Melanocyte Stimulating Hormone Activity* of Different Pituitary Preparations.† (23577)

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Pituitary extracts seem to contain 3 distinct principles which possess MSH-activity (1). One represents the intrinsic activity present in the ACTH molecule(2-5). The other 2 principles named *alpha*-MSH and *beta*-MSH are virtually free of ACTH activity and have been isolated mainly from "posterior" (posterior and intermediate) lobe extracts. Smaller amounts of these principles have been found in the anterior lobe extracts of certain species (*e.g.*, pig, man)(6,7). Chemically the 3 MSH active principles are distinct(6,8,9). Thus *alpha*-MSH is a basic polypeptide (pK = 10-11) while *beta*-MSH is an acid polypeptide (pK = 5-6). Furthermore *beta*-MSH is precipitated with acetone at pH 6.5 while *alpha*-MSH remains in the filtrate(6,7). Oxycellulose appears to adsorb all three principles but solvent partition between dilute acid and butanol separates *alpha*-MSH in the hydrophilic fraction and *beta*-MSH and ACTH in the butanol fraction(6, 10). Recent studies have elucidated the amino acid sequence of ACTH and *beta*-MSH. A common sequence of 7 amino acids is found in both the *beta*-MSH and ACTH molecules and it is said to account for the MSH activity of these 2 polypeptides. This heptapeptide sequence consists of: Met. Glu. His. Phe. Arg. Try. Gly.(4,5). The biological activities of the various chemically distinct active principles have not been analyzed to determine whether there are any qualitative differences among the actions of the various principles. Similarly only rough approximations of the quantitative differences of their activity have been given(6).

In the present studies various pituitary extract fractions were assayed to compare quantitative and qualitative bioassay characteristics of the different active principles found in these preparations.

Materials and methods. The bioassay method has been previously described in detail(11). Briefly the method involves photoelectric reflectance measurements from the dorsal surface of intact light-adapted *Rana pipiens* before and one hour after the administration of the test substance in the dorsal lymph sac. Four-point balanced assays were performed and the results were analyzed statistically(12). This method has been found to give reproducible results over a period of a year with an average precision index (lambda) of 0.39(11). A commercial ACTH preparation (Armour Labs, lot no. L 60311) was used as the provisional "standard" of comparison in all tests. It contained 1.6 I.U./mg of ACTH. Various concentrations of the same preparation served as the "unknown" in the "blind" tests. The Posterior Pituitary Powder (2 USP units/mg) and the Pitressin Powder (92 pressor units/mg) were kindly supplied by Parke, Davis & Co. The commercial preparations of Pitressin (lot no. G-D1294C) and Pitocin (lot no. E-1482G) were utilized; they contained 0.5% chlorobutanol as preservative. The oxycellulose purified ACTH(13) was kindly supplied by Dr. E. B. Astwood. It contained approximately 80 I.U./mg of ACTH. The butanol and hydrophilic fractions were prepared in Dr. Astwood's laboratory by partition of oxycellulose ACTH between dilute acid and butanol(10). The butanol fraction contained most of the ACTH activity (100-200 I.U./mg) while the acid fraction was virtually free of such activity (less than 1 I.U./mg). All solutions were made in 0.25% acetic acid. Doses were injected into the dorsal lymph sac in a volume of 0.1 ml. Control animals received 0.1 ml of 0.25% acetic acid. In addition control animals with the

* "Melanocyte stimulating hormone" (MSH) is adopted here because it has found general acceptance among American authors, although the present writers have certain reservations regarding some connotations of this terminology.

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Pitressin and Pitocin injected groups received chlorobutanol in amounts equivalent to that injected with Pitressin and Pitocin. Following the one hour reflectance measurement, all animals were injected with a dose (32 μg/frog) of the provisional "standard" known to produce maximal response. The maximal response was measured one hour later. To facilitate comparison the results are also expressed in terms of the ED_{50} which is defined as the dose producing a photoelectrically measured response of ½ or greater of the maximal response to any dose in 50% of the animals. In arriving at the percent response of each animal the average initial and maximal reflectance measurements of each group were considered.

Results. To evaluate the accuracy of the method experimentally, 3 bioassays were made with solutions of the "standard" the concentrations of which were unknown to the persons performing the assay. The actual concentrations were disclosed after arriving at an experimental estimate from the analysis of the bioassay data. Four-point balanced assays with 5-15 animals per dose were made against known solutions of the same material. The results of these 3 tests indicated that the concentrations determined by bioassay differed from those actually present by an error ranging from -6% to +7%.

Similar 4-point balanced bioassays with 10 animals per dose were made with several pituitary fractions available. The potency ratios between the various preparations and the "standard" were calculated statistically and are given in Table I. In the same table are shown the ED_{50}s *i.e.*, the dose of each preparation which produced half-maximal or greater darkening (measured photoelectrically) in 50% of the animals. In similar tests oxytocin (Parke, Davis & Co., Pitocin) did not produce any significant melanophore response in doses up to 500 μg/frog.

Dose response lines were constructed for all the preparations tested. The slopes of these lines did not differ significantly suggesting a qualitatively similar mode of action.

Utilizing an approximately ED_{50} amount of each preparation, time-response curves were obtained by measuring light reflectance before and every 15 minutes after the injection for a period of 2-3 hours. These tests were performed in 5 animals per group. No significant differences were found among the time-response curves of the different pituitary preparations tested. All curves were similar to those previously published for the standard (11). As would be expected, boiling the "oxycellulose-ACTH" preparation in dilute NaOH for 10 minutes altered significantly its time-response curve showing a prolongation of the effect. This alkali-treated "oxycellulose-ACTH" had a potency ratio nearly five times greater than the untreated material.

TABLE I. MSH Activity of Pituitary Preparations.

Preparation	Relative potency,* stand./unknown	ED_{50}, μg/frog	Activity units, MAU/g
"Standard" (Armour ACTH)	1.00	.93	1.1×10^6
Oxycellulose purified ACTH	1.6	.58	$1.7 \times$ "
Butanol fraction of oxycellulose ACTH	.16	5.8	1.7×10^5
Hydrophylic fraction of oxycellulose ACTH	1.7	.55	1.8×10^6
Des. post. pit. powder‡	.17	5.6	1.8×10^5
Pitressin solution‡	.78	1.2†	$8.3 \times$ "
Pitressin powder‡	.58	1.6	$6.3 \times$ "

* The 95% confidence limits in each case are of the order of 30% to 200% of reported values. Thus differences smaller than about ⅓ to twice a given value cannot be considered as statistically significant.

† In terms of vol the ED_{50} of the Pitressin solution corresponds to approximately 5×10^{-3} ml of the commercial preparation.

‡ Parke, Davis & Co.

Discussion. It is apparent from the "blind" bioassays that the method may be relied upon within a ±10% accuracy. In addition the method permits the calculation of the 95% confidence limits from the internal evidence of each bioassay.

The present results suggest that the different pituitary principles act in a qualitatively similar manner on melanophores, otherwise differences in the dose-response and/or time-response curves would be expected.

Quantitatively, the butanol fraction containing mainly ACTH, and perhaps *beta*-MSH, possesses 1/10th of the MSH activity present in an equal weight of the hydrophilic fraction which contains the *alpha*-MSH. Thus the intrinsic MSH activity of ACTH can not be greater than 1/10th the activity of *alpha*-MSH. Lee and Learner(6) reported the activity of highly purified ACTH as 1/100th that of highly purified MSH.

The activity of a given preparation can only be expressed in reference to a provisional "standard." However the "standards" used by different laboratories vary greatly in their MSH activities so as to be of no value for comparative purposes among laboratories. The use of the USP Posterior Pituitary Powder as a reference standard can not be unquestionably accepted since: 1) this preparation is not standardized for its MSH activity and different lots may vary in their MSH content, 2) the stability of the MSH activity in the preparation is not known and 3) such a comparison would imply that the active fraction in the unknown has a qualitatively identical MSH activity with that present in the USP Powder.

Our results do suggest that all preparations have a qualitatively similar MSH activity which would justify the use of a common standard for all extracts. Until this point is definitely established (with chemically pure preparations), however, it would seem more appropriate to refer to the MSH potency of the various extracts in terms of their biological activity rather than by comparison with an arbitrarily chosen standard. To facilitate comparison it is proposed that a provisional unit of activity be adopted which can be defined as the amount of an active fraction producing a response (darkening) equal to or greater than ½ maximal response in 50% of the treated animals. This unit can be called melanophorotropic activity unit (MAU). Such a unit, although it is defined in terms of activity and therefore lacks in accuracy, can still be useful until a universal MSH standard is established. The definition is sufficiently rigid to permit valid semi-quantitative comparisons of the results obtained in different laboratories.

Rough calculations from the published reports indicate that the proposed unit is approximately equivalent to that used by Sulman and associates(14) nearly 10 times greater than the unit used by Lerner and associates(15), and roughly 1,000 times smaller than that of Landgrebe, Waring and associates(7). The potencies of the preparations tested are given in activity units (MAU) in Table I.

In terms of activity units the frog bioassay method is sensitive to as little as 0.01 MAU. However 10 to 40 times larger amounts are required for accurate bioassay. From the data of Lee and Lerner(6) it can be calculated that this assay method can detect 10^{-5} μg of purified *alpha*-MSH and 10^{-3} μg of pure ACTH peptide. This corresponds to approximately 10^{-4} I.U. of ACTH. Thus if the MSH activity is intrinsic to the ACTH molecule as shown recently(4,5), it would seem that the frog melanphore assay method could be utilized as a sensitive bioassay of ACTH provided that the other pituitary MSH active fractions are completely eliminated from the extracts by chemical means.

Summary. 1) Bioassays of various pituitary preparations were made to compare quantitative and qualitative characteristics of the different MSH active principles known to be present in such preparations. Results suggest that the 3 chemically distinct pituitary fractions possessing MSH activity (*i.e., alpha*-MSH, *beta*-MSH, and ACTH) act on melanophores in a qualitatively similar manner. It is estimated that the intrinsic MSH activity of ACTH can not be greater than 1/10th that of *alpha*-MSH. 2) A melanophorotropic activity unit (MAU) is proposed which is defined as the amount of active principle producing ½ maximal response (or greater) in 50% of the treated animals. The approximate relationship of MAU to "units" used by other investigators is discussed. The frog bioassay is sensitive to 0.01 MAU and therefore could be used to measure as little as 10^{-3} μg of pure ACTH (corresponding to 10^{-4} I.U. of ACTH) provided this fraction can be completely separated from the other MSH active principles by previous chemical treatments, and assuming that ACTH has an in-

trinsic MSH activity as shown recently.

1. Sulman, F. G., and Eviator, A., *Acta Endocrinol.,* 1956, v23, 120.
2. Bell, P. H., *J. Am. Chem. Soc.,* 1954, v76, 5565.
3. Dixon, H. B. F., *Biochim. et Bioph. Acta,* 1956, v19, 392.
4. Harris, J. I., and Roos, P., *Nature* (Lond.) 1956, v90, 178.
5. Geschwind, I. I., Li, C. H., and Barnafi, L., *J. Am. Chem. Soc.,* 1956, v78, 4494.
6. Lee, T. H., and Lerner, A. B., *J. Biol. Chem.,* 1956, v221, 943.
7. Landgrebe, F. W., Ketterer, B., and Waring, H., in *The Hormones,* G. Pincus and K. V. Thiman, ed., Academic Press, N. Y., vIII, chp. ix, 1955.
8. Lerner, A. B., and Lee, T. H., *J. Am. Chem. Soc.,* 1955, v77, 1066.
9. Porath, J., Roos, P., Landgrebe, F. W., and Mitchell, G. M., *Biochim. et Bioph. Acta,* 1955, v17, 598.
10. Raben, M. S., Rosenberg, I. N., and Astwood, E. B., *Fed. Proc.,* 1952, v11, 126.
11. Deutsch, S., Angelakos, E. T., and Loew, E. R., *Endocrinology,* 1956, v58, 33.
12. Emmens, C. W., *Principles of Biological Assay,* Lond., Chapman and Hall, 1948.
13. Astwood, E. B., Raben, M. S., Payne, R. W., and Grady, A. B., *J. Am. Chem. Soc.,* 1951, v73, 2969.
14. Sulman, F. G., *J. Clin. Endocrinol. and Metab.,* 1956, v16, 755.
15. Lerner, A. B., Shizume, K., and Bunding, I., *ibid.,* 1954, v14, 1463.

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Correlation of Pigmentation and Bacteriophage Sensitivity in Coagulase Positive Staphylococci. (23578)

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In the past decade several investigators have demonstrated correlations between bacteriophage type of staphylococci and antibiotic resistance, habitat or clinical disease. Sensitivity to bacteriophage seems prevalent in the coagulase positive staphylococci, although Rountree(1) and Rippon(2) have reported phages that attack coagulase negative strains. The typing phages are generally limited in range to the coagulase positive staphylococci, with 60-70% of strains typable by test dilutions of phage and the majority of the remaining strains showing some degree of sensitivity to the undiluted phages. Attempts to correlate phage sensitivity with properties other than those mentioned above have generally been unsuccessful. The present report describes the association between pigmentation and phage sensitivity first observed during phage typing of staphylococci isolated from clinical specimens.

Methods. Two series of staphylococcal cultures* freshly isolated from routine clinical specimens were streaked, as received, on plates of Staphylococcus Medium No. 110(3) and incubated at 30°C for 2 days. Typical isolated colonies were subcultured on trypticase soy agar slants for further study. The original Medium No. 110 plates were scored for pigment production and incubated an additional 2 days at room temperature (25°C). In scoring, "pigmented" included the range from deep orange to pale orange or yellow, and "white" included the various shades of white and off-white. The additional incubation period enabled us to observe a number of mixtures in which slight differences in pigmentation emerged on the third or fourth day. The 2 components of such mixtures were treated as separate strains rather than color variants if they also differed in phage pattern or other properties than pigment. Tests for coagulase production were performed by the hospital laboratory and their findings were rechecked by the slide method with diagnostic plasma (Warner-Chilcott). Mannitol fermentation and proteolytic activity on Staphylococcus Medium No.

* Obtained from Parkland Memorial Hospital and Baylor University Hospital, Dallas.