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The Experimental Generation of Antibodies to α -Melanocyte Stimulating Hormone and Adrenocorticotrophic Hormone *

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Sensitivity, reproducibility, and specificity are requirements for a suitable assay for hormones. The quantitation of α -MSH¹ is currently based on a technique devised in 1954, which utilized isolated pieces of frog skin (1). In the presence of α -MSH, frog skin darkens with a measurable decrease in the reflection of light from the skin. Although this assay possesses great sensitivity (less than 10^{-11} g of α -MSH), it does not have the degree of specificity necessary to discriminate among the several substances that darken frog skin, such as ACTH and α - and β -MSH. This lack of specificity has prevented application of the *in vitro* frog skin assay to clinical problems.

Immunochemical techniques have become useful for the measurement of several small polypep-

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¹ Abbreviations: MSH, melanocyte stimulating hormone; ACTH, adrenocorticotrophic hormone; RSA, rabbit serum albumin; anti-ACTH, serum from rabbits that have received injections of rabbit serum albumin conjugated with ACTH; anti-MSH, serum from rabbits that have received injections of rabbit serum albumin conjugated with α -MSH; eicosapeptide "ACTH," the synthetic N-terminal eicosapeptide of ACTH.

ptide hormones, including insulin (2), glucagon (3), parathormone (4, 5), and ACTH (6, 7). These techniques depend on the availability of antibodies to the hormones. Alpha-MSH is a small polypeptide with a molecular weight of 1,665, and the occurrence of antibodies to this peptide has not been described. We report the generation of antibodies to α -MSH and ACTH in rabbits and suggest that the use of antisera may confer immunologic specificity to the bioassay of α -MSH, and may be useful for direct immunochemical measurement of the hormone.

Methods

Antigenic compounds were synthesized by conjugating rabbit serum albumin (RSA) with either α -MSH or ACTH by means of the carbodiimide reagent [1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide], as previously described for bradykinin and other peptides (8). Five mg of albumin, 5 mg of α -MSH or ACTH, and 100 mg of the carbodiimide reagent were dissolved, in that order, in 0.3 ml water. After 30 minutes a cloudy, gelatinous precipitate formed. The entire mixture was then subjected to dialysis against water for 48 hours. The precipitated material was used for immunization. Antigens for complement fixation were prepared in the same way, except that a different carbodiimide [1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-p-toluenesulfonate] was used to reduce interference by possible antibodies to the reagents. Three mg of the albumin-hormone conjugate (RSA-MSH or RSA-ACTH) was emulsified in Freund's adjuvant and injected into the toe pads and leg muscles of New Zealand albino rabbits. After 3 weeks, 2 mg of the compound in adjuvant was injected intraperitoneally, and 2 weeks later serum was collected. Further booster injections were administered intraperitoneally at monthly intervals. Antibodies were detectable after the first boost. Each of the sera used in these studies was obtained after the fifth boost from a single bleeding of one rabbit. Sera were not pooled.

Antibodies were detected by complement fixation, us-

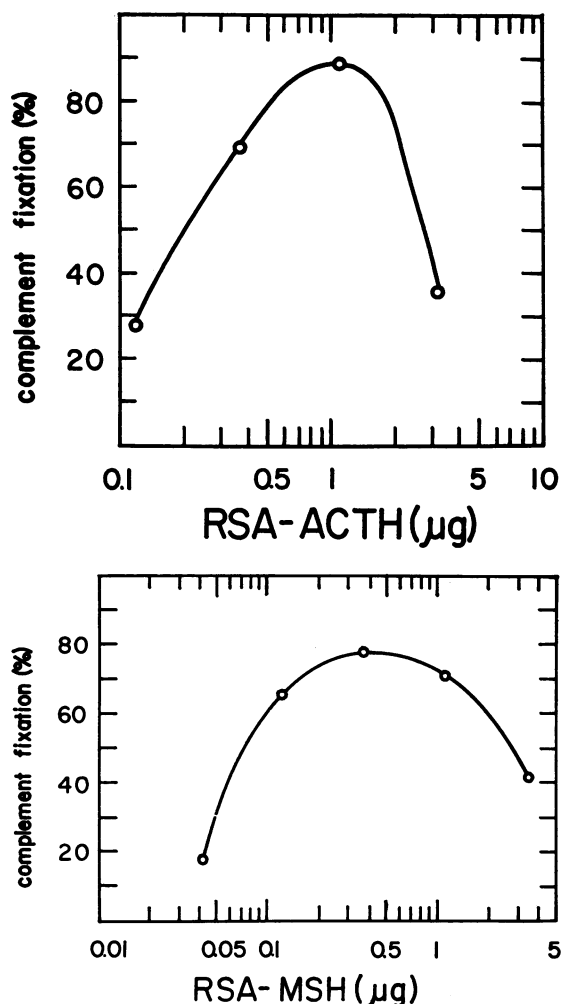


FIG. 1. COMPLEMENT FIXATION BY RABBIT SERUM ALBUMIN-ADRENOCORTICOTROPIC HORMONE (RSA-ACTH) AND MELANOCYTE STIMULATING HORMONE (RSA-MSH) AND THE ANTISERA INDUCED BY THESE COMPLEXES. These assays were performed as described in the text. One ml of diluted antiserum was used for each point. The dilution of anti-ACTH was 1:100 and of anti-MSH was 1:25.

ing the technique of Wasserman and Levine (9), and specificity was assessed by cross-reactivity of the antisera with other hormone-albumin complexes and by "hapten inhibition" of the complement fixation reaction by free peptides. The dilutions of antisera and the amounts of antigen required for maximal complement fixation were confirmed in three separate experiments, one of which is shown in Figure 1 for each antigen-antibody system. The hapten inhibition studies were performed several times. One complete study is summarized in Table I; the others confirmed the relative amounts of hormones required for inhibition of complement fixation by the two antisera.

Frog skin bioassay for darkening activity. Four skin samples were obtained from each frog and mounted on rings as previously described (1). Each compound was tested on a total of four samples from different regions of four frogs. In this way the darkening activity of four substances could be compared in a latin square. The frogs were decapitated and skinned at 7:30 a.m. The skins were trimmed, mounted on rings, placed in beakers, and washed in five changes of Ringer's solution. Peptide was added at 11:00 a.m. A darkening unit was defined as the degree of darkening produced by 0.04 μ g of a lyophilized water extract of beef posterior pituitary powder (1). Peptides were assayed before use. In each experiment, 10 U of the above posterior pituitary standard was added to a group of skins. The standard, unlike the purified peptide, was not mixed with buffer at room temperature for 30 minutes before being added to the frog skin. For this reason, occasionally the 10-U standard produced more darkening than 20 U of purified MSH that had been at room temperature for 30 minutes. Reflectance, which decreases as the skin darkens, was measured by a Photovolt 610 reflectance meter. The meter was calibrated as previously described (1), except that sensitivity control was adjusted to 65 for a white enamel disk with a green filter. Antiserum was added to 0.2 ml Ringer's solution in cellulose nitrate tubes containing the desired amount of peptide. After 30 minutes at room temperature, the mixture was quantitatively transferred to beakers containing the frog skin.

Bioassays for ACTH. Hypophysectomized rats (80 to 100 g), anesthetized with ether, received ACTH or a mixture of ACTH and antiserum that had been preincubated for 30 minutes at room temperature. The injections were made into a tail vein. Fifteen minutes later, the rats were decapitated and plasma was collected. Plasma steroids were measured by the method of Guillemin, Clayton, Lipscomb, and Smith (10).

TABLE I
Inhibition of complement fixation by unconjugated peptides*

Fixation inhibited	Inhibitor	Amount for 50% inhibition
RSA- α -MSH†: Anti-MSH (1 ml)	α -MSH	μ g 10
	β -MSH	>500
	ACTH	>500
	Eicosapeptide "ACTH"	>500
RSA-ACTH: Anti-ACTH (1 ml)	α -MSH	2.3
	β -MSH	7.5
	ACTH	0.85
	Eicosapeptide "ACTH"	1.15

* The fixation points were the points of maximal fixation shown in Figure 1. Free hormone was added with the antigenic complex; otherwise the tests were performed according to the text and Wasserman and Levine (9). Points of 50% inhibition were derived from plots of log concentration of hormone against $\log y/1 \times y$ where y = per cent inhibition. Data have been corrected to apply to the reaction of 1 ml of undiluted antiserum.

† RSA = rabbit serum albumin; MSH = melanocyte stimulating hormone; ACTH = adrenocorticotrophic hormone; "ACTH" = synthetic N-terminal eicosapeptide of ACTH.

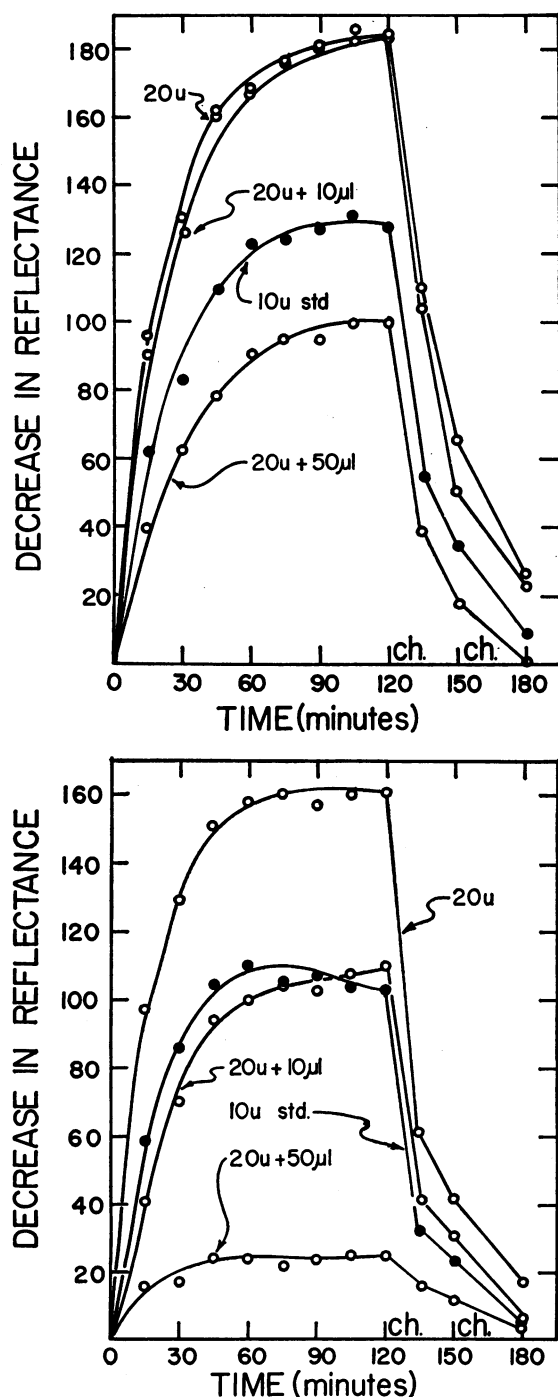


FIG. 2. INHIBITION BY ANTISERUM OF THE FROG SKIN DARKENING EFFECT OF α -MSH. Two-tenths ml Ringer's solution containing 20 U α -MSH (2.5×10^{-5} μ g) was mixed where indicated with antiserum in cellulose nitrate tubes at room temperature. Thirty minutes later, the contents of each tube were quantitatively transferred to a beaker containing the skin specimen. The time of transfer is zero time in the Figure. Reflectance readings were

TABLE II

Effects of antibodies to ACTH and MSH on response to ACTH of adrenal tumor cells in tissue culture*

ACTH μ g	Antiserum μ l	OD 250 $\times 1,000$		Inhibition by antiserum	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
0.05	0	136			
0.10	0	186	400		
0.10	Anti-ACTH (100)	70	153	62	62
0.10	Anti-ACTH (33)	152		18	
0.10	Anti-ACTH (11)	191		0	
0.10	Anti-MSH (100)	179	480	4†	0

* Mouse adrenal tumor cells were dispersed with trypsin and incubated as a monolayer in tissue culture plates with nutrient medium. Two days later, the medium was replaced by a balanced salt solution containing bovine serum albumin, 20 mg per ml, and bovine ACTH with or without antiserum, as indicated. The antiserum-ACTH mixtures were pre-incubated for 1 hour at 0° C before addition. After 2 hours (experiment 1), or 7 hours (experiment 2), the salt solution was removed from the plates and extracted with dichloromethane. The absorbancy of the dichloromethane at $240 m\mu$ was used as a measure of Δ^4 -3-ketosteroid production. The figures represent the total optical density units per culture plate. Each represents the mean of two closely matching plates. Inhibition by antiserum was read from a linear plot of ACTH against response. In this assay, 400 OD mU represents 10 μ g of steroid.

† The apparent effects of anti-MSH were not significant.

Mouse adrenal tumor cells were incubated in culture as described by Buonassisi, Sato, and Cohen (11). ACTH and serum were added to the culture dishes on the day after the tumor was plated. Steroid secretion was measured by extracting the medium 2 hours later with dichloromethane. The solvent was evaporated, the residue redissolved in ethanol, and the absorbancy measured at $240 m\mu$. Results of two experiments are recorded in Table II.

Materials. Highly purified α -MSH (porcine) free of ACTH was obtained by elution from a carboxymethylcellulose column. ACTH (porcine) free of α -MSH² and eicosapeptide "ACTH"³ were gifts. The rabbit albumin,⁴ the carbodiimides,⁵ and the hypophysectomized rats⁶ were purchased.

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⁴ Pentex, Kankakee, Ill.

⁵ Ott Chemical Co., Muskegon, Mich., and Aldrich Chemical Co., Milwaukee, Wis.

⁶ Hormone Assay Laboratories, Chicago, Ill.

made at 15-minute intervals. Each curve represents the sum of reflectance changes for four separate skin specimens. The activity of α -MSH used in this experiment was 8×10^{11} U per g; 20 U represented 2.5×10^{-5} μ g. The 10-U standard (STD) was the crude extract of pituitary described in the Methods. The standard did not sit at room temperature for 30 minutes but was added directly to the beakers. In the upper portion of the Figure, 10 and 50 μ l of anti-MSH were used where indicated. In the lower, 10 μ l and 50 μ l of anti-ACTH were used. The Ringer's solution was changed at 120 and 150 minutes, as indicated by "ch."

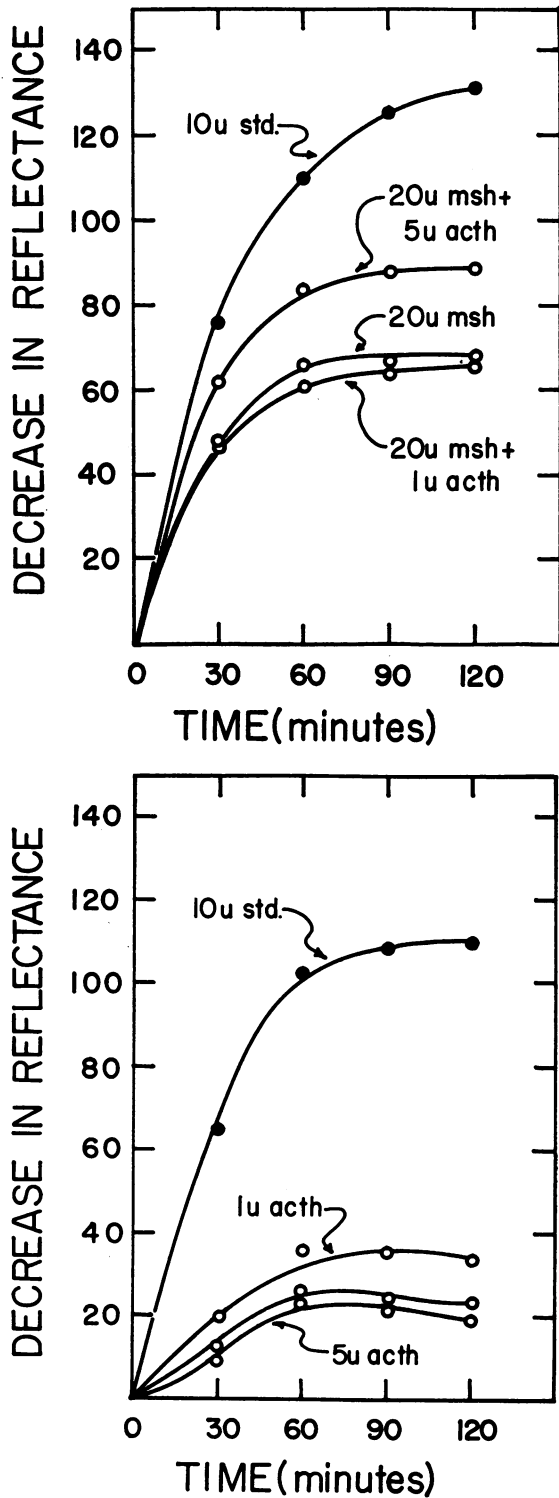


FIG. 3. INHIBITION OF FROG SKIN DARKENING BY ANTI-MSH. Peptides and antiserum were mixed as in Figure 2; zero time is the time of the addition of peptide to the frog skins. The darkening activity of the ACTH was

Results

The antisera induced by RSA-MSH and RSA-ACTH fixed complement with homologous complexes, as illustrated in Figure 1. The titers for complete fixation with the homologous antigenic complexes under standard conditions were 1:25 for anti-MSH and 1:100 for anti-ACTH. Anti-MSH did not cross-react with RSA-ACTH, but anti-ACTH did fix complement in the presence of RSA-MSH at a titer of 1:50. Similar specificity characteristics were evident in hapten inhibition assays (Table I). The reaction of RSA-MSH with anti-MSH was inhibited by α -MSH but not by β -MSH, ACTH, or the eicosapeptide "ACTH." In contrast, the reaction of RSA-ACTH with anti-ACTH was inhibited by ACTH, eicosapeptide "ACTH," and α - and β -MSH.

Anti-ACTH showed a greater affinity for MSH than did anti-MSH itself. The reaction of 1 ml of anti-ACTH with RSA-ACTH was inhibited 50% by 2.3 μ g α -MSH, but the reaction of 1 ml anti-MSH with RSA- α -MSH required 10 μ g of α -MSH for 50% inhibition. The smaller amount of hapten required for inhibition of complement fixation reflects the greater ability of anti-ACTH to bind free α -MSH.

Anti-MSH and anti-ACTH decreased the darkening effect of α -MSH as indicated in Figure 2. In the experiment shown in the upper Figure, 20 U of α -MSH (2.5×10^{-5} μ g) was mixed with either 10 μ l or 50 μ l of anti-MSH. Ten μ l of anti-MSH produced no inhibition of MSH darkening, whereas 50 μ l produced approximately 50% inhibition. Anti-MSH inhibited the darkening effect of α -MSH but not the darkening effect of β -MSH or ACTH. This is in agreement with the immunologic specificity tabulated in Table I. The Figure represents single experiments carried out on 2 dif-

7×10^7 U per g; the activity of α -MSH was 8×10^{11} U per g. The units noted on the Figure refer to darkening activity, not adrenocorticotrophic activity. In the upper section of the Figure, the curves with open circles represent the darkening activity of combinations of α -MSH and ACTH. In the lower section, with the same combinations of peptides, 20 μ l of anti-MSH has been mixed for 30 minutes. The curves with open circles represent reactions containing 20 U α -MSH (2.5×10^{-5} μ g), and 0 U, 1 U, or 5 U of ACTH, as indicated. The curves with closed circles in the upper and lower sections represent a 10-U standard of crude pituitary extract that has not been mixed with antiserum.

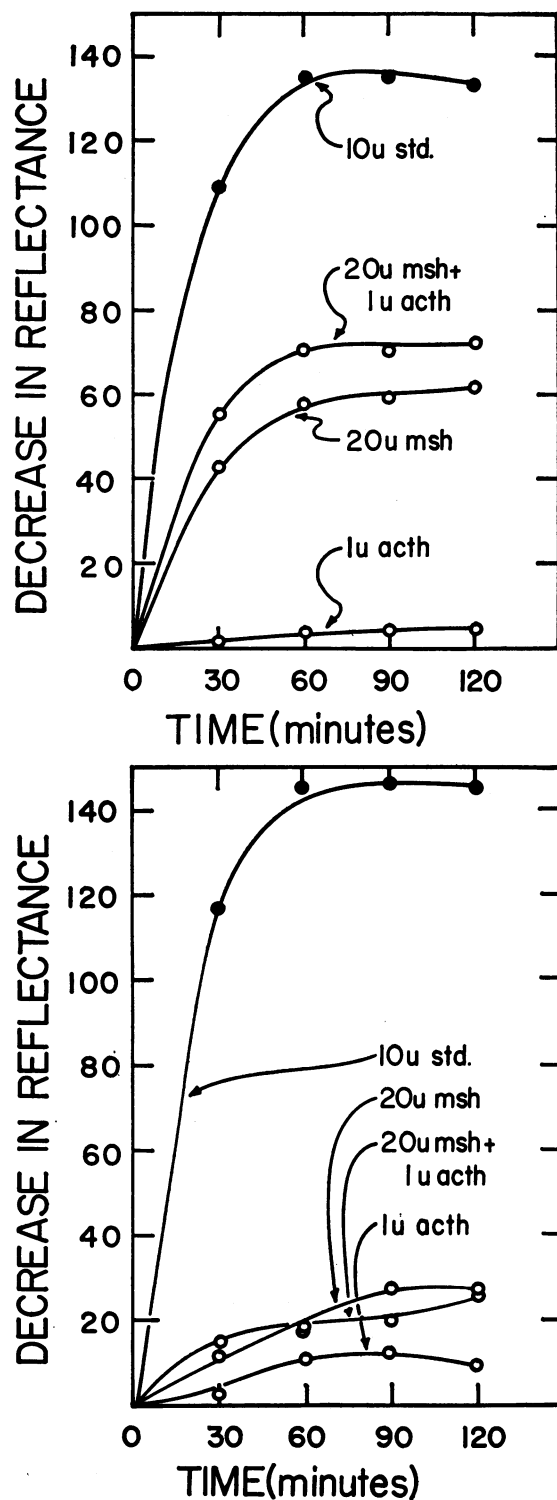


FIG. 4. INHIBITION OF FROG SKIN DARKENING BY ANTI-ACTH. In the upper part of the Figure no antiserum is used. The 10-U standard effects a greater darkening response than 20 U of α -MSH because the standard has not been incubated for 30 minutes at room temperature.

TABLE III
Inhibition by anti-ACTH of plasma steroid response to ACTH in the rat

Material injected	Plasma steroids after 15 minutes
Saline (7)*	$\mu\text{g}/100 \text{ ml} \pm \text{SE}$ 7.4 ± 0.6
ACTH, 0.05 μg , plus normal serum 0.02 ml (5)	34.3 ± 3.1
ACTH, 0.05 μg , plus anti-ACTH 0.02 ml (6)	7.3 ± 1.2

* Number in parentheses indicates the number of animals injected.

ferent days. Each experiment has been performed more than 6 times with no substantial variation.

Anti-ACTH was a more potent inhibitor of α -MSH darkening than was anti-MSH (Figure 2). Ten μl of the anti-ACTH produced 40% inhibition and 50 μl produced 90% inhibition of the darkening ability of 20 U of α -MSH (2.5×10^{-5} μg). When the Ringer's solution in the beaker containing the frog's skin was replaced at 120 and 150 minutes, there was prompt lightening of the skins. The greater ability of anti-ACTH to inhibit α -MSH, compared to that of anti-MSH, is consistent with the data in Table I, which indicate that anti-ACTH has a higher affinity for α -MSH than does anti-MSH.

In several experiments, 50 μl of either anti-MSH or anti-ACTH failed to inhibit the darkening activity of 0.3 μg of ACTH. Similarly, 50 μl of either antiserum did not inhibit 1×10^{-4} μg of β -MSH.

Serum from a rabbit immunized with RSA-insulin conjugate was used as a "control" in many of the preceding experiments. Twenty or 50 μl of insulin antiserum did not inhibit the darkening effect of 20 U (2.5×10^{-5} μg) α -MSH.

Although ACTH darkens frog skin, its darkening potency is approximately 10^4 less on a weight basis than that of α -MSH. If the antibody to MSH could not distinguish between α -MSH and ACTH, then an amount of ACTH insufficient to darken frog skin might combine with the antibody to MSH and interfere with the reaction between MSH and its antibody. The data in Figure 3 indicate that relatively enormous amounts of ACTH do not interfere with the inhibition of α -MSH

The lower part of the Figure duplicates the upper, except that antiserum is added to the three experiments represented by open circles.

TABLE IV
Summary of antibody effects

Assay	Anti-MSH	Anti-ACTH
Darkening of frog skin produced by:		
α -MSH	Inhibition	Inhibition
ACTH	No inhibition	No inhibition
β -MSH	No inhibition	No inhibition
Response of mouse adrenal tumor culture to ACTH	No inhibition	Inhibition
Complement fixation with:		
RSA-ACTH	No	Yes
RSA- α -MSH	Yes	Yes
Inhibition of homologous antibody-antigen by:		
ACTH	No	Yes
α -MSH	Yes	Yes
β -MSH	No	Yes

darkening by anti-MSH. This observation reflects both the low avidity of anti-MSH for ACTH noted in Table I and the degree of antibody excess needed for neutralization of biological activity of α -MSH.

Furthermore, the darkening effect of α -MSH is inhibited by anti-ACTH even in the presence of ACTH (Figure 4), a finding that could not be predicted from the data in Table I.

In Table III the inhibition by anti-ACTH of the response of hypophysectomized rats to intravenously administered ACTH is demonstrated. Twenty μ l of anti-ACTH neutralized 0.05 μ g of ACTH. Similar results were obtained when adrenal ascorbic acid depletion was measured instead of steroid secretion. A similar equivalence of anti-ACTH and ACTH was observed when the antiserum was used to neutralize the effect of ACTH on steroid secretion by cultured cells from mouse adrenal tumor (Table II). In this experiment 100 μ l of anti-ACTH inhibited by 75% the response to 0.1 μ g of porcine ACTH. Anti-MSH did not inhibit ACTH in this assay.

The several effects of anti-MSH and anti-ACTH are summarized in Table IV.

Discussion

Lack of specificity of the *in vitro* frog skin bioassay for α -MSH has prevented its general application to clinical problems. The experiments reported here permit the use of antiserum to confer immunologic specificity to the highly sensitive bioassays for α -MSH. Frog skin darkening activity due to α -MSH in human plasma, for example, could be differentiated from that produced by ACTH or β -MSH by inhibition by highly specific anti-MSH.

This study compared the reactivity of immunologic and biologic systems with α -MSH and ACTH. The ability of both antisera to neutralize the biological activity of α -MSH correlated well with their binding of α -MSH as measured by hapten inhibition. This may indicate that these antibodies and the biological receptor react with the same part of the peptide.

The nonspecificity of anti-ACTH is probably related to the inclusion within ACTH of an amino acid sequence common to α -MSH. If one of the sites in RSA-ACTH that induced antibodies in rabbits includes part of this sequence, those antibodies would also react with the similar sequence in α -MSH. On the other hand, the specificity of anti-MSH may be related to the terminal N-acetyl serine present in α -MSH but not in β -MSH or ACTH. Inclusion of this amino acid in the immunogenic site of α -MSH might result in antibodies specific for α -MSH relative to ACTH and β -MSH.

The conjugation of small polypeptides to albumin by reaction with carbodiimides provides a convenient method of synthesis of immunogenic complexes, which induce antibodies specific for the polypeptide moiety. In the case of α -MSH, specific antibodies were rapidly induced by the hormone-RSA conjugate. Unconjugated ACTH, on the other hand, can induce antibodies when injected into rabbits or guinea pigs (6, 7, 12, 13). Antibodies formed in response to unconjugated ACTH are more specific than those produced in the current study. In fact, Imura, Sparks, Grodsky, and Forsham (13) concluded that their antibodies to ACTH reacted with the part of the ACTH molecule that does not contain the α -MSH sequence. There probably are two or more immunogenic sites in the ACTH molecule. Differences in results may reflect individuality of the antibody-producing animals, as shown by Arquilla and Finn in guinea pigs producing antibodies to insulin (14). Alternatively, the differences may result from the use of conjugates. Conjugation to albumin may preferentially expose the sites of ACTH resembling MSH to the antibody producing mechanisms.

Anti-ACTH inhibits the adrenocorticotrophic function of ACTH but not its skin darkening effect. This observation could be explained if the affinity between the melanocyte and ACTH were greater than the affinity between the peptide and

the antibody. Such a proposal would, however, also require the adrenal cortex to have a relatively lower affinity for ACTH than that of the antibody.

Complement fixation in the presence of RSA-ACTH and anti-ACTH was inhibited by both β -MSH and ACTH, which indicates that the antibody binds these molecules. Darkening effects of these hormones, however, were not inhibited by the antiserum, which may indicate that the hormone-antibody complex is active at the melanocyte target or that the melanocyte target has very high binding affinity for the hormone compared to the binding by antibody. A large excess of antibody was required for the neutralization of the biological effect compared to the proportions indicated by the immunological data. This supports the idea that melanocytes bind the hormones strongly, compared to their binding by antisera. In *in vivo* experiments, the antisera were found to prolong the effects of α -MSH in frogs, with variable evidence of neutralization (15).

The binding of free hormone indicated by the hapten inhibition data permit use of these antisera for immunoassay of α -MSH. The differential abilities of the antisera to neutralize effects of the hormones on frog skin permit their use to add specificity to this sensitive bioassay.

Summary

Adrenocorticotrophic hormone (ACTH) and melanocyte stimulating hormone (α -MSH) were conjugated to rabbit serum albumin with carbodiimide reagents, and the induction of antibodies to these complexes in the rabbit is reported.

Complement fixing antibodies to α -MSH were found to be very specific, and they did not cross-react with β -MSH or ACTH. They neutralized the skin darkening effect of α -MSH on frog skin *in vitro*. Antiserum to ACTH was less specific, and it reacted with ACTH, the synthetic N-terminal eicosapeptide of ACTH (eicosapeptide "ACTH"), and both α - and β -MSH. Anti-ACTH neutralized the biological effects of ACTH on rat adrenal gland and adrenal tumor cells and the effect of α -MSH on frog skin. Neither antiserum in the small amounts used neutralized the darkening effect of β -MSH or ACTH.

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