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Significance of Intermedin Activity in Adrenocorticotropic Hormone Preparations.* (19650)

WILLIAM O. REINHARDT, IRVING I. GESCHWIND, JERKER O. PORATH,† AND CHO HAO LI.

From the Division of Anatomy, and the Hormone Research Laboratory, Dept. of Biochemistry, University of California, Berkeley.

Recent investigations by Johnsson and Högberg(1) and by Sulman(2,3) have suggested that intermedin and adrenocorticotropic hormone (ACTH) were closely related or identical, and that assay of melanophore expansion activity in the frog could be employed as a convenient test for ACTH. In addition, the physico-chemical properties(4) of the melanophore expanding hormone are in many respects similar to those reported for ACTH. The following experiments were designed to demonstrate whether the adrenal ascorbic acid depleting activity of various ACTH preparations is correlated directly with intermedin activity.

Assay methods. Hypophysectomized frogs, *Rana pipiens,* were employed for intermedin assay in most of these studies. In one series of experiments *Rana catesbiana* was employed. Quantitation of melanophore expansion was made on an arbitrary 1+ through 4+ scale, in addition to the determination of the minimal effective dose (m.e.d.) necessary to produce a 1+ reaction. Readings were made at half hour intervals for a 4 hour period after injection of the test solution (0.1 ml) into the dorsal lymph sac. Adrenocorticotropic hormone preparations were assayed by the adrenal ascorbic acid depletion test(5).

Results. The investigation proceeded along 5 separate lines. The first approach involved the determination of both types of activity in a number of ACTH preparations(6). The results of this series of experiments are presented in Table I. It is obvious that no direct correlation exists between the ACTH and intermedin activities of the various preparations. As a matter of fact, the few preparations tested show an inverse correlation.

TABLE I. Adrenocorticotropic and Intermedin Activities of ACTH Preparations.

Preparation, ACTH	ACTH activity, I.U./mg	Intermedin activity (m.e.d.), μg
L 2317 H	35	.5
M	30	.1
L 2316 C*	200	5

* Partial acid hydrolysate of L 2317 M(6).

The second series of experiments was undertaken in the hope of demonstrating a chemical separation of the adrenocorticotropic and intermedin activities. Fractions with highly potent adrenocorticotropic activity, obtained from a method using charcoal chromatography (7), exhibited a marked concentration of intermedin activity as well, and no separation was achieved. Definite separation was obtained, however, with fractions isolated from cellulose columns by a discontinuous pH gradient. As has been reported previously(8), almost all of the adrenocorticotropic activity resides in the second of three peaks obtained by this method. Intermedin activity, on the other hand, was almost completely concentrated in the first peak (Table II). Thus, an effective

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† Permanent address, Biokemiska Institutionen, Uppsala Universitet, Uppsala, Sweden.

TABLE II. Distribution of Adrenocorticotropic and Intermedin Activities in the Fractions Obtained by Employing a Discontinuous pH Gradient on Cellulose Columns.

Peak*	Exp.	ACTH activity, I.U./mg	Intermedin activity (m.e.d.), μg
1	a	<4	< .5
	b	3	< .5
	c	6	< .5
	d	<4	<1
	e†	3	<1
	f	<3	<<1
	g	4	.1
2	a	40	>5
	b	120	5
	c	180	5
	d†	130	>1
	e	60	>1
3	a	<4	10
	b	<4	>2
	c	4	>2
	d	5	>2
	e	4	5
	f†	10	>1
	g	8	>1

* See reference (8).

† These solutions were assayed in *Rana catesbiana*.

TABLE III. Migration of Adrenocorticotropic and Intermedin Activities During Zone Electrophoresis on Paper.*

Fraction	ACTH activity, I.U./mg	Intermedin activity†
Anode	<7	3+
Origin	30	2+
Cathode I	70	1+
II	<7	±

* Borate buffer, pH 8.5, was used; a potential of 6 volts/cm paper was applied 19 hr, at 5°C.

† All solutions were assayed at 1 μg level. An arbitrary 1+ - 4+ scale of increasing intensity of darkening was employed for grading assay response.

separation of the bulk of the two activities has occurred.

Partial separation of the two activities was also obtained by employing zone electrophoresis on paper(9). For the experimental conditions employed (pH 8.0-9.0), the bulk of the ascorbic acid activity is to be found at the origin and in its neighboring cathode areas(6). When solutions obtained by extracting various sections of such papers were assayed for melanophore expanding activity (Table III), the region of greatest activity was found in the anode fraction where not more than a small percentage of the total ACTH activity could be demonstrated, whereas the cathode fraction immediately adjacent to the origin (Cathode I) contained the greatest ACTH activity, but little of the intermedin activity.

TABLE IV. Effect of Alkali-Heat Treatment on the Adrenocorticotropic and Intermedin Activities of an ACTH Preparation.

Preparation	Exp. No.	ACTH activity, I.U./mg	Intermedin activity (m.e.d.), μg
Control	1	30	>1
	2	30	2
Alkali-heat treatment	1	1.5	<1
	2	1.4	< .5

Another experimental evaluation employed was the determination of the effect on adrenocorticotropic activity of conditions known to activate intermedin(10). In these experiments 3 mg of an ACTH preparation was dissolved in 10 ml of 0.1 N NaOH. The solution was placed in a boiling water bath for 5 minutes, and then immediately neutralized and cooled. A control solution was treated identically, except that the heat treatment was omitted. Both solutions were then diluted and simultaneously assayed for intermedin and adrenocorticotropic activities at the same dose levels. The results are shown in Table IV.

Potentiation of intermedin activity has apparently occurred. The control solution was negative at the 0.5 and 1.0 μg levels, whereas the boiled preparation gave a 2+ response at as low a level as 0.5 μg. Under these conditions, however, marked inactivation of the adrenocorticotrophic hormone took place, and only a small percentage of its original activity survived the alkali-heat treatment.

The last approach to the determination of ACTH and intermedin association involved assay for each of these activities in the separated anterior and intermediate lobes of the pituitary of the bullfrog, *Rana catesbiana*. With the frog under ether anesthesia, and dissection performed under the binocular microscope, a clear separation of these 2 lobes was attained. The lobes were frozen on dry ice immediately upon removal from the donor,

then homogenized in 0.9% saline and assayed for the respective concentrations of the 2 activities.

Per unit of wet weight, the adrenal ascorbic acid depleting activity of the anterior lobes was at least twice that of the intermediate lobes, whereas the melanophore expanding activity of the intermediate lobe was approximately 60 times as great as that of the anterior lobe. Thus, the m.e.d. of the anterior lobes in this latter test was 700 μg, while the m.e.d. of the intermediate lobe was 12 μg. When considered in conjunction with the difference in adrenal ascorbic acid depleting activities, the relative difference between the activities in the 2 lobes is of the order of 100 times.

Discussion. The bulk of the data presented above is interpreted as a refutation of the claims of Johnsson and Högberg(1) and of Sulman(2,3) who purport to demonstrate the identity of ACTH and intermedin. Sufficient variation in intermedin activity has been found among a number of ACTH preparations, all of which were prepared from whole sheep pituitaries, to indicate that correlative studies are necessary. None of the aforementioned investigators has presented data demonstrating a correlation between the 2 activities.

The best demonstration of the non-identity of ACTH and intermedin would have to come from an actual chemical separation of the 2 hormones. Some success in this direction has been reported here with the use of a chromatographic procedure and filter paper electrophoresis. It is not here claimed that an ACTH preparation has been obtained in this laboratory which is free of melanophore expanding activity, but present evidence indicates that such a separation can be achieved. Raben, Rosenberg, and Astwood(11) have also recently reported an effective separation of the 2 activities.

Charcoal columns give a highly purified ACTH preparation, but the intermedin activity of such preparations is also greatly enhanced. As an isolated example, the activity of such preparations would lend support to the claims of a connection between the 2 activities.

It should be pointed out that the method of separation attempted by Johnsson and Högberg(1), *i.e.*, paper chromatography using phenol-water as a solvent, is recognized to be a system of limited usefulness for the purification of ACTH(12). Johnsson and Högberg (1) reported in addition that both hormones are inactivated by nitrite, formaldehyde, trypsin, and various tissue homogenates. These criteria cannot be considered sufficiently specific to characterize any polypeptide (see, for example, the effect of some of these reagents on posterior lobe principles(13)). On the other hand, potentiation of intermedin activity by heat treatment in an alkaline medium has effectively demonstrated the differing sensitivity of the 2 hormones to such a procedure, for little of the ACTH activity is retained after treatment.

An unequal distribution of the 2 hormones can also be demonstrated in the lobes of the pituitary gland by assaying the separated excised anterior and intermediate lobes of the frog. Determination of the concentration of both activities in each lobe has shown their non-identity.

It would appear that most of the presently available methods employed to purify ACTH also purify intermedin. Thus, the ACTH and pitressin preparations employed by Sulman possess more melanophore expansion activity than does his intermedin preparation(2). It may be noted that, in our experience, as little as 8 milliunits of pitressin (Parke-Davis) has given a positive intermedin response. Such preparations possess about 0.003 I.U. of ACTH activity per 10 I.U. of pitressin activity(14). Sulman(3) has found that a similar preparation of pitressin has an m.e.d. for intermedin activity of 500 milli-units. Furthemore, it can be speculated that most intermedin preparations must be heavily contaminated with ACTH, as evidenced by the ACTH-like clinical and physiological activities reported by Johnsson and Högberg(1), following the administration of such preparations to patients.

Summary. The validity of claims reporting an identity of ACTH and intermedin is questioned by experiments in which assays by the melanophore expanding test in the hypophy-

sectomized frog and the adrenal ascorbic acid depleting test in the hypophysectomized rat were employed. The results demonstrate: 1. Lack of correlation between the 2 activities in various ACTH preparations. 2. Partial separation of intermedin and ACTH activities using a discontinuous pH gradient on cellulose. 3. Differential migration of activities during zone electrophoresis on filter paper. 4. Potentiation of intermedin and inactivation of ACTH by alkali-heat treatment. 5. Differential concentration of ACTH and intermedin activities in the anterior and intermediate lobes of the frog pituitary.

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Effect of Various Allergens and Antibiotics on Experimental Poliomyelitis.* (19651)

ALBERT MILZER AND FREDDIE NICHOLSON.

From the Medical Research Institute, Michael Reese Hospital, Chicago.

Last year we reported that the inoculation of pertussis vaccine, diphtheria toxoid or *Salmonella typhimurium* vaccine would significantly decrease the incubation period before onset of paralysis in mice infected with the Lansing strain of poliomyelitis virus(1). Since then we and others(2,3) have been able to confirm these results. Dean *et al.*(3) reported that mice injected in a foreleg with pertussis vaccine alone or combined with diphtheria-tetanus toxoids were more frequently paralyzed in the injected limb following infection with mouse encephalomyelitis virus than the unvaccinated controls. The present studies were carried out to determine the effect of various allergens and antibiotics on the incubation period of mice infected with the Lansing strain of poliomyelitis virus. The allergens tested included house dust, mixed tree pollen, mixed grass pollen, ragweed pollen, mixed mold spores, and horse serum. The antibiotics tested were dihydrostreptomycin, terramycin, and penicillin.

Materials and methods. Groups of Swiss mice (Plymouth strain) weighing 13 to 15 g were inoculated intracerebrally with approximately 10 LD_{50} units of the Lansing strain of poliomyelitis virus. The identity of the Lansing strain used in this laboratory was verified periodically by neutralization tests with hyperimmune Lansing antiserum. Control mice inoculated with nutrient broth were also included in every experiment. The various allergens tested were obtained through the courtesy of the Allergy Laboratory of Michael Reese Hospital and were used in the following concentrations: 1. House dust (Endo) 1:400, 2. Mixed tree pollen 1:500, 3. Mixed grass

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