Sensitive Bioassay Method for Measuring Antidiuretic Hormone in Mammalian Plasma. (24605)

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Accurate measurement of antidiuretic hormone (ADH) titers in mammalian plasma has long been desired. Insensitivie assay methods and non-specific responses have plagued previous attempts to measure the circulating level of ADH under various physiologic conditions. In several existing methods, the assay animal has been a normal dog or a dog with experimentally produced diabetes insipidus(1,2,3). While these preparations are suitable for assay of relatively rich extracts of the neurohypophysis or hypothalamic nuclei, a more sensitive method is required for measurement of the circulating level of ADH in biologic fluids. Van Dyke(2) has stated that the rat assay is better suited for estimation of small quantities of ADH and that intravenous injections must replace the less specific intraperitoneal or subcutaneous routes. Assays of antidiuretic hormone in rats have been performed using both conscious and anesthetized animals. Ginsburg and Heller(4) used conscious rats in which a metal cannula was placed in the bladder, and a polyethylene tube in the jugular vein on day prior to assay. Urine collection consisted of prodding the animals into emptying their bladder every 5 or 10 minutes. It is extremely questionable if this procedure can be relied upon to produce complete emptying of bladder. Furthermore, animals were hydrated by stomach tube at frequent intervals. Considering the well known hypothalamic-neurohypophyseal interrelationships, the psychic influence of prodding and hydration would undoubtedly effect the endogenous release of ADH. For this reason, sensitivity of this method of bioassay must be questioned. Jeffers, Livezey and Austin(5), and Blackmore and Chester(6) rendered rats unconscious with ethanol. Their choice of anesthetic had the dual function of maintaining a steady state of anesthesia, as well as inhibiting endogenous release of ADH(7). A constant degree of hydration was maintained by administering fluid *via* a polyethylene tube passed into the stomach. This procedure presented a better physiologic approach to the problem of ADH bioassay. All previous methods(4,5,6) considered a decrease in urine flow as the criterion of antidiuresis. An important modification of this practice was suggested by Crawford and Pinkham(8) and by Thorn(9) in that urine density, rather than urinary flow rates, was used as index of response. This is especially important when spontaneous variations in urine flow are observed under control conditions. These variations may be due to either changes in glomerular filtration rate or solute load presented to the distal tubule. It is important to note that changes in urine specific gravity reflect only changes in absorption of water in the renal tubules. This alone could account for greater sensitivity in bioassay of ADH. It is also possible to measure specific gravity simply and with a high degree of accuracy. It was our purpose to combine the best features of existing methods of ADH assay, to verify previous data using standard solutions of vasopressin, and then to adapt the procedure further to measure circulating levels of antidiuretic hormone in mammalian plasma.

Methods. Holtzman strain female albino rats (220-280 g) were used as assay animals. On the afternoon prior to assay, the animals were given 3% of body weight of tap water at 40°C as suggested by Blackmore and Chester(5). All food was withheld during the night preceding the assay, but water was allowed *ad lib.* On day of assay, animals were prepared as described by Thorn(9). Following hydration with tap water and anesthetization with 12% ethanol (5% of body weight *via* stomach tube), the bladder was exposed with a suprapubic incision and cannulated by piercing upper portion with a 14 gauge hypodermic needle and threading a small piece of polyethylene tube down through the bladder and out the urethra. The bladder was then tied off above entrance of ureters to close the incision and reduce bladder dead space. The right exter-

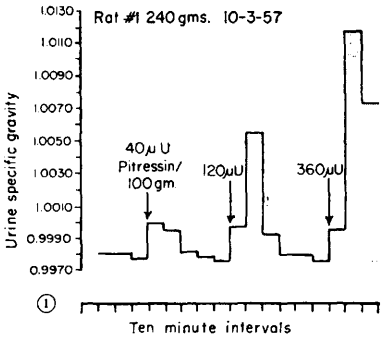
TABLE I. Antidiuretic Response to Doses of Reference Vasopressin.

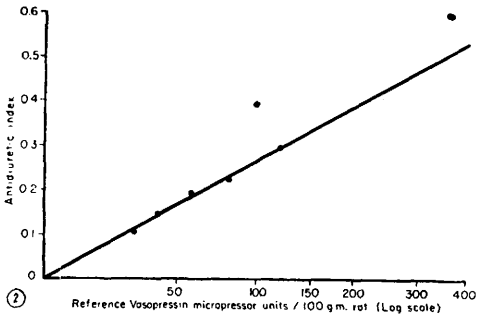
Dose of reference vasopressin, μpressor units/100 g rat	30	40	60	80	100	120	360
No. of observations	11	17	16	29	17	9	7
Mean antidiuretic index	.1088	.1454	.1910	.2213	.3901	.2964	.5945
Stand. dev. (±)	.0548	.0761	.0938	.0284	.0706	.0583	.0533

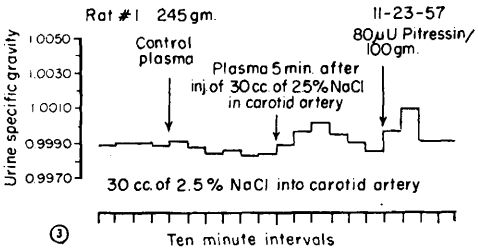
nal jugular vein was cannulated with fine polyethylene tubing for injection of all standard and unknown solutions. The stomach was cannulated *via* the esophagus for maintainance of constant degree of hydration and anesthesia with a 2% ethanol solution. At least 3 assay animals were prepared for every assay. It was sometimes necessary to discard an animal due to lack of sensitivity to standard solutions of vasopressin, or hematuria due to bladder surgery. Urine was collected in calibrated tubes under kerosene, and urine specific gravity was determined by the falling drop method(10). Degree of antidiuresis was expressed as antidiuretic index *i.e.* percentage change in urine specific gravity calculated from average of two 10 minute samples preceding injection, and the average of two 10 minute samples immediately following injection. One lot of Pitressin (Parke Davis & Co. R 983M, a lysine vasopressin prepared from combination of hog and beef pituitaries) served as reference material. Working standards were prepared fresh daily by dilution of the stock with physiologic saline. The volume of either standard or unknown solution injected for assay was fixed at 0.7 cc, the final amount regulated by dilution. After each injection, an additional 0.1 cc of heparin saline solution (1:100) was flushed through the tube to wash in all the test material. At least 60 minutes was allowed between injections to avoid any possible tachyphylaxis. Dogs were used as experimental animals. The neurohypophysis of the dog is drained by internal jugular veins; these veins contain blood richest in antidiuretic hormone. Due to extremely variable size of these veins, and the difficulty in sampling blood from the vein while maintaining a patent flow, blood samples were taken from catheter in the superior vena cava just above the right atrium. The catheter was placed using fluoroscopy. Ginsburg and Heller(4) reported blood from right heart of rat or from veins directly draining the posterior pituitary had equal levels of ADH activity. These authors reported that blood readily lost its detectable ADH activity upon standing, and thus should be rapidly injected for assay after withdrawal from animal. Blood to be assayed for ADH activity, was heparinized and immediately centrifuged after withdrawal from dog. 0.7 cc of dog plasma was slowly injected into external jugular vein of the rat and flushed in, as described above. In no case was time of blood withdrawal from dog to injection of plasma into the rat greater than 13 minutes. The usual time elapsed varied from 3 to 8 minutes. In all cases, the same rat was used for determination of ADH activity in control and experimental plasma samples. Thus, all variations in individual sensitivity were eliminated.

Results. Dose *vs.* response data for micropressor units of vasopressin *vs.* antidiuretic index is shown in Table I. A typical assay for standard doses of vasopressin is shown in Fig. 1. The immediate response to injection of standard vasopressin reflects the advantage of intravenous injections. The response is over in 30-40 minutes following injection, and thus a different standard or unknown can be tested every 60 minutes in the same rat. Antidiuretic indices obtained with this animal are included in the overall standard curve of log dose *vs.* response in Fig. 2. Linear results obtained by plotting antidiuretic index *vs.* log dose are clearly indicated.

Verney's classic experiment(11) was repeated wherein 30 cc of 2.5% NaCl solution was injected into the carotid artery of hydrated dog with average control urine flow of 1.28 ml/min. Five minutes later, the dog's plasma was assayed for level of circulating ADH when urine flow had fallen to a rate approximately 0.62 ml/min. Increase in specific gravity of rat's urine is much greater following injection of plasma after activation of the







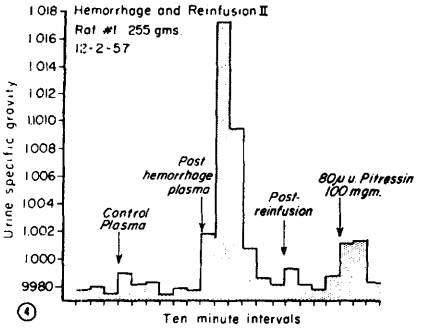


FIG. 1. Change in specific gravity of rat urine following intrav. inj. of reference vasopressin.

FIG. 2. Standard curve: Antidiuretic index *vs* log dose of reference vasopressin.

FIG. 3. Change in specific gravity of rat urine following intrav. inj. of dog plasma.

FIG. 4. Change in specific gravity of rat urine following intrav. inj. of dog plasma.

osmoreceptors, than the control plasma (Fig. 3). Thus, increase in circulating ADH occasioned by action of hypertonic salt solution on the osmoreceptors, resulting in reduction of urine flow of about 59%, is herein measured.

Sensitivity of this method of bioassay is indicated by series of experiments on hemorrhage. Following control determinations, the dog was hemorrhaged 25% of its estimated blood volume, then further blood samples were taken for determination of ADH activity. Following reinfusion of all heparinized withdrawn blood, plasma ADH levels were again determined. Results are given in Table II.

TABLE II. Antidiuretic Hormone (Microunit Equivalents Vasopressin/1.0 cc Plasma), 4 Experiments.

C_1	C_2	H_1	H_2	R_1	R_2
95	106	3764	750	29	15
22	14	3814	501	30	4
305	169	1350	3552		
33	73	1964	806	61	161

C = Control, H = Post hemorrhage, R = Post reinfusion.

When compared by analysis of variance, there is statistical significance to the 0.1% level between determinations taken during hemorrhage and those taken in control periods and following reinfusion. Fig. 4 is a typical assay experiment.

Discussion. Results obtained when comparing antidiuretic index to doses of reference vasopressin are in excellent agreement with Thorn(7). This is especially evident when different strains of animals and different lots of reference vasopressin were used. The report of Blackmore and Chester(5) of 45-105 microunit equivalents of vasopressin/cc of normal dog plasma is in close agreement with results obtained in this experiment. Again, differences in strains of animals and lots of vasopressin can account for much of the small difference.

Highly significant increase in circulating level of ADH following hemorrhage, had been reported by Ginsburg and Heller(9). However, these authors used unanesthetized animals for their assay, which was a highly un-

satisfactory method for reasons mentioned above. Our method is far more sensitive and can be adapted to measurement of circulating ADH under various physiologic conditions.

Summary. A method of bioassay for small amounts of antidiuretic hormone in plasma of a dog is presented. The method utilizes rats rendered unconscious with ethanol, under constant water load, and with intravenous injections of all standards and unknowns. Change in specific gravity of rat urine expressed as antidiuretic index is used as criterion of potency. This method appears to be the most sensitive bioassay for ADH available in that small dose changes of reference vasopressin, or endogenous circulating ADH, can be differentiated.

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Hemosiderin. Iron Extraction Studies.*† (24606)

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Preparations of hemosiderin from horse spleens vary considerably in iron concentrations(1). Schwietzer(2) stated that part of hemosiderin iron may be present in crystalline form (limonite) as FeO(OH). After removing iron from hemosiderin with reducing agent, Greenberg(3) noted presence of apoferritin, thus indicating that this protein was originally present as ferritin. No other data are available concerning the form in which iron is present. This investigation is concerned with solubility of iron-containing compounds of hemosiderin in a chelating agent.

Methods. Hemosiderin was isolated from frozen horse spleens by fractional sedimentation as previously described(1). The procedure was modified by washing final product 4 times with distilled water. The washed hemosiderin pellet was changed to pasty consistency by addition of a few drops of water and the material stored in refrigerator in stoppered container. An aliquot of each sample was analyzed for total solids, iron and nitrogen. About 600 mg of "wet" hemosiderin were transferred to polyethylene bottle and 4 ml 9.2% disodium ethylenediamine tetraacetate (Na_2EDTA)/mg of iron were added. This mixture, to which 1 ml toluene was added, was continually agitated by magnetic stirrer. After centrifuging, 0.5 ml aliquots were removed, as a rule, after 1, 5, 15 and 25 days of stirring, for determination of iron. To release apoferritin from hemosiderin, we removed iron according to procedure of Greenberg(3). About 10 g of "wet" hemosiderin, equivalent to about 2 g of dried material, were suspended in dialyzing bag containing 60 ml of 1 M acetate buffer (pH 4.6), the bag then placed in 500 ml stoppered graduate filled with $Na_2S_2O_4$-acetate buffer and the cylinder rotated at slow speed. The reducing solution was changed every 12 hours. At end of 3 days the dialysate was practically free of iron. Subsequently, the material was dialyzed

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