–3737, 1960.
53. Hofmann, K., Yajima, H., Yanaihara, N., Liu, T. and Lande, S.: Studies on polypeptides. 18. Synthesis of a tricosapeptide possessing full biological activity of natural ACTH. J. Am. chem. Soc. 83: 487–489, 1961.
54. Horowitz, S. B.: Energy requirements of melanin granule aggregation and dispersion in the melanophore of *Anolis carolensis*. J. Cell. Comp. Physiol. 51: 341–357, 1958.
55. Inoue, S.: The effect of colchicine on the microscopic and sub-microscopic structure of the mitotic spindle. Expl. Cell Res. Suppl. 2: 305–318, 1952.
56. Kappeler, H.: Synthese von L-gln-L-his-L-phe-L-arg-L-try-gly. Helv. Chim. Acta 44: 476–491, 1961.
57. Kappeler, H. and Schwyzer, R.: Synthese eines Heptapeptides mit starker CRF-Wirkung. Helv. Chim. Acta 43: 1453–1459, 1960.
58. Kinosita, H.: Studies on the mechanism of pigment migration within fish melanophores with special reference to their electrical potentials. Ann. Zool. Japan 26: 115–127, 1953.
59. Kinosita, H.: Electrophoretic theory of pigment migration within fish melanophores. Ann. N. Y. Acad. Sci. 100: 992–1004, 1963.
60. Kohn, R. R.: The influence of pituitary posterior lobe preparations on the action of phenolases *in vitro*. Biol. Bull. mar. biol. Lab., Woods Hole 103: 304–305, 1952.
61. Krivoy, W. A. and Guillemin, R.: On a possible role of β-MSH in the central nervous system of mammalia: an effect of β-MSH in the spinal cord of the cat. Endocrinology 69: 170–175, 1961.
62. Lande, S., Kulovich, S. and Lerner, A. B.: Unpublished observations.

63. LANDE, S., LERNER, A. B. AND UPTON, G. V.: Pituitary peptides. Isolation of new peptides related to β-MSH. J. biol. Chem. 240: 4259–4263, 1965.
64. LANDGREBE, F. W. AND MITCHELL, G. M.: A purified extract of the intermediate lobe of the pituitary. J. exp. Physiol. 39: 11–16, 1954.
65. LANDGREBE, F. W. AND WARING, H.: Biological assay and standardization of melanophore expanding pituitary hormone. Quart. J. exp. Physiol. 33: 1–18, 1944.
66. LEE, T. H. AND BUETTNER-JANUSCH, V.: On the mechanism of sodium hydroxide modification of α-MSH. J. biol. Chem. 238: 2012–2015, 1963.
67. LEE, T. H. AND LERNER, A. B.: Isolation of MSH from hog pituitary gland. J. biol. Chem. 221: 943–959, 1956.
68. LEE, T., LERNER, A. B. AND BUETTNER-JANUSCH, V.: Isolation and structure of α- and β-MSH's from monkey pituitary gland. J. biol. Chem. 236: 1390–1394, 1961.
69. LERNER, A. B.: Mechanism of hormone action. Nature, Lond. 184: 674–677, 1959.
70. LERNER, A. B.: Hormonal control of pigmentation. Ann. Rev. Med. 11: 187–194, 1960.
71. LERNER, A. B., CASE, J., TAKAHASHI, Y., LEE, T. AND MORI, W.: Isolation of melatonin, the pineal gland factor that lightens melanocytes. J. Am. chem. Soc. 80: 2587, 1958.
72. LERNER, A. B., CASE, J. D., WATARU, M. AND WRIGHT, M. R.: Melatonin in peripheral nerve. Nature, Lond. 183: 1821, 1959.
73. LERNER, A. B., LANDE, S. AND KULOVICH, S.: Biologic properties of racemized α-MSH, β-MSH and ACTH. Exerpta Medica int. Congr. Series No. 83: 392–397, 1965.
74. LERNER, A. B. AND LEE, T. H.: Isolation of homogeneous MSH from hog pituitary gland. J. Am. chem. Soc. 77: 1066–1067, 1955.
75. LERNER, A. B. AND MCGUIRE, J. S.: Melanocyte-stimulating hormone and adrenocorticotropic hormone. Their relationship to pigmentation. New Eng. J. Med. 270: 539–546, 1964.
76. LERNER, A. B., SHIZUME, K. AND BUNDING, I.: The mechanism of endocrine control of melanin pigmentation. J. clin. Endoc. Metab. 14: 1463–1490, 1954.
77. LERNER, A. B. AND TAKAHASHI, Y.: Hormonal control of melanin pigmentation. Rec. Progr. Horm. Res. 12: 303–313, 1956.
78. LERNER, A. B. AND WRIGHT, M. R.: *In vitro* frog skin assay for agents that darken and lighten melanocytes. Meth. Biochem. Anal. 8: 295–307, 1960.
79. LI, C. H.: Adrenocorticotropins. 19. Esterification of ACTH. J. biol. Chem. 235: 1383–1385, 1960.
80. LI, C. H., SCHNABEL, E. AND CHUNG, D.: The synthesis of L-his-L-phe-L-orn-L-try-gly and L-his-L-phe-D-orn-L-try-gly and their melanocyte-stimulating activity. J. Am. chem. Soc. 82: 2062–2067, 1960.
81. MALAWISTA, S. E.: On the action of colchicine. The melanocyte model. J. exp. Med. 122: 361–384, 1965.
82. MARSLAND, D. A.: Mechanism of pigment displacement in unicellular chromatophores. Biol. Bull. mar. biol. Lab., Woods Hole 87: 252–260, 1944.
83. MATTHEWS, S. A.: Observations on pigment migration within the fish melanophore, J. exp. Zool. 58: 471–486, 1931.
84. MCGUIRE, J., MCGILL, R., LEEMAN, S. AND GOODFRIEND, T.: The experimental generation of antibodies to α-MSH and ACTH. J. clin. Invest. 44: 1672–1678, 1965.
85. MCGUIRE, J. and MOLLER, H.: The differential responsiveness of dermal and epidermal melanocytes to hormones. Endocrinology 78: 367–372, 1966.
86. MERRIFIELD, R. B.: Solid phase synthesis. III. An improved synthesis of bradykinin. Biochemistry, N. Y., 3: 1385–1390, 1964.
87. MOLLER, H. AND LERNER, A. B.: MSH inhibition by acetylcholine and noradrenaline in the frog skin bioassay. Acta Endocr. Copenh. 51: 149–160, 1966.
88. NEY, R. L., OGATA, E., SHIMUZU, N., NICHOLSON, W. E. AND LIDDLE, G. W.: Structure-function relationships of ACTH and MSH analogues. Int. Congr. Series No. 83: 1184–1191, 1965.
89. NOVALES, R. R.: The effects of osmotic pressure and sodium concentration on the response of melanophores to intermedin. Physiol. Zool. 32: 15, 1959.
90. NOVALES, R. R.: The role of ionic factors in hormone action on the vertebrate melanophore. Am. Zool. 2: 337–353, 1962.
91. NOVALES, R. R.: Responses of cultured melanophores to α-MSH, melatonin and epinephrine. Ann. N. Y. Acad. Sci. 100: 1035–1047, 1963.
92. OTSUKA, H. AND INOUYE, K.: Synthesis of peptides related to the N-terminal structure of corticotropin. III. Synthesis of histidylphenylalanyl-arginyltryptophan. Bull. chem. Soc. Japan 37: 1465–1471, 1964.
93. PICKERING, B. T. AND LI, C. H.: Adrenocorticotropins. 29. The action of sodium hydroxide on adrenocorticotropin. Arch. Biochem. Biophys. 104: 119–127, 1964.
94. RABEN, M. S.: The nature of the effect of alkali on intermedin. J. clin. Endocr. 15: 842–843, 1955.
95. REICHLIN, S.: Neuroendocrinology. New Engl. J. Med. 269: 1182–1191, 1963.
96. RICHARDS, F. M. AND VITHAYATHIL, P. J.: The preparation of subtilisin-modified ribonuclease and the separation of the peptide and protein components. J. biol. Chem. 234: 1459–1465, 1959.
97. ROSEN, F. AND NICHOL, C. A.: Corticosteroids and enzyme activity. Vit. and Horm. 21: 135–214, 1963.
98. RUDMAN, D.: Adipokinetic action of polypeptides and amine hormones upon the adipose tissue of various animal species. J. Lipid Res. 4: 119–129, 1963.
99. SCHALLY, A. V., ANDERSON, R. N., LONG, J. M. AND GUILLEMIN, R.: Isolation of α- and β-MSH on a preparative scale. Proc. Soc. exp. Biol. Med. 104: 290–293, 1960.
100. SCHALLY, A. V., BOWERS, C. Y., KUROSHIMA, A., ISHIDA, Y., REDDING, T. W. AND KASTIN, A. J.: Hormonal activities of beef and pig hypothalamus. Abstracts, XXIII Int. Congr. of Physiol. Sci., Tokyo, September 1–9, 1965.

101. SCHALLY, A. V., BOWERS, C. Y., CARTER, W. H. AND HEARN, I. C.: Isolation of gram amounts of arginine vasopressin, oxytocin and α-MSH. Arch. Biochem. Biophys. 107: 332–335, 1964.
102. SCHALLY, A. V., LIPSCOMB, S. AND GUILLEMIN, R.: Isolation and amino acid sequence of α₂-CRF from hog pituitary glands. Endocrinology 71: 164–173, 1962.
103. SCHNABEL, E. AND LI, C. H.: Synthesis of L-his-D-phe-L-arg-L-try-gly and its MSH activity. J. Am. chem. Soc. 82: 4576–4579, 1960.
104. SCHNABEL, E. AND LI, C. H.: The synthesis of gly-his-phe-arg-try-gly and its MSH activity. J. biol. Chem. 235: 2010–2012, 1960.
105. SCHWYZER, R.: Synthetische Analoge des Hypertensins. I. Einleitung. Helv. chim. Acta 44: 667–674, 1961.
106. SCHWYZER, R., COSTOPANAGIOTIS, A. AND SIEBER, P.: Zwei Synthesen des α MSH mit Hilfe leicht entfernbarer Schutzgruppen. Helv. chim. acta 46: 870–889, 1963.
107. SCHWYZER, R., ISELIN, B., KAPPELER, H., RINICKER, B., RITTEL, W. AND ZUBER, H.: Die Synthese des β-MSH mit der Aminosaurensequenz des bovinen Hormons. Helv. chim. Acta 46: 1975–1996, 1963.
108. SCHWYZER, R. AND KAPPELER, H.: Synthese von Zwischen produkten fur den Aufbau corticotrop wirksamer Polypeptide. Helv. chim. Acta 44: 1991–2002, 1961.
109. SHIZUME, K. AND IRIE, M.: The role of the liver in the inactivation of MSH in the body. Endocrinology 61: 506–513, 1957.
110. SMITH, P. E.: Experimental ablation of the hypophysis in the frog embryo. Science 44: 280–282, 1916.
111. SMITH, P. E. AND GRAEBER, J. B.: A differential response of the melanophore stimulant and oxytocic autocoid of the posterior hypophysis. Anat. Rec. 27: 187, 1924.
112. SNELL, R. S.: Effect of the melanocyte stimulating hormone of the pituitary on melanocytes and melanin in the skin of guinea pigs. J. Endocr. 25: 249–256, 1962.
113. SNELL, R. S.: Effect of corticotropin on the activity of mammalian epidermal melanocytes. J. Endocr. 28: 79–85, 1963.
114. SNELL, R. S.: Effect of the α-MSH of the pituitary on mammalian epidermal melanocytes. J. invest. Derm. 42: 337–347, 1964.
115. SNELL, R. S.: Effect of melatonin on mammalian epidermal melanocytes. J. invest. Derm. 44: 273–275, 1965.
116. SNELL, R. S. AND BISCHITZ, P. G.: Effect of large doses of estrogen and estrogen and progesterone on melanin pigmentation. J. invest. Derm. 35: 73–82, 1960.
117. SPAETH, R. A.: Evidence proving the melanophore to be a distinguished type of smooth muscle cell. J. exp. Zool. 20: 193–215, 1916.
118. TALEISNIK, S. AND ORIAS, R.: An MSH-releasing factor in hypothalamic extracts. Am. J. Physiol. 208: 293–296, 1965.
119. VAN DE VERDONK, F. C. G.: Mechanism of hormone action in color change in amphibians. Gen. comp. Endocr. 2: 623–634, 1962.
120. WALLER, J. P. AND DIXON, H. B. F.: Selective acetylation of the terminal amino acid group of corticotropin. Biochem. J. 75: 320–328, 1960.
121. WARING, H. AND LANDGREBE, F. W.: *In* The Hormones, ed. by G. Pincus and K. V. Thimann, vol. 2, p. 427, Academic Press, Inc., New York, 1950.
122. WASSERMAN, E. AND LEVINE, L.: Quantitative micro-complement fixation and its use in the study of antigenic structure by specific antigen-antibody complex inhibition. J. Immunol. 87: 290–295, 1961.
123. WEBER, H. H.: ATP and motility of living systems. Harvey Lect. 49: 37, 1953–54.
124. WILSON, I. B.: Acetylcholinesterase. *In* The Enzymes, ed. by Boyer, Lardy and Myrback, vol. 4, pp. 501–520, Academic Press, Inc., New York, 1960.
125. WRIGHT, R. M. AND LERNER, A. B.: On the movement of pigment granules in frog melanocytes. Endocrinology 66: 599–609, 1960.
126. WYMAN, L. C.: Blood and nerve as controlling agents in the movements of melanophores. J. exp. Zool. 39: 73–132, 1924.
127. YAJIMA, H. AND KUBO, K.: Synthesis of D-his-L-phe-L-arg-L-try-gly and L-his-L-phe-L-arg-D-try-gly and their physiological properties in frog melanocytes. Biochim. biophys. Acta 97: 596–597, 1965.
128. YAJIMA, H. AND KUBO, K.: Peptides. II. Synthesis and physiological properties of all D histidylphenylalanylarginyltryptophylglycine, an optical antipode of an active fragment of α-MSH. J. Am. chem. Soc. 87: 2039–2044, 1965.
129. YAJIMA, H. AND LANDE, S.: Manuscript in preparation.