COLORIMETRIC DETERMINATION OF ACTH

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Summary: A sensitive colorimetric method has been developed to measure changes of ACTH in pituitaries under control, stress and stress-recovery conditions. The method employs the quantification of the single tryptophan molecule contained in ACTH by means of the blue Schiff's base produced by p-dimethylaminobenzaldehyde. The method is sufficiently sensitive that ACTH may be determined directly in many stressful conditions rather than by cumbersome bioassay or radiolabeling procedures.

The measurement of ACTH, adrenocorticotrophic hormone, presently entails lengthy indirect bioassay procedures. The older activity oriented methods have measured ACTH by adrenal ascorbic acid depletion (1), plasma corticosterone changes and corticosterone production from adrenal slices and isolated cells *in vitro* (2). More recently, the principles of hemagglutination inhibition (3), radioimmunoassay (4), and radioreceptor assay (5), have ingeniously been used to determine the hormone.

We have developed a sensitive direct colorimetric method for ACTH that quantifies the ACTH molecule. Our method depends on the colorimetric quantification of the single tryptophan molecule contained in the hormone chain. This procedure was developed to determine cofactor relationships to ACTH activity. We have found a strong correlation between the trace element zinc and ACTH activity (6). Subsequently, zinc deficiency has been directly linked to ACTH activity (7), and this method has been devised to allow the comparison of ACTH activity with actual hormone production.

Our colorimetric procedure is based on the production of the blue Schiff's base by the interaction between tryptophan and p-dimethylaminobenzaldehyde

in acid solution. Schwyzer and Sieber (8), in describing the total synthesis of ACTH, demonstrated that there is only one tryptophan molecule in the 39 amino acid chain of this low molecular weight protein. Others (9, 10) have shown that ACTH from various species varies in amino acid composition, but always contains one tryptophan molecule, so that direct correlation always exists between the ACTH and tryptophan content.

In carrying out our procedure, the ACTH is first isolated by liquid chromatography and then converted to the colored complex. Filtered acetone-HCl homogenates of whole pituitary glands are processed by the chromatographic procedure of Island *et al* (11), using the ion exchange resins Amberlite CG-50 and C-25 (medium) SP-Sephadex. The Amberlite resin is stripped with 10 ml of 50% acetic acid and this elutant passed through the Sephadex. This second column is then washed with a series of aqueous pyridine-acetic acid solutions, and the column eluted with 50 ml of 1.0 M NH_4OH. The ammoniacal solution is flash evaporated and the residue taken up in 1.0 ml of 0.01 HCl. This test solution is added to a freshly prepared solution of 30 mg p-dimethylamino-benzaldehyde in 9.0 ml of 19 N H_2SO_4, according to procedure H of Spies and Chambers (12). The mixture is shaken, cooled to 25^0 C, and allowed to stand in the dark for one hour. The color is then developed by the addition of 0.1 ml of 0.045 percent aqueous sodium nitrite solution, the mixture shaken, and the trasmittance finally read at 590 mμ after standing for 30 minutes.

Commercial porcine ACTH (chromatographically isolated peptide, Sigma Chemical Co.) was used to prepare controlsolutions to test the accuracy of the procedure. Recovery of the hormone by the chromatographic techniques outlined above showed no significant loss or enhancement of ACTH in amounts ranging from 10 to 1000 ng, and was always greater than 90 percent. Figure 1 illustrates a comparison of a standard curve as determined by both a bioassay method and the colorimetric procedure.

ACTH concentrations in the whole pituitaries of 36 adult male Spraque-Dawley rats, weighing 300-425 gms, were determined and expressed in ng/mg

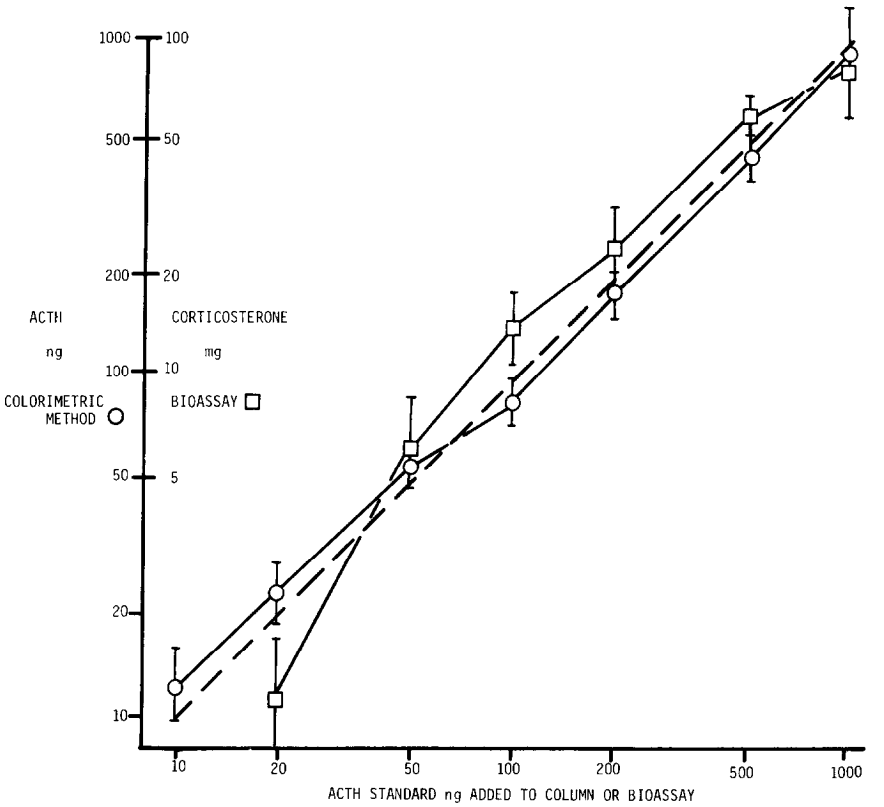

Figure 1. Standard curve comparing bioassay technique and colorimetric method of determining ACTH. Range of ACTH solutions is from 10 ng to 1000 ng chromatographically isolated peptide. Each point is the mean of six determinations ± S.E. and is plotted on log-log paper.

fresh tissue. To demonstrate the effectiveness of the procedure in separating differences under experimental conditions, the 36 animals were divided into control, stress and stress-recovery groups of 12 each. All animals were anesthesized with 25 mg/kg pentobarbital sodium, and a catheter was inserted into the right carotid artery for the withdrawal of blood. Oligemic hypotension, a maintained mean arterial pressure of 50 mm Hg for one hour, was used as stress, and two hours post-reinfusion as stress-recovery. The rats were then sacrificed by an air embolism of the heart, each pituitary removed, and homogenates prepared. ACTH concentrations in the whole pituitaries of these 36 animals were determined by both a bioassay (2), and the colorimetric ACTH method. The bioassay was carried out in 36 additional rats where a

portion of the pituitary homongenate was injected, blood from the adrenal vein sampled and corticosterone extracted and measured (2). A statistical correlation was computed between the methods. To compute the correlation between tests, it was necessary to equate units of change. Therefore, the results of the stress and stress-recovery groups were calculated as % control and ranked. A non-parametric Spearman r_s correlation (13), was computed based on the ranks of the two groups.

The results of the bioassay and colorimetric procedures for the determination of ACTH in three experimental groups are presented in Table 1. The colorimetric method demonstrates very significant differences ($F= 1918$; $df= 2/33$; $p < 0.0001$) between the three groups, as determined by a one-way analysis of variance. The Student-t test was used for identifying significant differences among combinations of means, and indicated that the stress group had a significant mean increase in ACTH (257 ng/mg) over both the control group (122 ng/mg) ($t= 17.35$; $p < 0.0001$) and the stress-recovery group (107ng/mg) ($t= 19.97$; $p < 0.0001$). The control group, likewise, varied significantly from the stress-recovery group ($t= 2.78$; $p < 0.01$). The ACTH activity determined by the corticosterone based bioassay procedure (2) also showed significant differences

TABLE 1

COMPARISON OF ACTH LEVELS DETERMINED BY BIOASSAY AND COLORIMETRY ($\bar{x} \pm$ S.D.) OF GROUPS OF WHOLE RAT PITUITARIES UNDER CONTROL, STRESS AND STRESS-RECOVERY CONDITIONS.

ACTH Method	CONTROL	STRESS	STRESS-RECOVERY
COLORIMETRY (ACTH, ng/mg)	122 ± 9.0	257 ± 14.8	107 ± 8.0
BIOASSAY (2) (Corticosterone, mg/100 ml)	14.2 ± 6.5	22.3 ± 6.2	11.5 ± 5.1

between experimental groups as determined by a one-way analysis of variance ($F= 49.93$; $df= 2/33$; $p < 0.001$).

The specificity for ACTH was shown in correlations between the bioassay and the colorimetric method. In comparing the values of the stress group, the correlation between the two procedures was $r_s = + 0.66$. Likewise, the stress-recovery group showed a positive correlation of $r_s = + 0.77$.

The application of the colorimetric reaction of tryptophan to the determination of ACTH marks the first direct quantification of the ACTH molecule. Bioassay and radiological means have only measured the displacement by, or effects of, ACTH. The colorimetric method can be useful in more fully describing cofactor effects on the activity of ACTH. Sandstead *et al* (14) inferred a relationship between ACTH and mineral elements when they reported that, in zinc-deficient Egyptian dwarfs, exogenous doses of ACTH elicited little adrenal response. Zinc supplementation to the diets of these dwarfs seemed to improve their pituitary-adrenal response and suggested that zinc was required for proper ACTH activity. Zinc chelation of metalo-ACTH complexes, likewise, demonstrated a dependent relationship between mineral elements and ACTH (7). The zinc cofactor was shown to be necessary for active glucosteroidogenesis *in vitro*. The coupling of a colorimetric test for ACTH with a bioassay procedure could greatly expand the understanding of storage and release mechanisms of this vital hormone.

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