HORMONAL STIMULATION OF MELANOGENESIS IN TISSUE CULTURE*

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The classic concept of the role of intermedin in the evocation of pigmentary phenomena was first developed by Zondek and Krohn in 1932 (1). Recently, interest in the chromatophorotropic effect of ACTH has led to the conclusion that contamination of various ACTH preparations with intermedin produces clinical hyperpigmentation (2). However, the inconsistent action of intermedin in melanogenesis has been demonstrated by many workers (3). This in part may result from the close structural similarities of ACTH and the intermedins which have precluded their complete chemical separation. Thus, the so-called intermedin effects of ACTH preparations and ACTH effects of intermedin preparations must be evaluated in a completely comparable and parallel series of experiments. In addition, the presence of endogenous pituitary hormones and other humoral factors in both intact experimental animals and humans, has complicated the interpretation of biological assays and clinical findings with ACTH and intermedin. Thus, for the critical evaluation of the purported role of intermedin in melanogenesis, it is of paramount importance to maintain a uniform endocrine environment in which various ACTH and intermedin preparations are to be tested. Fulfillment of these requirements has been the basic concept of the present study.

Recently, intermedin has been found to consist of two polypeptides, α-intermedin and β-intermedin (4), both of which have been evaluated in this report. In addition, as Pickford and Kosto (5) have suggested that prolactin also may affect melanogenesis, the action of this hormone has also been evaluated in tissue culture. The present study demonstrated that α-intermedin, β-intermedin, and prolactin fail to stimulate melanogenesis in goldfish integument grown in tissue culture, whereas only ACTH stimulates melanogenesis under similar conditions.

The pigment cell terminology of this report is that utilized at the Third and Fourth Conference on the Biology of Normal and Atypical Pigment Cell Growth. Their definitions are as follows:

Melanocyte: a melanin-producing and melanin-containing cell.

Melanophore: a pigment effector cell in lower animals.

Melanogenesis, melanin formation, and melanin synthesis: increase in the amount of melanin and refer neither to the darkening of any pre-existing melanin granules nor to the redistribution of melanin granules in the pigment cells.

Melanin dispersion and melanin aggregation: rearrangements of preexisting melanin granules in a pigment-containing cell (e.g. melanophores) so that these granules are distributed throughout the cytoplasm (dispersion) or localized in a compact perinuclear mass (aggregation).

To clarify the discussion in this report, the original terminology applied to the hormone originating from the pars intermedia of the pituitary, intermedin, has been retained. Other names have been applied to this polypeptide, for example, a term which is currently widely used, melanocyte-stimulating hormone or MSH.

METHODS AND MATERIALS

The details of roller tube tissue culture technic employed in this study have been described by Hu *et al* (6) for the *in vitro* cultivation of human skin. The caudal fins of completely xanthic (non-melanotic) goldfish were excised, washed as described below, cut into pieces approximately 1 x 2 mm in dimensions, and embedded in a plasma clot on a rectangular coverslip (12 x 50 mm). These explants were placed in culture tubes containing 2 ml of a medium consisting of human ascitic fluid, diluted chick embryonic extract and Gey's balanced salt solution. These cultures were maintained at a constant temperature of 37° C for 72 hours.

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To inhibit bacterial contamination and bacterial growth, the pieces of fin were washed in two changes of Gey's balanced salt solution containing 166 mcg neomycin sulfate per ml. The nutrient supernatant fluid also contained the same concentration of antibiotic.

Freshly prepared solutions of the hormones were immediately incorporated in both clot and supernatant fluid. The hormone preparations employed and the hormone concentrations utilized are indicated in Table I, II, III, IV, V and VI. In addition, ACTH (146RS3) was subjected to enzymatic, acid and alkali hydrolysis as indicated in Table VII, and then tested as above.

To obtain reliable results, the experiments were repeated 3 to 5 times, each utilizing 4 to 6 different fish. Control cultures were made from each fish in all experiments. Data on control cultures were omitted from the tables for the reported findings regarding induced melanogenesis were always made in comparison to the controls. The caudal fin of each fish was used only once to avoid possible variation after fin regeneration. Thus, approximately 150 fish were used to provide the material for more than the 7000 explants cultured in the present study.

Various methods of microscopic examination were employed: (a) phase contrast study and time-lapse cinematography of living cultures, (b) dopa reaction (7), (c) unstained cultures after methyl alcohol fixation, and (d) May-Gruenwald-Giemsa (8) stained preparations.

The presence of melanogenesis was ascertained as follows: (1) whenever melanin containing cells were seen in hormone treated cultures, while no melanin containing cells occurred in control tubes free of hormone, or (2) when there were definitely more pigment containing cells in hormone treated cultures as compared with controls. In addition, the size, degree of ramification, and intensity of pigment color (as a gauge of their melanin producing ability) of the melanocytes in treated and untreated cultures were studied.

RESULTS

Cytological Studies

The growth of goldfish fin explants in tissue culture is rapid and luxuriant. After twenty-four hours, a sheet-like epithelial outgrowth is obvious, and with time increases in size. Partial retraction and liquefaction of the clot and the formation of small empty spaces in various areas of the outgrowth usually occur in from 48 to 72 hours; the cells of the outgrowth appear normal and healthy for at least another 24 hours.

The outgrowth is predominantly epithelial in nature, however, scattered fibrocytes occur between the epithelial cells. In hormone treated cultures and in those few control cultures in which occasional pigment cells are visible, the melanocytes are usually found immediately adjacent to the explant and only rarely move into the outgrowth itself (Fig. 1). The melanocytes which are found in the outgrowth are smaller than those found in the explant itself. The details of the cytological findings are presented below.

(*A*) *May-Gruenwald-Giemsa Preparations.* This stain demonstrates the extent of epithelial outgrowth well. The four types of cells seen in the outgrowth are:

1. *Epithelial cells* in the form of sheets (Fig. 1).
2. *Fibrocytes* scattered in between the epithelial cells or in thin sheets (Figs. 1, 2).
3. *Presumptive melanocytes* which are small rounded cells, whose morphology and staining reactions resemble those of young pigment cells found in cultures of human skin (9). The cells most commonly have short, stubbed processes, a scanty amount of occasionally granular cytoplasm and an oval or round, intensely red staining nucleus. The nuclei of epithelial cells and fibrocytes stain a definitely less intense red color (Fig. 2).
4. *Large melanocytes* were found in large numbers in ACTH treated cultures and only rarely found in control cultures. The morphology of these cells is variable. Some are bipolar with long slender processes (Fig. 2); others are stellate and highly branched (Fig. 3), while still others are broad and plaque-like. Although their nuclei are not much larger than those of the epithelial cells, the entire melanocytes including processes, are several times larger than either epithelial cells or fibrocytes. In addition, multinucleated melanocytes may often be present. The delineation between melanocyte and melanophore was not attempted for this was not considered pertinent to the present study.

(*B*) *Unstained Cultures.* Because of their intracellular black melanin granules which contrast with the unstained background, the melanocytes are readily visible in unstained cultures (Figs. 4, 5). As indicated above, these cells are usually found on and immediately adjacent to the explant. These cells represent the population of melanocytes which contain pigment granules. Since the propigment cells do not contain melanin granules, they are not visualized in this type of a

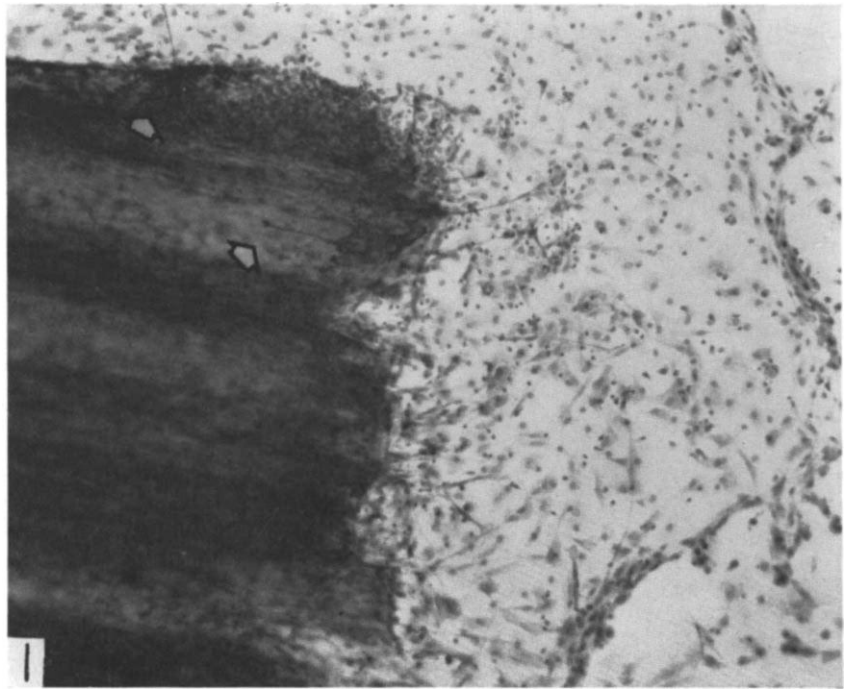


FIG. 1. Caudal fin culture. ACTH treated. May-Gruenwald-Giemsa. 100 ×. Outgrowth of epithelia cells in sheets with fibrocytes, melanocytes and small round cells scattered in between. Note the large heavily pigmented polydendritic cells in the dark, solid explant portion.

preparation. These fixed, but unstained preparations, are more satisfactory for the examination of melanocytes than any method used. Therefore, in the later experiments, only this type of preparation was utilized.

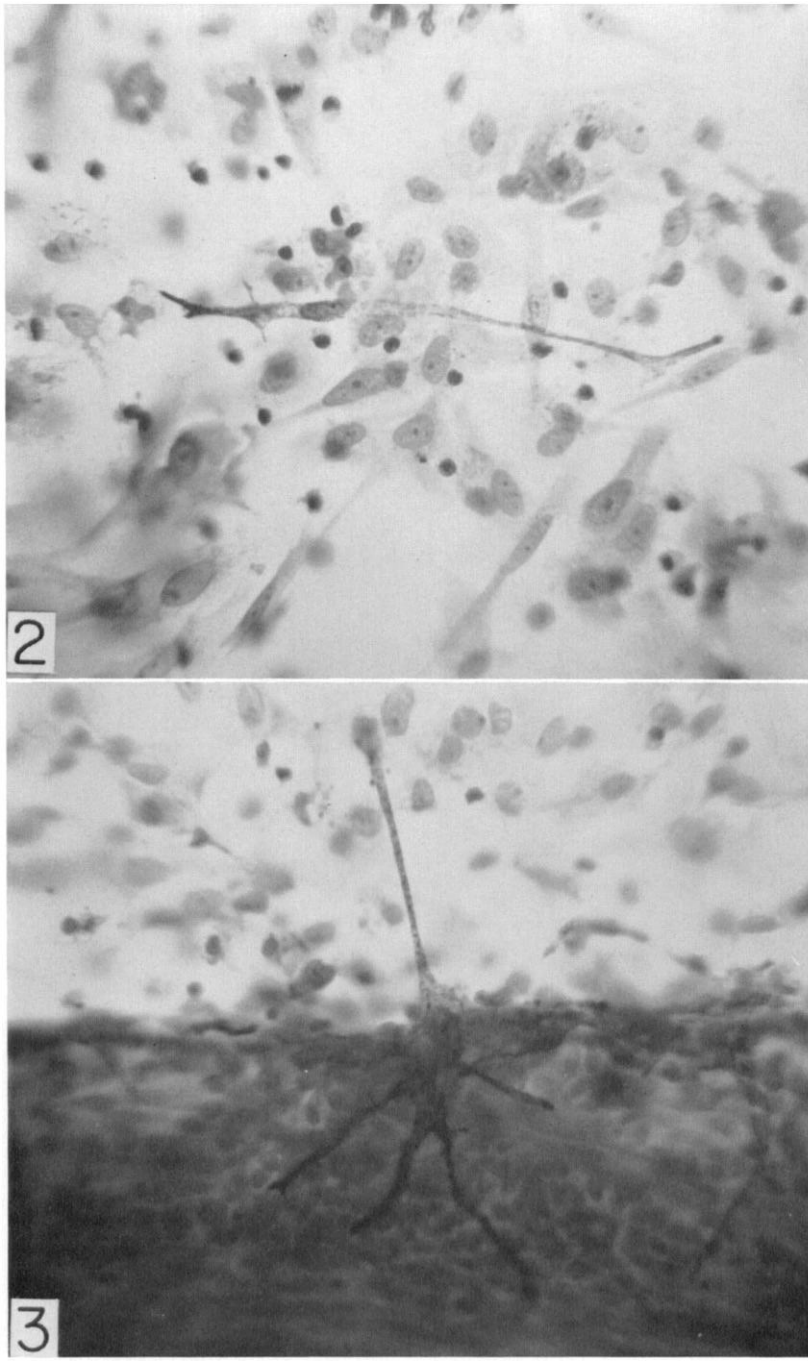
(*C*) *Dopa reaction.* The dopa reaction has no advantage over the above technics; on the other hand, it has several disadvantages. Firstly, in processing the cultures during this cytochemical reaction, the explants frequently loosen from the clots and are lost. In addition, most of the melanocytes following ACTH treatment contain melanin granules, and it is impossible to determine whether the intracellular granules are black prior to or after exposure to the dopa reagent. Thus, it is not possible to accurately define a positive dopa reaction. In addition, dopa positive cells do not occur in the control cultures. Consequently, under such conditions the dopa reaction does not contribute more information regarding new melanin formation. Because of these technical drawbacks, the dopa reaction was omitted from the later experiments.

(*D*) *Phase contrast examination of living cultures.* All previously described cell types are visible in the living state. In addition, the following cells are seen:

1. *Lipophores.* These cells are visible only in the living state. Their yellow to red carotenoid pigment is soluble in organic solvents; hence their color is lost following fixation and dehydration. As these cells are not pertinent to the present study further discussion of them is omitted.
2. *Motile cells.* Careful examination reveals the amoeboid movement of actively motile cells. This motility is obvious with use of time-lapse cinematography. Such cells contain cytoplasmic inclusions of either rather large, refractile globules or small dark granules (Fig. 6). Such cells are not always identifiable in fixed preparations.

Endocrine Studies

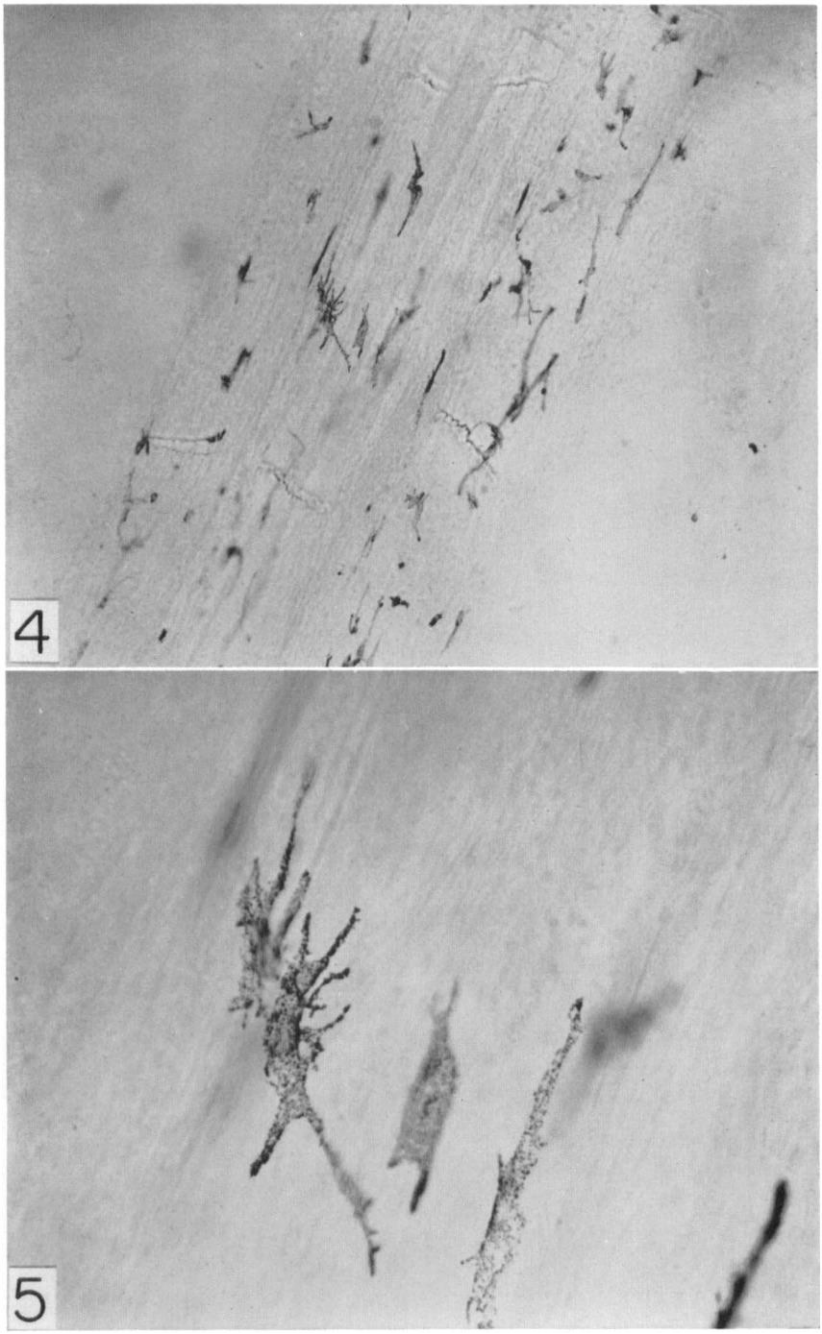
The effects of ACTH, intermedin and prolactin upon melanogenesis and the appearance of melanocytes are summarized in Table II, III, IV, V and VI. The ACTH concentrations tested vary from 2×10^{-7} to 21.6 IU per ml of medium. The concentrations of intermedin tested range from



FIGS. 2 and 3. Caudal fin culture. ACTH treated. May-Gruenwald-Giemsa. 430 ×

FIG. 2. One long melanocyte with a "foot-plate-like" structure at end of one dendrite. Many small round cells with distinct deeply staining nuclei and little cytoplasm. A few of these cells contain melanin granules, and appear to be young melanocytes. Epithelial sheet and a few fibrocytes are seen in the background.

FIG. 3. Three melanocytes at edge of explant. The largest one is seen in the center with one long process extending into the outgrowing epithelial sheet.



FIGS. 4 and 5. Caudal fin culture. Fixed and unstained. Culture treated with ACTH, 0.02 IU/ml medium for 72 hours.

FIG. 4. Numerous spindle, stellate, and polydendritic melanocytes on the explant. Note the black intracytoplasmic melanin granules in good contrast with the unstained light background. 100 ×

FIG. 5. High magnification of Fig. 4. 430 ×

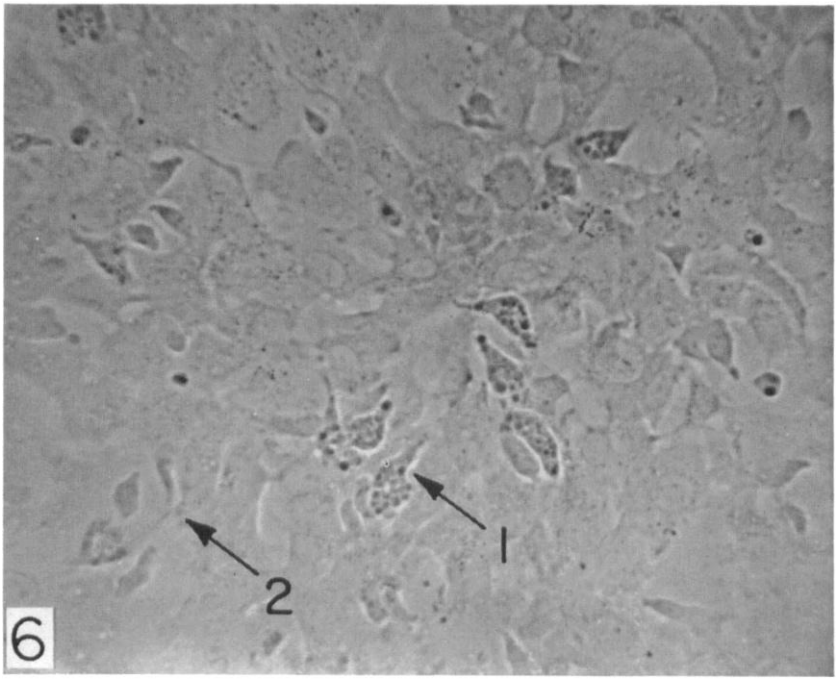


FIG. 6. Living untreated culture of goldfish caudal fin. Phase contrast microscopy. 430 ×. Epithelial sheet forms the lighter background. The darker cells are motile cells with either (1) large round refractile bodies, or (2) fine dark granules.

0.1 Frog skin units (10), FSU, to 1.7×10^6 FSU per ml of medium. As indicated in Table II, melanin formation occurs at the minimal intermedin concentration of 1.4×10^4 FSU/ml. On the other hand, the minimal amount of ACTH necessary to induce melanogenesis is 2×10^{-3} IU/ml. As the concentrations of these hormones are obviously represented in different biological units, it is not possible to compare them. In addition, as these hormones do not occur in a constant ratio in each of the various preparations utilized in this study, it is only possible to compare their actions at various concentrations depending upon the preparation used.

The data indicate that intermedin concentrations are not directly correlated with melanogenesis. For example, melanocytes appear in cultures made with an ACTH preparation (146RS3) containing 2×10^{-3} IU ACTH and 60 FSU intermedin per ml (intermedin being present as impurity in the ACTH preparation), and consistently in concentrations of 300 FSU intermedin and 1×10^{-2} IU ACTH. While with another intermedin preparation (D216-155C) containing 1.4×10^4 FSU intermedin and 2×10^{-4} IU ACTH per ml (ACTH being present as impurity in the intermedin preparation) melanin formation does not occur at all times. Therefore, if intermedin is the hormone responsible for melanin formation in goldfish, it is difficult to reconcile the fact that melanogenesis fails to uniformly occur after the use of 1.4×10^4 FSU of a different preparation, an increase of 233 times the intermedin concentration. Further, the results with the two intermedin preparations of Armour Laboratories demonstrate this point rather well (Table IV). Melanogenesis occurs at 1.7×10^3 FSU intermedin (AL 1037) containing 2.8×10^{-4} IU ACTH, and at 1.4×10^4 FSU intermedin (D216-155C) with 2×10^{-4} IU ACTH. At these concentrations the latter preparation more frequently showed a lesser melanogenic action than the former, thus again correlating ACTH concentrations, and not intermedin concentrations, with melanogenesis.

The melanogenic activities of two intermedin preparations, LR-I and D216-155C, (Tables IV and V, respectively) are comparable. However, LR-I reveals no detectable ACTH activity as indicated by ascorbic acid depletion method, while D216-155C reveals 2 IU/mg of ACTH activity (Table I). The significance of this finding

TABLE I

Assay data of hypophyseal hormones used in tissue culture studies of goldfish skin

Hormone Preparation	Animal Origin	Supplier	Intermedin (MSH) FSU/mg[2]	ACTH IU/mg[1]	Luteotropic Activity Unit/mg	Other Activities
ACTH:						
146RS3	Porcine	Armour	1×10^5	3.6		? trace of thyrotropin or gonadotropin
907-109-A4[3] (Corticotropin A)	Porcine	Armour	2×10^5	100.0		
cc99H'B_2D[4]	Ovine	Geschwind & Li	1×10^4–1×10^5	78.0		
L2821A (α-Cort.)	Ovine	Geschwind & Li	1×10^4–1×10^5	80.0		
Intermedin (MSH):						
D216-155C[5]	Porcine	Armour	1.4×10^8	2.0		
AL 1037[5]	Porcine	Armour	6.0×10^5	0.1		Histamine activity 0.5 gamma/mg
Intermedin (MSH)[6]	Porcine	Lee & Lerner	1.0×10^6	No data		
LR-I[7] (β-intermedin, β-MSH)	Bovine	Geschwind & Li	1–2×10^6	0		
CCD4 (β-intermedin, β-MSH)	Porcine	Geschwind & Li	4.0×10^6	0.001		
α-intermedin (α-MSH)	Porcine	Lee & Lerner	6.0×10^6	No data		Vasopressin activity
Prolactin:						
L2651AP	Ovine	Geschwind & Li	2.0×10	0.02	30–35	

[1] IU: International Unit based on ascorbic acid depletion.

[2] FSU: Frog Skin Unit as defined by Shizume *et al*, 1954.

[3] Dr. W. F. White of Armour Laboratories informed us that this sample had not been assayed for intermedin activity, but several comparable materials had been assayed in Dr. A. B. Lerner's laboratory and all values were approximately 2×10^8 FSU/gm.

[4] This preparation has not been assayed for intermedin activity, but according to Dr. I. I. Geschwind the activity is probably between 10^7 to 10^8 FSU/gm.

[5] Alkali treated.

[6] Purified by acetic acid extraction, ether purification, oxycellulose adsorptions and a short counter-current distribution in 2-BuOH-0.05% trichloroacetic acid.

[7] A counter-current purified intermedin preparation with no detectable ACTH activity.

is of particular interest for it is now recognized that ACTH has several actions which cannot be evaluated by the ascorbic acid depletion assay. Thus, the melanogenic activity of ACTH may not depend upon the ACTH potency as indicated by this assay technic.

An β-intermedin preparation, CCD4, which has ACTH activity of less than 1×10^{-3} IU/mg, is very different from LR-I in regard to melanogenic activity. The former preparation is active in intermedin and ACTH dilutions far below those which were expected to show melanogenic activity (Table V). Again, these findings are explicable only if the ascorbic acid depletion potency has little correlation to the melanogenic activity of ACTH.

The experimental findings utilizing a porcine intermedin prepared by Lee and Lerner (Table I), indicate that the melanogenic activity is comparable to that of D216-155C. A preparation of α-intermedin also prepared by Lee and Lerner, is somewhat more active than both D216-155C and LR-I (Tables IV and V). It was purified by carboxyl methyl cellulose column chromatography, contained a small amount of vasopressin but apparently was free of β-intermedin.

TABLE II

The effects of ACTH and intermedin upon melanogenesis in tissue culture of goldfish skin

ACTH 146RS3			Intermedin D216-155C		
Intermedin[1] FSU/ml	ACTH[1] IU/ml	Melanogenesis[2]	Intermedin FSU/ml	ACTH IU/ml	Melanogenesis
6×10^5	21.6	+			
3×10^5	10.8	+			
3×10^4	1.08	+			
6×10^3	2×10^{-1}	+			
3×10^3	1×10^{-1}	+			
6×10^2	2×10^{-2}	+	1.4×10^6	2×10^{-2}	+
3×10^2	1×10^{-2}	+	2.8×10^5	4×10^{-3}	+
6×10	2×10^{-3}	±	1.4×10^5	2×10^{-3}	+
6	2×10^{-4}	−	1.4×10^4	2×10^{-4}	±
			1.4×10^3	2×10^{-5}	−

[1] See Footnotes 1 and 2, Table I.

[2] + Indicates the presence of melanin-containing melanocytes in 90–100% of the cultures. The melanocytes are usually larger, more darkly pigmented and possess more branched dendrites than those in the control cultures.

± Indicates the presence of melanocytes in about 50% of the cultures.

− Like untreated control cultures.

Alkali treatment of intermedin preparations potentiates the intermedin effect while destroying at least the contained ascorbic acid and ketogenic activities of ACTH. An alkali treated intermedin, AL 1037, like the α-intermedin, appears to be more active than D216-155C, although the latter is also alkali treated. This difference, however, is not very great and probably is within the range of technical limitations. It may be of little or no significance (Table IV).

Surprisingly, the melanogenic activities of the three highly purified ACTH preparations are not as great as that of the less purified preparation (146RS3). All three purified hormones produce positive results at intermedin concentration of 6×10^3 FSU/ml. According to data obtained from earlier experiments (Table II), intermedin at this concentration is not expected to have melanogenic activity. However, on the basis of ACTH potencies, the ascorbic acid depleting activities of the three purified hormones are at least 200 times higher than that of the less purified material. As these hormones were obtained from two different laboratories, the method of preparation may be a differential factor. However, this may not be an adequate explanation, for the two preparations from the Armour Laboratories also yield differential findings, as the highly purified porcine preparation (907-109-A4) appears less effective in stimulating melanogenesis than the less purified porcine material (146RS3). This result appears inexplicable in terms of either intermedin or ACTH. For example, in comparing these ACTH preparations, dilutions are made so that the concentration of intermedin (as impurity) in each is 6×10^3 FSU/ml. If the melanogenic effect is attributable to ACTH alone, a greater degree of pigment formation should be expected in the highly purified preparation (at the above intermedin level, 4.0 IU ACTH per ml). Melanogenesis is observed in both preparations, but the degree of melanosis invariably is more extensive and the pigment containing cells more numerous in the less purified preparation (at the above intermedin level, 0.2 IU ACTH per ml). The findings are consistent when a series of concentrations of both preparations are tested (Table III). The apparent decrease in melanogenic activity of highly purified ACTH preparations also occurs in two ACTH preparations supplied by Drs. Geschwind and Li (Table III). There appears to be a definite loss of melanogenic activity during purification of ACTH. This loss is not detectable by the usual bioassay technic which is based upon the depletion of adrenal ascorbic acid. It is possible that ACTH may lose some of its activities, i.e. melanogenic factor, during the process of purification while retaining its adrenal ascorbic

TABLE III

Comparative action of four ACTH preparations upon melanogenesis in tissue culture

146RS3			907-109-A4			cc99H'B$_2$D			L2821A		
Intermedin[1] FSU/ml	ACTH[1] IU/ml	Melano-genesis[2]	Intermedin FSU/ml	ACTH IU/ml	Melano-genesis	Intermedin FSU/ml	ACTH IU/ml	Melano-genesis	Intermedin FSU/ml	ACTH IU/ml	Melano-genesis
6×10^5	21.6	+									
3×10^5	10.8	+									
						1–10 $\times 10^3$	7.8	+	1–10 $\times 10^3$	8.0	+
			6×10^3	4.0	+						
									2.5–25 $\times 10^2$	2.0	−
3×10^4	1.08	+									
						1–10 $\times 10^2$	7.8×10^{-1}	−			
			6×10^2	4×10^{-1}	±						
6×10^3	2×10^{-1}	+	3×10^2	2×10^{-1}	−				2.5–25 $\times 10$	2×10^{-1}	−
3×10^3	1×10^{-1}	+									
						1–10 $\times 10$	7.8×10^{-2}	−			
			6×10	4×10^{-2}	−						
6×10^2	2×10^{-2}	+	3×10	2×10^{-2}	−				2.5–25	2×10^{-2}	−
3×10^2	1×10^{-2}	+									
						1–10	7.8×10^{-3}	−			
6×10	2×10^{-3}	±	3	2×10^{-3}	−				0.25–2.5	2×10^{-3}	−
						1–10 $\times 10^{-1}$	7.8×10^{-4}	−			
6	2×10^{-4}	−									
						1–10 $\times 10^{-2}$	7.8×10^{-5}	−			

[1] See footnotes 1 and 2, Table I.

[2] See footnote 2, Table II.

TABLE IV

Comparative action of three intermedin preparations upon melanogenesis in tissue culture

D216-155C			AL 1037			Intermedin (Preparation of Lee & Lerner)		
Intermedin[1] FSU/ml	ACTH[1] IU/ml	Melanogenesis[2]	Intermedin FSU/ml	ACTH IU/ml	Melanogenesis	Intermedin FSU/ml	ACTH IU/ml	Melanogenesis
			1.7×10^{6}	2.8×10^{-1}	+			
			3.4×10^{5}	5.6×10^{-2}	+			
1.4×10^{6}	2×10^{-2}	+	1.7×10^{5}	2.8×10^{-2}	+	1.3×10^{3}	No data	+
2.8×10^{5}	4×10^{-3}	+	3.4×10^{4}	5.6×10^{-3}	+			
1.4×10^{5}	2×10^{-3}	+	1.7×10^{4}	2.8×10^{-3}	+	1.3×10^{5}		+
1.4×10^{4}	2×10^{-4}	±	1.7×10^{3}	2.8×10^{-4}	+	1.3×10^{4}		±
1.4×10^{3}	2×10^{-5}	−	1.7×10^{2}	2.8×10^{-5}	±	1.3×10^{3}		−
			1.7×10	2.8×10^{-6}	−			
			1.7	2.8×10^{-7}	−			

[1] See footnotes 1 and 2, Table I.
[2] See footnote 2, Table II.

TABLE V

Comparative action of α- and β-intermedin upon melanogenesis in tissue culture

LR-I (β-intermedin)			CCD4 (β-intermedin)			α-Intermedin (Lee & Lerner)		
β-intermedin[1] FSU/ml	ACTH[1] IU/ml	Melanogenesis[2]	β-intermedin FSU/ml	ACTH IU/ml	Melanogenesis	α-intermedin FSU/ml	ACTH IU/ml	Melanogenesis
			8×10^{5}	2×10^{-4}	+			
			4×10^{5}	1×10^{-4}	+			
$1–2 \times 10^{5}$	0	+	2×10^{5}	5×10^{-5}	+			
			8×10^{4}	2×10^{-5}	+	6×10^{4}	No data	+
$1–2 \times 10^{4}$	0	+	4×10^{4}	1×10^{-5}	+			
			8×10^{3}	2×10^{-6}	+	6×10^{3}		+
$1–2 \times 10^{3}$	0	±	4×10^{3}	1×10^{-6}	+			
$1–2 \times 10^{2}$	0	−	8×10^{2}	2×10^{-7}	+	6×10^{2}		±
$1–2 \times 10$	0	−						
						6×10		−
						6		−
						6×10^{-1}		−

[1] See footnote 1 and 2, Table I.
[2] See footnote 2, Table II.

acid depleting potency. Of course, other pituitary hormones may also be involved here. Complete assay data on all the preparations used are not available. As indicated in Table I, AL 1037 has a histamine activity of 0.5 gamma per mg, 146RS3 may have trace amounts of thyrotropin or gonadotropin and α-intermedin (Lee and Lerner) has some vasopressin activity. However, Chavin (11) has demonstrated that intact and hypophysectomized goldfish do not become melanized after administration of TSH, gonadotropins, oxytocin or vasopressin.

TABLE VI

Effect of luteotropin (L2651AP) upon melanogenesis in tissue culture

Luteotropin Unit/ml	Intermedin[1] FSU/ml	ACTH[1] IU/ml	Melanogenesis[2]
30–35	20	2×10^{-2}	±
3–3.5	2	2×10^{-3}	−

[1] See footnotes 1 and 2, Table I.
[2] See footnote 2, Table II.

TABLE VII

Effects of various hydrolytic procedures upon melanogenic activity of ACTH (146RS3) on goldfish skin in tissue culture, and comparison with reported findings dealing with other ACTH factors and intermedin

Treatment	Ascorbic Acid Depleting Activity	Ketogenic Activity	Intermedin Activity	Melanogenic Activity
0.1 N NaOH, 100 C, 20 min.	Total loss (Engel and Engel, 1957)	Total loss (Engel and Engel, 1957)	No loss (potentiation effect)	No loss
6N HCl, 37.4 C, 24 hrs	Total loss (Li, 1948)			Total loss
1N HCl, 100 C, 1 hr			Little or no loss (Bagnara, 1958)	Total loss
0.05% pepsin, 0.05M HCl, 37.4 C, 4–5 hrs	Little or no loss (Li, 1948)			Definite loss
1% pepsin, 0.05% HCl, 37 C, 24 hrs			Little or no loss (Bagnara, 1958)	Total loss
0.1% trypsin, pH 7.0, 37 C, 5 hrs			Almost total loss (Bagnara, 1958)	Total loss

Ovine prolactin at a concentration of 30 to 35 units per ml of medium only occasionally induces the appearance of melanin-containing melanocytes *in vitro* (Table VI). At this concentration the prolactin preparation contains 0.02 IU ACTH and 20 FSU intermedin/ml. At a concentration of 3 to 3.5 prolactin units/ml the results were consistently negative. Therefore, these data fail to confirm Pickford and Kosto's (5) *in vivo* findings dealing with *Fundulus* even though the concentrations of the same hormone preparation† used in the present experiment are comparable to those of above authors. Because of the limited supply of prolactin, the combined effects of intermedin and prolactin upon melanogenesis could not be investigated at this time. The ACTH present in the prolactin experiments appears to be less effective melanogenically than expected, for no pigment formation is observed at concentrations of 2×10^{-3} IU ACTH per ml, and only occasional pigment formation at 2×10^{-2} IU ACTH per ml. These concentrations may be compared with those of the less purified ACTH, 146RS3, with which 2×10^{-2} IU ACTH per ml are found to be consistently effective in producing melanogenesis. A similar loss of melanogenic activity may occur during the isolation of lactogenic hormone as in the purification of ACTH. In any case, under the described experimental conditions, prolactin, despite the ACTH contamination, is not effective in stimulation of melanogenesis.

In order to determine whether enzymatic digestion, acid or alkali treatment will affect the melanogenic potency of ACTH, lot 146RS3 was subjected to the treatment as indicated in Table VII. The results indicate that the melanogenic activity of ACTH is not altered by alkali treatment but is lost following trypsin digestion and acid hydrolysis.

Interestingly, alkali treatment is known to potentiate the intermedin effect. Bagnara (12) has shown this to be the case in the preparation (146RS3) used in the present study. However, no definite increase in the melanogenic action of the alkali treated hormone occurred when tested in tissue culture.

While Bagnara demonstrated little or no loss of intermedin activity following 1% pepsin digestion for 24 hours, the present results indicate an almost total loss of melanogenic activity of the hormone following pepsin digestion as described by Li (13) or Bagnara (12). After partial acid hydrolysis (0.02% ACTH in 1 N hydrochloric acid at 100° C for 1 hour), there is a total loss of melanogenic activity, in contrast to the almost

† Drs. Geschwind and Li who have supplied the prolactin used in the present study have indicated that this is the same preparation utilized by Pickford and Kosto.

complete retention of intermedin potency reported by Bagnara (12) after similar treatment of the same hormone preparation. Thus, it is apparent from these differential reactions that the ACTH factor causing melanogenesis is separate and distinctly different from the melanin-granule-dispersing intermedins.

DISCUSSION

The darkening effect of intermedin upon frog skin is the result of the dispersion of the melanin granules in the melanophores. This observation can only be made upon cells containing melanin, since intermedin causes the dispersion of the pigment granules peripherally in the cytoplasm of the melanophores ("expansion" of the cells). The changes in light reflectance reading, the basis of the Frog Skin Unit (FSU), indicate only the changes in the degree of dispersion of the melanin granules in the melanophores. This change is rapid and does not indicate the synthesis of new melanin. The present study deals with the demonstration of *newly formed* melanin granules in cells of ACTH treated tissue cultures of goldfish melanocytes. Although it is not possible to histochemically demonstrate the existence of melanin precursors in goldfish propigment cells, ACTH activates pigment synthesis in these cells and thus appears to be responsible for the conversion of a colorless propigment cell into the melanin containing cell, the melanocyte. The mechanism by which ACTH produces pigment in goldfish melanocytes in unknown, although the possibility exists that ACTH activates an enzyme system or even releases an inhibitory mechanism preventing pigment formation. The tissue culture method is most suitable for studying the direct effect of ACTH on propigment cells. Utilizing this technic, the only difference between the experimental and control cultures is the presence or absence of ACTH. Neural, hormonal, chemical and physical factors remain constant. This is in contrast to the situation which exists in an intact animal in which a given stimulus, i.e. ACTH, elicits a series of physiological responses in the organism. In the latter situation, it is impossible to distinguish the direct from the indirect effects. It is to be expected, therefore, that agreement of *in vitro* and *in vivo* results may not always occur.

ACTH directly stimulates the formation of pigment in the dermal melanocytes of goldfish *in vitro* when the propigment cells are maintained in a uniform endocrine environment. Despite the fact that pigment formation occurs in the intermedin treated cultures, the evidence indicates that trace contamination of the intermedin preparations with ACTH is actually responsible for the observed pigmentation. With the use of conventional assay methods, ACTH contamination is apparently not detected in highly purified intermedin preparations, such as lot LR-I. However, melanin-containing melanocytes do appear in cultures treated with this purified preparation. It is evident, therefore, that the presently available ACTH assay methods are not always sufficiently sensitive for detection of the minute quantities of ACTH required for stimulation of melanogenesis in goldfish melanocytes. It is suggested, therefore, that the *in vitro* melanogenic action of ACTH may provide an extremely sensitive bioassay for this hormone.

ACTH has been found to have a variety of adrenocortical and extra-adrenal effects. In this regard, both the weight and ascorbic content of the adrenal cortex have long been known to be controlled by separate adrenocorticotropic "factors," the adrenal weight factor, AWF, and the ascorbic acid depleting factor, AAF (14, 15, 16). These factors do not occur in proportionally equivalent ratios in the ACTH preparations tested by the above authors. A wide variety of other actions of ACTH upon the cortex and extra-adrenal tissues have been summarized by Li (17) and Engel (18). Interestingly, among the other actions of ACTH, these authors as well as Chavin (11), have suggested that ACTH itself has a melanin-granule-dispersing activity, on the basis of biochemical and physiological evidence. In fact, ACTH may not be a uniform hormone product. For example, the recent study of Newman *et al* (19) suggests that specific varieties of ACTH may be secreted by the hypophysis as a function of the neural area from which a given stimulus may originate. If confirmed, this may offer a partial explanation of the multifaceted activity of ACTH. Despite the presently available information concerning the multiple activities of ACTH, the ascorbic acid depletion bioassay procedure is routinely used in the determination of ACTH potency. Thus, in any work dealing with even highly purified ACTH, the number of variables is unknown. Perhaps the questions raised in the present study, as well as in others, may be resolved to some degree by the

quantitative determination of the known ACTH "factors" in the preparations utilized, for the melanogenic action of ACTH may closely follow some amino acid sequence other than that demonstrated by adrenal ascorbic acid depletion. This is apparent from the tissue culture findings (Table III) in which a less purified preparation (146RS3) was twenty times more effective in stimulating melanogenesis than the more highly purified preparation (907-109-A4) when potency is based on ascorbic acid depleting activity and intermedin concentrations are identical. However, on a weight basis, the latter is 1.4 times more effective in stimulating melanin synthesis than the former preparation, thus indicating that not only is the purified material, indeed, more potent, but also that the melanogenic activity is not related to the ascorbic acid depleting activity of ACTH.

Present evidence indicates that highly purified ACTH may not be the material of choice for basic endocrine studies dealing with melanogenesis. Engel and Engel (20) reported that the treatment of purified ACTH with 0.1 N sodium hydroxide at 100° C for 20 minutes resulted in the loss of ascorbic acid depleting potency and ketogenic activity. However, such treatment does not abolish intermedin activity. The results of the present study also indicate the preservation of the melanogenic activity of goldfish melanocytes following such alkali treatment. This lends support to the concept that the segment of the ACTH molecule possessing melanogenic activity may not be identical to that portion of the molecular structure responsible for adrenal ascorbic acid depletion and ketogenesis (Table VII). In addition, Hungerford *et al* (21) and Johnsson and Hogberg (22) have found that alkali-treated ACTH loses its ascorbic acid depleting action but its eosinopenic activity is still demonstrable. It appears quite probable that the various amino acid sequences of the polypeptide known as ACTH may each have some specific biological action which may be dependent upon but varying in their degree of dependence upon the integrity of the entire molecule as presently known. It is conceivable, therefore, that the molecular structure of ACTH is actually more complex than presently realized, for the procedures involving low pH and heat which are routinely utilized in the extraction and purification of pituitary material may partially hydrolyze the "natural" ACTH molecule. Similar but more extreme treatment of the "purified" hormone has been shown by many workers (17) to alter the amino acid sequences. Further consideration of this problem in regard to ACTH structure, as well as that of other pituitary hormones, may clarify the manifold problems in this area.

The close structural and chemical similarity of intermedin and ACTH makes their complete separation difficult. In addition, as indicated above, the purification of the hormonal end product may lead to partial or complete loss of the principle responsible for melanogenesis. From the data obtained to date, ACTH appears to be the principle agent in melanogenesis in goldfish, intermedin playing a secondary role, if any, in this process. It should be noted, however, an augmentation of ACTH action in melanogenesis by intermedin has not been demonstrated. In considering hormonal interaction in melanogenesis, the findings of Pickford and Kosto (5) are of interest. Their results indicate that prolactin potentiates the response of killifish (*Fundulus heteroclitus*) melanocytes to intermedin, permitting the formation of new pigment cells in response to a dose level of intermedin which is ineffective when administered alone. It is possible that the prolactin as well as the intermedin used in their experiments contained small amounts of ACTH melanogenic factor. The parallel bioassays suggested above might be instrumental in explaining these apparent discrepancies and in breaking the complex termed ACTH into its component physiological parts.

Evocation of melanogenesis in *Fundulus* as described by Pickford and Kosto (5) requires a very high dosage of intermedin. For example, 100 FSU per gram weight of animal barely produced melanogenesis, while 1,000 FSU per gram of animal produced maximal effect. The latter dosage may well be above physiological limits and if so, the results may fall into the category of pharmacological or paradoxical effects. Earlier, Chavin (3) showed that 1×10^6 FSU of β-intermedin injected into xanthic goldfish did not evoke melanogenesis in this species. The appearance of melanocytes and melanin granules, therefore, may be a more specific response in the goldfish than in *Fundulus*. Recently, Chavin (11) demonstrated that the intermedin contained in the pituitaries of xanthic goldfish was approximately 20 units (23) per gram of fish. When fish were maintained over a black background for 115 days, a 66% increase in intermedin occurred. Despite the gradual increase in endogenous intermedin,

melanogenesis did not occur. It is also of interest in this respect that Chavin (24) has found that the intermedin content of the pituitaries of saline stressed fish is in the same range as that of the control fish; the former are melanized but the latter are not. These findings indicate that endogenous intermedin is not the hormone which stimulates melanogenesis.

The melanogenic action of ACTH is direct and without adrenocortical mediation. Thus, the hormonal balance between the pituitary secretion of ACTH and the adrenal cortex may determine the degree of melanoderma. In clinical hypopituitarism where little ACTH is synthesized, a decrease in pigmentation occurs; in hypoadrenalism, increased pigmentation results from the overproduction of ACTH in response to the low blood adrenal corticoid levels. Clinical hyperpigmentation following the injection of ACTH originally was believed to be a result of the intermedin contamination in the ACTH preparation. No positive evidence is available to indicate that the increase in pigmentation following intermedin administration is not due to the contamination of intermedin by a molecular variety or moiety of ACTH.

The melanin-granule-dispersing effect of intermedin shown so consistently by various workers in frog or fish pigment cells has been established beyond doubt. But it is important to note that such effects were observed mainly in the pigment-effector cells, the melanophores. These cells are specialized, of a type found only in cold-blooded vertebrates but not in higher animals. In tissue culture, these cells were not seen in mitosis; they were usually degenerate, and were not replaced on prolonged incubation *in vitro*. Utilizing perfusion technic, Hu and Mori (25) failed to observe pigment dispersion effect of intermedin in frog melanocytes grown in tissue culture. These pigment-containing cells, on the basis of their morphological characteristics, were believed to be melanocytes instead of melanophores. It is conceivable that these two types of cells respond differently to hormonal stimulation. The effect of intermedin on frog melanophores appears to be rapid, the reaction taking place within a matter of minutes. The response of fish propigment cells and melanocytes to ACTH in tissue culture was found to be much slower. Even though melanin-containing polydendritic cells were present in tissue culture at the end of 48 hours incubation, invariably many more were found at the end of 72 hours. It seems apparent that the response of melanocytes to the effect of the melanogenic factor is relatively slow and requires several days to reach the maximal response. Due to technical difficulties it has not been possible to observe the prolonged action of ACTH on cultured fish melanocytes maintained in a perfusion chamber. Further study is in progress. The evidence obtained to date indicates rather convincingly that the effects of hormones upon melanophores and melanocytes are different. Intermedin is effective in stimulating dispersion of pigment granules in melanophores, while ACTH is effective in inducing melanogenesis in melanocytes.

SUMMARY

Because of difficulties of complete separation of the ACTH and intermedin activities in even the most highly purified hormone preparations, and the relatively scanty experimental evidence dealing with melanogenesis in a completely controlled endocrine environment, the actions of various hormones reported to affect melanin synthesis in the skin were studied. The effects of ACTH, α-intermedin, β-intermedin and prolactin (luteotropin) upon the appearance of melanocytes and stimulation of melanin synthesis in completely xanthic goldfish skin grown in tissue culture were compared. The intermedins and prolactin were found to have some stimulatory effect upon melanogenesis *in vitro*, but only resulting from the inherent ACTH contamination. Only ACTH was found to stimulate melanin formation. However, the ACTH preparations used varied in melanogenic potency. Enzymatic digestion as well as acid or alkaline hydrolysis studies of ACTH demonstrated that the melanogenic activity of ACTH was not correlated with the ascorbic acid depleting factor, the ketogenic factor or the intermedin activity. Thus, some portion of the ACTH molecule other than those responsible for the above activities stimulates melanogenesis. The current practice of indicating ACTH potency in terms of adrenal ascorbic acid depleting activity does not indicate the melanogenic potency of the preparations.

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REFERENCES

1. Zondek, B. and Krohn, H.: Hormon des Zwischenlappens der Hypophyse (Intermedin). III. Zur Chemie, Darstellung und Biologie des Intermedins. Klin. Wchnschr., **11:** 1293–1298, 1932.
2. Lerner, A. B., Shizume, K. and Bunding, I.: The mechanism of endocrine control of melanin pigmentation. J. Clin. Endocrinol., **14:** 1463–1490, 1954.
3. Chavin, W.: Pituitary-adrenal control of melanization in xanthic goldfish *Carassius auratus* L. J. Exper. Zool., **133:** 1–46, 1956.
4. Harris, J. I. and Lerner, A. B.: Amino acid sequence of the α-melanocyte-stimulating hormone. Nature (Lond.), **179:** 1346–1347, 1957.
5. Pickford, G. E. and Kosto, B.: Hormonal induction of melanogenesis in hypophysectomized killifish (*Fundulus heteroclitus*). Endocrinology, **61:** 177–196, 1957.
6. Hu, F., Livingood, C. S. and Hildebrand, J. F.: The roller tube tissue culture technic in the evaluation of the primary irritancy producing capacity of topical medicaments and chemicals. J. Invest. Dermat., **26:** 23–39, 1956.
7. Laidlaw, G. F. and Blackberg, S. N.: Melanoma studies: II. A simple technic for the dopa reaction. Am. J. Path., **8:** 491–498, 1932.
8. Jacobson, W. and Webb, M.: The two types of nucleoproteins during mitosis. Exper. Cell Res., **3:** 163–183, 1952.
9. Hu, F., Staricco, R. J., Pinkus, H. and Fosnaugh, R. P.: Human melanocytes in tissue culture. J. Invest. Dermat., **28:** 15–32, 1957.
10. Shizume, K., Lerner, A. B. and Fitzpatrick, T. B.: In vitro bioassay for the melanocyte stimulating hormone. Endocrinology, **54:** 553–560, 1954.
11. Chavin, W.: Pituitary hormones in melanogenesis. Pigment Cell Biology, Chapter IV, pp. 63–83. Academic Press, New York, 1959.
12. Bagnara, J. T.: Hypophyseal control of guanophores in Anuran larvae. J. Exper. Zool., **137:** 265–283, 1958.
13. Li, C. H.: Biochemistry of adrenocorticotropic hormone—a review. Conf. Metabol. Aspects of Convalescence, Josiah Macy Jr. Foundation, Trans. 17th Meeting, pp. 114–137, 1948.
14. Dixon, H. B. F., Moore, S., Stack-Dunne, M. P. and Young, F. G.: Chromatography of adrenotropic hormone on ion-exchange columns. Nature, **168:** 1044–1045, 1951.
15. Reinhardt, W. O., Geschwind, I. I. and Li, C. H.: On the evidence suggesting a multiplicity of adrenocorticotropic hormones: an evaluation of bioassay methods. Acta Endocrinol., **8:** 393–424, 1951.
16. Moyer, A. W., van der Sheer, J., Ritter, R., Tesar, W. C., Logan, J. B., Oleson, J. J. and Cox, H. R.: Comparative assays on adrenocorticotropic hormone preparations. Proc. Soc. Exper. Biol. & Med., **79:** 1–3, 1952.
17. Li, C. H.: Hormones of the anterior pituitary gland. Part I. Growth and adrenocorticotropic hormones. Adv. Protein Chem., **11:** 101–190, 1956.
18. Engel, F. L.: Some unexplained metabolic actions of pituitary hormones with a unifying hypothesis concerning their significance. Yale J. Biol. & Med., **30:** 201–223, 1957.
19. Newman, A. E., Redgate, E. S. and Farrell, G.: The effects of diencephalic-mesencephalic lesions on aldosterone and cortisone secretion. Endocrinology, **63:** 723–736, 1958.
20. Engel, F. L. and Engel, M. G.: The ketogenic activity of corticotropin, a presumed extra-adrenal action. Endocrinology, **62:** 150–158, 1958.
21. Hungerford, G. F., Reinhardt, O. W. and Li, C. H.: Lack of correlation of eosinopenic and adrenal ascorbic acid-depleting activities of various adrenocorticotropic preparations. Proc. Soc. Exper. Biol. & Med., **81:** 320–323, 1952.
22. Johnsson, S. and Högberg, B.: The occurrence of melanophore hormone in serum from human subjects in different pathological conditions and its relation to corticotropin. Acta Endocrinol., **13:** 325–342, 1953.
23. Wright, P. A.: A convenient and rapid technique for assay of intermedin. Paper Mich. Acad. Sci., Arts, and Letters, **39:** 271–279, 1954.
24. Chavin, W.: unpublished.
25. Hu, F. and Mori, W.: unpublished.