LOCALIZATION OF ADRENOCORTICOTROPIC HORMONE BY HISTOCHEMICAL AND IMMUNOCHEMICAL METHODS

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PLATE 12

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INTRODUCTION

Many advances have been made in the isolation and physicochemical characterization of proteins, but little is known of their individual distribution in cells and tissues. Histochemical techniques have been developed for a small group of enzymes (1), but a far greater number of proteins, including many interesting enzymes and hormones, cannot be localized by existing techniques.

A method which, in principle, seemed applicable to the localization of any protein was that reported by Coons, Creech, Jones, and Berliner (2). These workers were able to localize foreign antigens in tissues by using fluorescent antibody solutions as specific histochemical reagents. Antipneumococcal serum protein was made highly fluorescent by chemical conjugation with fluorescein. Tissue sections were prepared from mice which had been injected with the pneumococcal antigen. The sections were flooded with the fluorescent antibody solution, washed to remove excess antibody, and examined under the ultraviolet fluorescence microscope. Certain structures were seen to be stained with the yellow-green fluorescence of the antibody. Control experiments showed that the staining was a specific immunochemical reaction. In a series of more recent reports, Coons and his associates (3–6) have employed the method for the localization of several bacterial and viral antigens in tissues, with excellent results.

The present report describes the adaptation of the fluorescent antibody technique to a study of the distribution of certain native protein antigens in cells and tissues, and records in detail the procedures and findings of an attempt to localize adrenocorticotropic hormone (ACTH) in the anterior pituitary gland.

EXPERIMENTAL

A prime requisite for the localization of any substance by the method described is that it must be an effective antigen, capable of giving rise to a specific antiserum. Since the biochemical nature of ACTH is uncertain, it was assumed

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initially that ACTH might occur in the pituitary gland and in certain hormone preparations as a potentially antigenic protein.

1. Production of Antiserum.—

Adrenalectomized rabbits were used in the attempt to produce an antiserum to hog ACTH. It was thought that the scarcity of experimental or manifest evidence for an antibody response, even to crude preparations of ACTH known to contain much inert protein, might be explained by the suppressive effect of adrenal stimulation on immune responses (7). Any such effect would be avoided by the use of adrenalectomized animals, for these should react to ACTH as to an inert foreign protein.

Accordingly, the adrenal glands were removed from male rabbits weighing approximately 6 pounds. The procedure was done in two stages through a lumbar retroperitoneal approach. Following removal of the second gland, each animal received one intramuscular injection of 2.0 mg. of desoxycorticosterone in oil. Each animal was then maintained with sodium chloride *ad libitum*, supplied in the form of a salt block, and a stock diet supplemented by carrots and lettuce. One adrenalectomized rabbit maintained in this way was used to produce an antiserum. Adrenal insufficiency was indicated by the continued intake of about 1 gm. of salt daily for 40 days, and by the absence of accessory adrenal tissue at autopsy. A total of 10.5 mg. of alum-precipitated ACTH[1] was administered in two courses by combined intraperitoneal and subcutaneous injection. Precipitin tests were strongly positive during the second course. The animal was bled from the heart 6 days after the last injection and 40 days after adrenalectomy. The serum was fractionated for γ_2-globulin by the ethanol method of Nichol and Deutsch (8). A comparable globulin fraction of normal rabbit serum was prepared in the same way.

2. Preparation of Fluorescent Conjugates.—

Conjugation with fluorescein was done by the methods of Coons and his associates (2, 3). In synthesis of the isocyanate, the mixed isomers of 4-aminofluorescein were used. The method of Hardy (9) was introduced to decrease side reactions. This gave an isocyanate preparation which, after distillation to remove phosgene and acetone, was completely soluble in dry acetone. The solution in acetone, which was used without further purification, has proved to be fairly stable and reactive even after one year of storage protected from moisture, light, and heat.

The serum globulin preparations, in 50 mg. portions, were conjugated with fluorescein isocyanate at 0° to −5°C. in the cold room. The products were purified by repeated ethanol fractionation at +2° to −8°C. Material insoluble in 0.15 M saline at pH 7.4 was discarded, and soluble fluorescent material was removed by precipitation of the protein conjugates in 40 per cent ethanol.

[1] Armour ACTH, Lot 60-61, prepared from hog pituitary. Potency: equivalent to International Standard Armour LA-1-A.

This process was repeated until no more fluorescence could be detected in supernatant solutions examined by strong ultraviolet light. The final products were dissolved in 2.0 ml. saline and lyophilized. Yields, corrected for salt content, were 30 to 40 mg. for each lot. When ready for use, the conjugates were dissolved in 8 ml. of 0.15 M saline with 2 ml. of 0.07 M phosphate buffer at pH 7.4 and were centrifugalized to remove a small residue of insoluble material.

3. Histochemical Techniques.—

The Altman-Gersh method of fixation by freezing-drying was used to prepare tissue sections with good cytological detail and presumably without loss of antigenicity (10). Thin slices of hog, sheep, and beef pituitary glands and of hog kidney were frozen at the slaughter-house in isopentane at −160°C. The tissues were dried from the frozen state, infiltrated with paraffin at 58°C. *in vacuo* for 5 to 10 minutes, imbedded, and sectioned at a thickness of 4 μ. Sections were mounted on thin slides coated with a thin layer of purified egg albumin. Finger pressure and brief warming were used to flatten the sections. Slides were passed rapidly through xylene and absolute methanol and were then left in methanol at 5°C. for 30 minutes.[2]

After hydration in 95 and 70 per cent ethanol, the slides were dipped briefly in saline buffered to pH 7.4, and excess fluid was then removed. The sections were covered by a few drops of fluorescent globulin solution. The slides were placed in covered Petri dishes with moist cotton to prevent drying and were left at 5°C. for periods up to 48 hours. To remove the excess fluorescent solution, the slides were rinsed and washed 10 minutes in 3 changes of cold buffered saline at pH 7.4. After passage through 70 and 95 per cent ethanol, the tissue section was flooded with a few drops of a mixture of equal parts of ethanol, glycerol, and 0.07 M phosphate buffer (pH 7.4).[3] This was drained off; the slides were wiped to remove any excess around the sections; reagent glycerol was added and coverslips applied.

Such preparations could be kept for several months with only gradual fading of fluorescence intensity if the coverslips were ringed with a melted polyethylene glycol.[4] In some cases, sections were mounted in immersion oil[5] which caused some loss in fluorescence intensity but resulted in a slight gain in resolution of cytological detail.

[2] In experiences with several different fluorescent antibody preparations, methanol was found to fix soluble cytoplasmic granules in cells of pituitary and kidney without detectably denaturing tissue antigens. It was superior to other fixatives, which completely or partially destroyed the reactivity of antigens.

[3] This aided in transition from ethanol to the glycerol-mounting medium, and provided a trace of buffer in the final preparation.

[4] Carbowax 1540w, Union Carbide and Carbon Corp.

[5] Shillaber's Grade A—very low fluorescence.

4. Fluorescence Microscopy.—

An ultraviolet source of high intensity was obtained by use of the 1000 watt AH-6 mercury lamp.[6] A compact water jacket with internal shielding was designed to fit within a standard microscope lamp housing. For most purposes, this was used with a 3 cm. cell containing acidified 10 per cent copper sulfate, and a No. 5840 Corning filter. This combination transmitted traces of violet in addition to the long ultraviolet light. A standard research microscope was used, with a cardioid darkfield condenser. Wratten filters 2B or K2-8 were fitted in the ocular assembly to absorb ultraviolet and traces of violet-blue light scattered by the section. The combination of darkfield condenser and ocular filters made it possible to use an immersion objective (N.A. 1.30) without a funnel stop, for resolution of cytological detail.

5. Staining by Fluorescent Globulin Conjugates.—

Sections of hog, sheep, and beef pituitary and of hog kidney were mounted on the same slide and treated with the fluorescent antiserum conjugate by the method outlined above. Other sections were similarly treated with the fluorescent normal serum conjugate.

On examination under the fluorescence microscope, weak traces of yellow-green fluorescence could be seen in sections treated with the normal serum conjugate. These traces were in blood vessels in all the tissues, but did not involve the parenchymal cells in any case.

In marked contrast to this, sections treated with the antiserum conjugate showed brilliant staining of certain cells of the hog anterior pituitary. No cells were stained in sheep or beef pituitary or in hog kidney. It was also noted that the entire vascular network was strikingly outlined in both hog and sheep tissues by strong staining of plasma protein within the vessels.

Control slides were treated with solutions of unconjugated normal and antiserum globulins and were then flooded with the antiserum conjugate. Staining was much weaker in the sections pretreated with antibody than in those pretreated with normal globulin.

From these preliminary results it appeared that the antiserum conjugate contained antibodies to hog plasma antigens, in addition to antibodies reacting with certain cells in hog pituitary. Therefore hog serum was added to the fluorescent antibody solution in small increments until no further precipitation was noted on standing and after centrifugation. In addition, frozen-dried hog kidney powder was added to remove any traces of antibody against hog connective tissue antigens, and the solution was again centrifugalized. The purified conjugate was then found to stain the scattered cells of hog pituitary with undiminished brilliance, whereas the intravascular spaces were very weakly stained. (Figs. 1 to 3.)

In frontal sections through the middle of the gland, the fluorescent cells

[6] General Electric Co.

were seen to be distributed throughout the anterior lobe but were found to be more numerous around the periphery and near the midline. The cells varied somewhat in size and shape. Their cytoplasm seemed filled by very fine, closely packed, fluorescent granules.

A record was made by charting and photography of fluorescent cells in several areas. After removal of the coverslip, the section was treated with dichromate and stained by the azan method. Serial sections were also used for identification of cells; alternate sections were stained by the fluorescent antibody and azan methods. In every instance the fluorescent cells were found to be basophils. Attempts to differentiate between types of basophils by the aldehyde-fuchsin method (11) were unsuccessful.

6. Immunochemical Testing of Fluorescent Conjugates.—

In an attempt to determine whether the purified antibody conjugate contained precipitins against the hormone itself, quantitative precipitin tests were

TABLE I

Precipitin Tests with Fluorescent Conjugates

No.	Conjugate used	Antigen	Total protein
		μg.	μg.
1	Antibody	50	45
2	Antibody	200	66
3	Normal globulin	50	5
4	Normal globulin	200	5

done against a highly active ACTH preparation. In these tests, a preparation of hog ACTH having a potency of approximately 120 times standard was used as the antigen.[7]

The purified antibody conjugate and the normal globulin conjugate were again centrifugalized and 0.50 ml. portions, each containing about 1 mg. of globulin, were mixed with the antigen in a final volume of 1.0 ml. (Table I). No precipitate was seen in the normal globulin controls after 1 hour at 37°C. and 24 hours at 2°C., but a definite precipitin reaction was noted in the antibody tubes after 1 hour at 37°C. After 24 hours at 2°C., the tubes were centrifugalized in the cold, washed with cold saline, and again centrifugalized. Protein in each tube was determined by the micro Folin method of Heidelberger and MacPherson (13), by comparison with a rabbit serum globulin standard (Table I). Repeated precipitin determinations gave similar results.

In these preliminary tests, about 10 to 20 per cent of the highly active ACTH preparation was precipitable by the purified antibody conjugate. This value

[7] Supplied by the Research Division, Armour and Co. Prepared as described by Lesh and coworkers (12).

is a rough estimate based on the assumption that tube 1 (Table I) contained an excess of antibody and that the combining ratio of antibody to antigen was about 5 to 1.

To determine whether this precipitation carried down hormone activity, assays were done[8] on a pair of supernatant solutions, corresponding to those from tubes 1 and 3 of Table I. Of the total hormone added to the normal globulin conjugate control, 60 per cent was found. In the supernatant from the antibody conjugate, 24 per cent of the original activity was found. This suggests that a rather large part of the hormone activity of this highly active ACTH preparation is associated with a relatively small fraction of precipitable antigen.

DISCUSSION

Finerty and Briseno-Castrejon (15) recently reviewed the problem of the origin of adrenocorticotropic hormone and reported experiments which suggested that the hormone was secreted by the acidophil cells of the pituitary. Halmi (11), from similar experiments, felt that the hormone was possibly produced by a subclass of basophil cells. Thus, indirect evidence based on differential cell counts and inferences based on experimental or clinical endocrinology have been inconclusive or contradictory.

The fluorescent antibody technique held the promise of a solution of the problem, provided certain conditions could be met: (*a*) ACTH must be shown to be an effective antigen, capable of giving rise to an antiserum which would retain its activity after conjugation with fluorescein; (*b*) control experiments must demonstrate that the antibody so prepared reacted with the hormone itself rather than with some inert protein contaminant in the ACTH preparation used for producing antiserum; (*c*) specific immunochemical staining must be produced in pituitary sections treated with the fluorescent antibody.

ACTH seemed to be a very effective antigen in the adrenalectomized rabbit used in this study. No attempt was made to produce antiserum in intact animals. However, Chase (16) and Gordon (17) injected intact mice and rats with different preparations of ACTH and obtained evidence of antibody formation and of serum antihormone activity. Interpretation of these results has been difficult because of the unsettled question of protein impurities present in ACTH preparations. The materials originally described as pure ACTH (18, 19) were proteins of molecular weight around 20,000. On the other hand, fractions of much higher hormone potency have been isolated by various methods and found to have average molecular weights in the range of 1200 to 10,000 (12, 20–22). Smith, Brown, Ghosh, and Sayers (23) have reviewed the properties of the earlier preparations and have concluded that these may be the hormone as it is stored in the gland. The more potent fractions of lower particle weight might

[8] By the Armour assay group, using the adrenal ascorbic acid depletion method (14).

then be formed by partial splitting of the naturally occurring protein (20) and discarding of inert fragments in subsequent purification steps. This would be analogous, as Smith and his coworkers have pointed out (23), to the process of partial peptic digestion of antitoxins, which produces smaller particles of higher specific activity. Alternatively, the hormone might be an extremely active polypeptide, small amounts of which might be mixed with an inert protein in the older preparations.

Evidence bearing on this problem was obtained by immunochemical testing of the antibody against one of the highly active ACTH preparations of low particle weight. It was reasoned that if the hormone itself were a very active non-antigenic polypeptide, and the antigen in the less active ACTH preparation used for antibody production an inert protein impurity, a highly purified ACTH preparation should have lost most of this inert protein; any traces remaining should be precipitable by the antibody without much loss of hormone activity from the supernatant solution.

On the other hand, if ACTH were a protein, the highly active ACTH fraction of low particle weight must be the result of partial splitting of the protein molecule. If this protein were the specific antigen which gave rise to the antibody used in this study, the active particles derived from it might still react with the antibody prepared against the intact protein. The resulting precipitate should carry down much of the hormone activity.

The results indicate that the antibody did precipitate a portion of the highly active ACTH preparation, and with it much of the hormone activity. This suggests that the active particles were indeed derived from a parent protein, and that this protein was the antigen which gave rise to the antibody used in this work.

Further study by more precise quantitative methods over the entire range of antigen-antibody proportions will be required for determination of the total fraction of precipitable antigen in the highly active ACTH preparation. At optimal proportions of antigen to antibody, this fraction may prove somewhat greater than the 10 to 20 per cent figure suggested from the data here reported. Additional hormone assays of the supernatants and of the precipitates should show more accurately what proportion of the hormone activity is in the precipitable fraction. The results reported here suggest that the precipitable fraction may be very active; the residue of smaller, non-precipitable polypeptide units may have a lower specific activity. This might indicate loss of hormone activity when the protein molecule is split beyond a certain point. Such a possibility would account for the failure of attempts to isolate smaller polypeptides of higher activity.

Answers to these and other questions must be looked for in further work. For the problem at hand, it is sufficient to note that the purified antibody reacted both with the active hormone and with cytoplasmic elements in the basophil

cells of hog pituitary. Because the hormone was shown to react with the antibody, because the tissue is known to contain the hormone, and because in the tissue only the basophils bound antibody, it appears that the basophils must contain the adrenocorticotropic hormone.

Whether the cells stained in this way included all the basophils is not known. Since the basophils are said to produce the gonadotropic and thyrotropic hormones as well (24, 25), further work should show either that the separate hormones are produced simultaneously or in different cycles by the same cells, or that they are made by distinct families of basophils.

The procedures and findings reported here for the localization of ACTH indicate that the fluorescent antibody method should be a useful technique for the localization of any protein antigen in tissues. Multiple antigens in the preparation used for antiserum production could, in this instance, be detected by the results of tissue staining. The antibodies corresponding to serum protein impurities in the original antigen could be differentially absorbed. Since it is likely that almost all hormones and enzymes isolated from tissues will prove impure by immunochemical, if not by the more commonly used physical, criteria, it is important to note that this is not an insurmountable barrier to localization by the antibody method.

The use of the freezing-drying method and controlled methanol fixation assures sections in which cytological artefacts are minimal and antigens are rendered insoluble without appreciable loss of capacity for binding antibody. Further experience not reported here has shown that staining of antigens fixed in this way can be achieved even with a non-precipitating type of antibody. Other technical modifications described in this report have been useful in increasing the sensitivity and specificity of the method.

SUMMARY

The fluorescent antibody technique was adapted to the localization of native protein antigens in cells and tissues. This method was applied specifically to the localization of adrenocorticotropic hormone in the pituitary gland.

An antiserum to hog ACTH was produced in an adrenalectomized rabbit. The γ_2-globulin fraction of the serum was conjugated with fluorescein. After purification, the fluorescent antibody solution stained selectively the cytoplasm of basophil cells of the hog pituitary. No cells of sheep or beef pituitary or of hog kidney were stained. A fluorescent globulin solution prepared from normal rabbit serum gave no selective staining in any of these tissues.

Immunochemical tests showed that the fluorescent antibody gave a precipitin reaction with a highly active ACTH preparation of low molecular weight. The supernatant solution from this reaction showed a loss of hormone activity.

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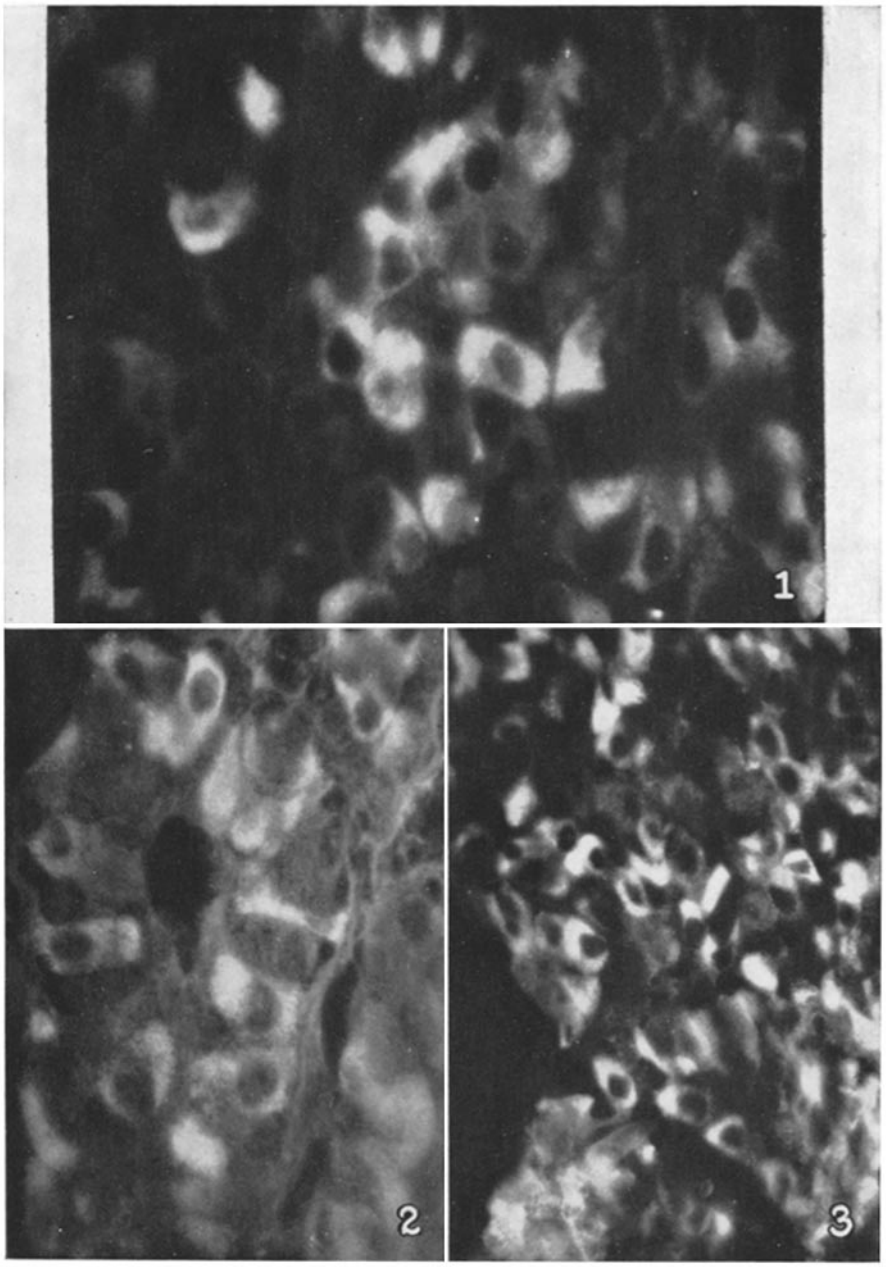
EXPLANATION OF PLATE 12

FIG. 1. Fluorescence photomicrograph of hog anterior pituitary stained by fluorescent antibody prepared against ACTH. Scattered cells show strongly stained cytoplasm against a background of faintly autofluorescent cells. × 900.

FIG. 2. Another area of same, showing arrangement of fluorescent cells near a small vessel. Note granular appearance of cytoplasm in several cells. × 900.

FIG. 3. Lower magnification, showing distribution of fluorescent cells near the periphery of the gland. × 475.



(Marshall: Localization of adrenocorticotropic hormone)