II. METHODS

METHODS

'In Vivo' Experiments

Mongrel dogs (15-20 kg) were deprived of food for 24 hours before the experiment and had access to water ad libitum. On the day of the experiment, α-chloralose was given as an anesthetic in order to cannulate the carotid artery, femoral artery, and femoral vein. The bladder was also catheterized to measure the urine output. After surgery the animal was placed on a sling to rest comfortably throughout the experimental period.

When the animal was awake again, an infusion of 0.6% NaCl and 1% glucose (6% body weight) was started via the femoral vein. The experimental period began when the animal achieved a urine output of 3-4 ml/min over three 15 min collections. Blood pressure was monitored continuously by means of a transducer introduced into the femoral artery. Blood was collected from a jugular vein for estimation of plasma ADH and osmolality. Drugs and osmotic solutions were injected through the carotid artery. This route was chosen to minimize hemodynamic changes caused by certain drugs, which by itself might have a profound influence on ADH release. Moreover, concentration of drug which enters the brain by intracarotid infusion exceeds that by an intravenous route so that smaller amounts of the drug are needed to bring about the desired effect.

The effect of agonists and antagonists on ADH release was studied as follows:

a) In the case of agonists (stimulants), a dose-response curve was obtained.

b) In the case of antagonists (blockers), the animal was first infused with 3 M NaCl (2.0 ml/min for 2 min.) to stimulate ADH release. After the animal was stabilized, as assessed by the urine output, the antagonist was infused through the carotid artery. After an appropriate time interval and stabilization period, the 3 M NaCl was infused again to see if the drug indeed decreased or blocked the ADH release, induced by the osmotic stimulation. The blood sample to estimate the ADH and plasma osmolality was collected 3 min. after the infusion of NaCl.

'In Vitro' Experiments

Preparation of isolated supraoptico-neurohypophysial (SONH) system

Male albino rats weighing 200-250 g were decapitated and the brain rapidly exposed by removing the overlying major parts of parietal and frontal bones. The posterior approach toward the pituitary fossa employed by Daniel and Lederis (1967) was used. Under a magnifying illuminated mirror the dura, cranial nerves and blood vessels were cut with ophthalmic scissors, while the cut surface of the medulla was gently lifted upward and forward with a moist cotton swab. The pituitary was freed from its attachment to the cranial floor. The brain with the pituitary was lifted forward, and the olfactory lobes were divided, and the whole specimen removed from the cranium and placed in ice cold isotonic medium, bubbled with oxygen. The tissue fragments adhering to the pituitary were removed under a dissection microscope with 40-fold magnification and the pituitary stalk inspected for damage. To avoid injury to the neural lobe, it was lifted upward by gripping the pars intermedia with fine forceps. Blood vessels connecting the adenohypophysis also were separated by blunt dissection. A tissue block 4 mm wide, 4 mm deep, and 5 mm long was taken which contained the SON, hypothalamus, pituitary stalk and neurohypophysis. The tissue was immediately transferred into the incubation medium. The whole process was completed in less than 10 min.

The isotonic medium used was a phosphate buffer, pH 7.4 (McIlwain and Rodnight, 1962) with the following composition, (mM): NaCl= 120; KCl=4.8; KH_2PO_4=1.2; $MgSO_4$=1.3; $CaCl_2$=2.8; $NaHCO_3$=26.0; glucose=10.0; 95% O_2 5% CO_2; pH 7.4; tonicity 300±3. The hypertonic media were prepared by adding appropriate amount of NaCl to the isotonic media. All the drugs used were dissolved in the isotonic media.

The preparation was incubated in 2.0 ml volume of medium which was bubbled with a gas mixture of 5% CO_2 in O_2 at 37°. The total duration of time the tissue was incubated never exceeded 2 hr and only one dose of drug was tested in each preparation. The incubation period consisted of two phases: 1) the stabilization period consisting of two half hour periods, 2) the experimental phase consisting of four 15 min periods when the effect of the antagonist was tested, and three 15 min periods when the agonist was tested. In the case of an agonist drug, the first experimental period was the control; the second, the time the tissue was in contact with the drug, and the third, the recovery period after the tissue was rinsed twice with the isotonic media lacking drug.

In the case of an antagonist, the antagonist was added in the first period. At the start of the second period, the drug as

well as the hypertonic solution was added. The third and fourth periods constituted the recovery phase when the tissue was rinsed twice with isotonic media to remove the effect of the drug and the hyperosmotic solution. Following each incubation period, the medium was collected and stored at -20°C until assayed for ADH.

Oxygen Uptake Study

To test the viability of the isolated SONH, oxygen consumption was measured by using oxygen saturation monitor (Yellow Spring). The glass chamber was filled with incubation buffer and saturated with oxygen. Then the preparation was incubated for 3 min and oxygen level in the chamber was monitored. This was done every 30 min up to a period of 150 min.

The plasma osmolality was measured by freezing point depression method. Plasma ADH level was measured by a radioimmunoassay for arginine vasopressin developed by me at The University of Texas Health Science Center at Dallas.

Development of Radioimmunoassay for Arginine Vasopressin

1. Preparation of antiserum. The successful preparation of vasopressin antiserum has allowed me to develop a highly sensitive and a specific radioimmunoassay for arginine vasopressin. Two methods of conjugation (thyroglobulin and bovine serum albumin) were used. Each method has produced excellent antibodies.

a. Conjugation of AVP with thyroglobulin (TG). 5.0 mgm of AVP was conjugated to 20 mgm of thyroglobulin with 50 mgm of carbodiimide (CDI). Approximately 50,000 cpm of [^{125}I] AVP was added to the above mixture to assess the amount of conjugated AVP. The mixture was stirred constantly at room temperature for 4 hours after which it was kept in a cold room and stirred overnight. After overnight stirring, an equal volume of 1 M hydroxylamine was added to reverse a possible tyrosine rearrangement in the presence of CDI. CDI may alter the aromatic ring of tyrosine, forming o-aryl iso-urea or N-aryl urea, and since tyrosine in position two of AVP might be in the locus for the antigenic determinant of the molecule, any modification of this amino acid was considered undesirable.

The mixture was stirred for another 30 minutes and counted for radioactivity. It then was dialyzed against 4,000 ml of deionized water for 72 hours, changing the water every 24 hours. The dialyzed mixture was again counted for radioactivity and the molar incorporation of AVP to thyroglobulin was calculated. Initial molar concentration of AVP and TG was AVP:TG = 145.5:1. Final molar incorporation was calculated by measuring the representative conjugation [^{125}I] AVP to the reaction mixture. The percent of conjugation was 52.1 and the ratio incorporated was TG:AVP = 1:73.2. It was diluted to a desired volume, distributed in small aliquotes and stored at -20°C until further use.

b. Conjugation of AVP with bovine serum albumin (BSA). 6.0 mgm of AVP was conjugated to 20 mgm BSA with 1 ml of 0.025 M glutaraldehyde. Representative conjugations were performed by adding a trace amount of [^{125}I] AVP to the reaction mixture. The mixture was stirred for 1 hour at room temperature, then counted for radioactivity from which the molecular incorporation of AVP to BSA was calculated. The dialyzed mixture was diluted to a desired volume and distributed in vials which were stored at -20°C until required. The conjugation was 94% while the molar incorporation ratio of BSA:AVP was 1:19.4.

c. Method of injection. New Zealand white rabbits were shaved on the posterior side from shoulder to pelvic area. One mgm equivalent of the respective conjugate was mixed thoroughly with an equal volume of Freund's adjuvant. This mixture was injected at multiple sites subcutaneously. Booster doses equivalent to 500 µg were given first biweekly for 6 weeks and thereafter once a month. Rabbits were bled starting from the 10th week to see if antibody against AVP was formed. A total of 50 rabbits were innoculated, with 3 rabbits giving titers of 1:100,000 or better.

The antiserum (PVK-13) used was diluted 1:10 in 0.02 M phosphate buffer (pH 7.4) and stored in glass vials at -20°C. Working

solutions (those used in the assay) were prepared at four month intervals by diluting the stored stock 100-fold with 0.02 M phosphate buffer, 0.14 M NaCl and control rabbit serum (10 µl/ml).

2. Iodination of arginine vasopressin. [^{125}I] AVP was prepared by the lactoperoxidase method. This method was chosen in favor of the classical Hunter and Greenwood technique (1962) to obviate the chloramine-T toxicity, as well as the deterioration of chloramine-T itself at times, by storage thus yielding a poor iodinated AVP.

a. Materials

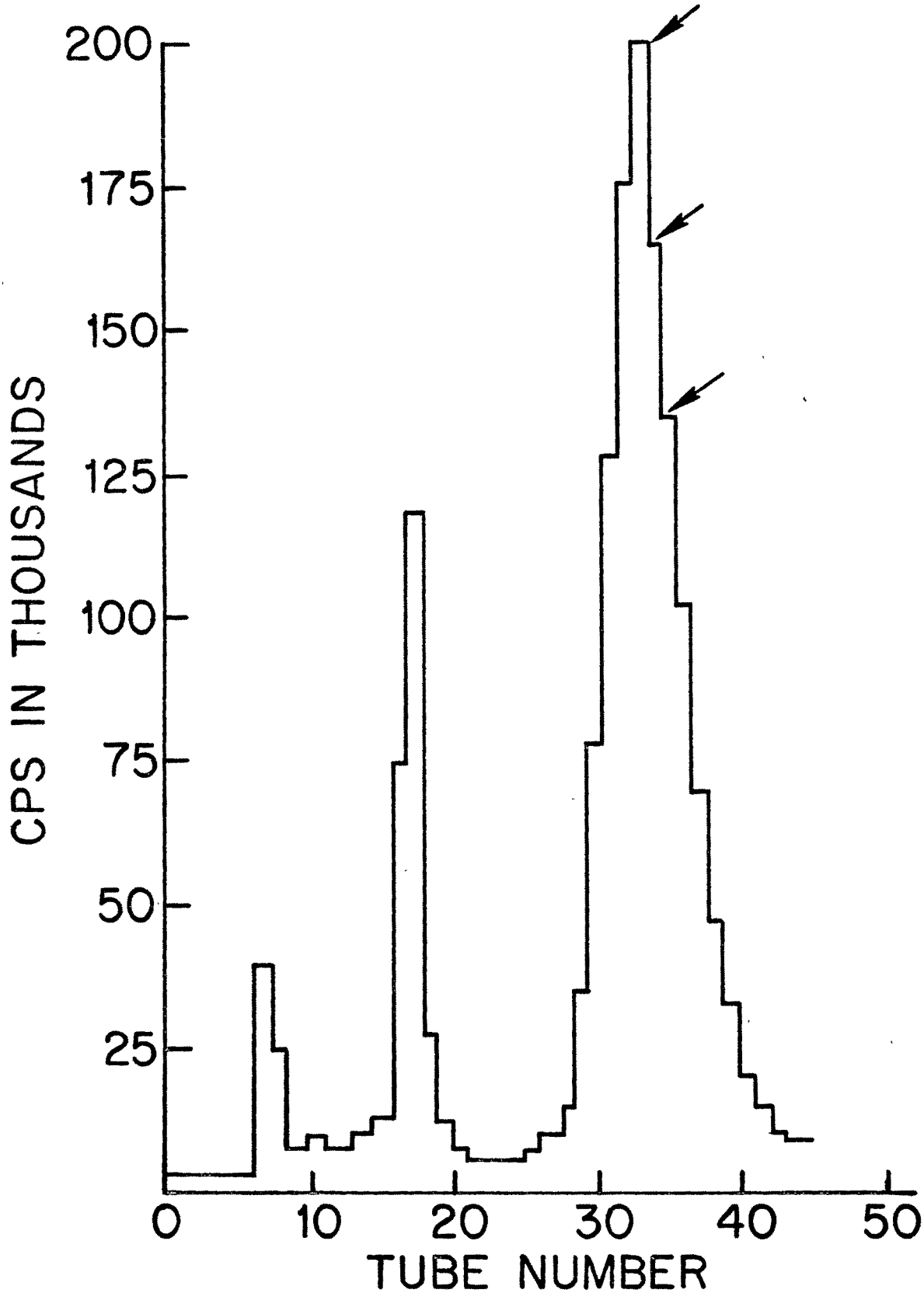
1. Lactoperoxidase: obtained from Calbiochem, with a specific activity of 122 I.U. mg^{-1} - 0.5 mg/ml in 0.5 M PO_4 buffer, pH 7.0
2. Hydrogen perioxide 30% (Mallinckrodt) 1:100 dilution
3. Na ^{125}I-iodide (New England Nuclear) 1 mCi
4. Arginine vasopressin (biopotency 267 I.U./mg, Schwarz/Mann)
5. Acetate buffer - pH 5.8 (0.2 M Acetic Acid; 0.2 M sodium acetate)
6. PO_4 buffer, 0.5 M pH 8.4 with 0.1% Na azide

b. Reaction. The iodination was carried out in a vial containing ^{125}I 1 mCi, acetate buffer 50 µl, AVP 10 µg in 10 µl; lactoperoxidate 5 µg in 10 µl; H_2O_2 10 µl (1:100 diluted).

The above reagents were added rapidly in succession, and vortexed for 30 seconds. At the end of 30 seconds, 0.5 ml of PO_4 buffer containing Na azide was added to the mixture which stopped the reaction. The mixture was then applied to the preequilibrated sephadex Gr-25 (S.F.) column and the fractions were eluted out by 0.25% acetic acid containing 1.25 mg/ml of BSA. One ml fractions were collected and they were counted to identify the peak. Four peaks were obtained from the sephadex column. Figure 3 represents elution pattern of radioactivity when the iodination mixture was chromatographed in a sephadex G-25 (S.F.). The first peak has very little immunoreactivity and may represent damaged labeled fragments of AVP or labeled protein which was present in the buffer. Peak 2 is smaller than Peak 1 and behaves like Peak 1 in its lack of immunoreactivity. Peak 3 is the most prominent peak and binds the antibody. The binding capacity is consistently in the neighborhood of 95%. In the beginning 1-2 tubes from the third peak were rechromatographed but since further purification of the labeled AVP was not obtained, the rechromatography procedure was omitted. The fourth peak, which is smaller than Peak 3, also initially has the

Figure 3

Purification of ^{125}I-labelled AVP by column chromatography using Sephadex, G-25 (SF). Labelled AVP was eluted by suing 0.25% glacial acetic acid containing 1.25 mgm/ml of BSA. 1.0 ml fractions were collected, counted for radioactivity and plotted. Two tubes from the descending portion of the third peak were used for RIA.


200
175
150
125
100
75
50
25
CPS IN THOUSANDS
0
10
20
30
40
50
TUBE NUMBER

capacity to bind the antibody. However, after 4-5 days incubation, most of the binding capacity of the fourth peak was lost while the labeled hormone from the third peak retained its antibody binding capacity for up to 3 months. Therefore, the two tubes from the descending portion of the third peak were used for the assay. It was found to be active for 4-6 weeks.

3. Sample Preparation

a. Sampling techniques. Venous blood is drawn from animals or patients into lithium heparinized tubes. Samples are kept at 4°C until centrifuged at 2500 rpm for 20 minutes in a refrigerated Sorvall Centrifuge (RC-3). Plasma is frozen immediately at -20°C for subsequent extraction and assay. Exposure of plasma to temperatures above 4°C is kept at a minimum after thawing for extraction.

b. Extraction. Two ml of acetone (nanograde) is added to 1 ml of plasma and centrifuged at 4°C at 3000 rpm for 30 min. The supernatant is completely poured into another test tube and mixed with 5 ml of petroleum ether (nanograde), vortexed and left to stand for 10 min. The top petroleum ether phase is removed and discarded. The lower aqueous acetone phase is blown to dryness under a mild air current at 0-4°C and stored at -20°C until assay time. This method is associated with a loss of 5-10% of the original AVP content.

4. Assay Procedure

a. Standard curve. Standard AVP is dissolved at a concentration of 1 mg/ml in 0.2 N acetic acid and stored in glass at -20°C. This preparation has shown no change in immunological potency in over 18 months. However, working solutions used to prepare the standard curve (1 µg/ml) undergo a significant decrease in immunological potency when stored for more than 3 months, and therefore, these are prepared at frequent intervals from the stock solution.

The standard curve is prepared by mixing 1000 cpm of [^{125}I] AVP, quantities of nonlabeled AVP varying from 0.5 to 10 pg/tube, antibody to a final dilution of 1:100,000, and diluent to the final volume of 0.5 ml. The diluent is a 0.5 M phosphate buffer, pH 7.4, containing 0.017 M EDTA, 0.14 M NaCl, 2.5 mg/ml BSA (pentex) and 47 µg/ml L-cystine.

b. Sampling handling. Dried plasma extract was resuspended in 1 ml of incubation buffer and 0.2 ml of this was substituted for the known quantities of AVP of the standard curve. Assays are done in triplicate. The tubes then are incubated for 7 days at 4°C.

c. Separation of bound from [^{125}I] AVP. After the incubation period, just prior to separation, 0.05 ml of rabbit gamma globulin (16 mg/ml, dissolved in 0.14 M NaCl) is added followed by the addition of 0.5 ml of saturated ammonium sulphate. The tubes

are vigorously shaken for 10 min at 4°C and then centrifuged at 3000 rpm for 45 min at 4°C. Rabbit gamma globulin precipiates together with the antibody bound hormone, leaving a clear supernatant containing the free as well as damaged hormones. Both fractions are counted for 10 min in an automatic Packard Gamma system (model 5312) with a 2" well counter and a counting efficiency of 60%. The percentage of binding is calculated for each sample, and the standard graph is plotted. Nonspecific precipitation of [^{125}I] AVP is determined by incubating without AVP antiserum.

5. Results

a. Assay sensitivity. PVK-13 antiserum when used as described can consistently detect a minimum of 0.5 pg/ml of the AVP standard, with the most sensitive and precise portion of the standard curve extending to about 3 pg.

b. Specificity. In an attempt to find a cross reactivity of the antiserum with LVP (Schwartz-Mann) and oxytocin, separate standard curve was made by using oxytocin and LVP at 100-fold higher concentrations than AVP. No detectable cross reactivity could be demonstrated.

c. Reproducibility and precision of the assay. This was assessed by two criteria, via interassay coefficient of variation and intraassay coefficient of variation. Triplicate assays on the

acetone extract for each of 50 different samples were performed. In all the samples with varying plasma AVP (1-10 pg/ml) the intraassay coefficient of variation averaged 7.5%.

The interassay coefficient of variation was determined by assaying the same on 20 different times. The average coefficient of variation was 13%.

d. Validity of the assay. Five different types of experiments were conducted to validate our RIA.

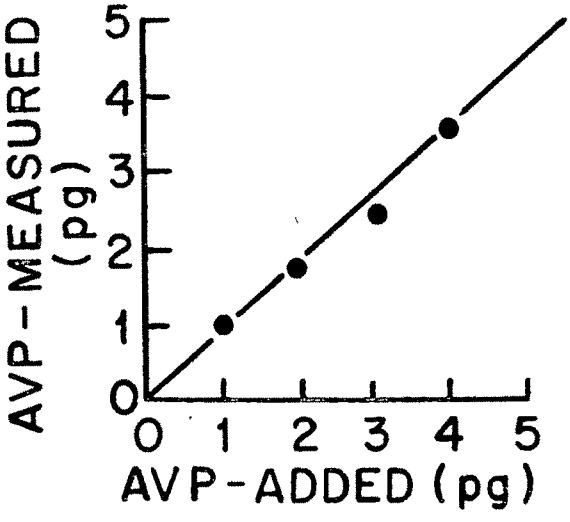
1. Experiments to estimate the recovery of known amounts of added hormone to plasma. For this purpose plasma pools were set up with endogenous hormone content of approximately 1 pg/ml. Unlabeled hormone was added to this pool to give a concentration of 1 pg/ml to 5 pg/ml of plasma. Recovery was estimated after substracting the endogenous hormone content simultaneously determined in samples without added hormone. The average recovery was 93% (Figure 4).

2. Recovery experiments by measuring plasma ADH after administration of a known amount of ADH to patients with pituitary diabetes insipidus. Three patients were studied. After administration of AVP, the immunologically measured plasma level of ADH rose promptly with time which was reflected by parallel changes in urine osmolarity (Uosm) and plasma osmolarity (Posm) (Fig. 5). This could be considered as a typical bioassay.

Figure 4

Estimation of recovery of known amounts of added to plasma. Plasma pools were set up with endogenous hormone content of approximately 1.0 pg/ml. Unlabelled hormone was added to this pool to give a concentration of 2-6 pg/ml of plasma, and assayed. Recovery was estimated after substracting the endogenous hormone content simultaneously determined in samples without added hormone. The average recovery was 93%.

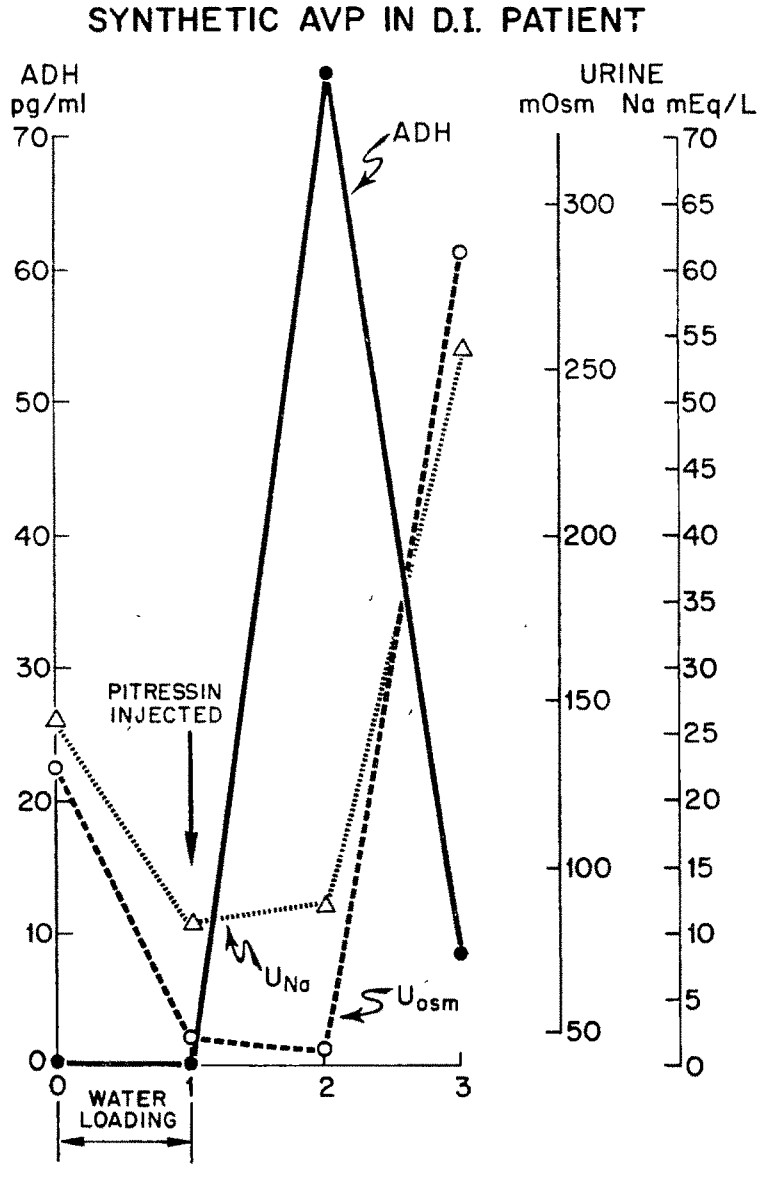
RECOVERY ASSAYS



(Recovery assays and controls done as part of each assay show 80-90% recovery achieved consistency)

Figure 5

Recovery experiments by measuring plasma ADH after administration of a known amount of ADH to patients with pituitary diabetes insipidus. Three patients were studied. After administration of AVP, the immunologically measured plasma level of ADH rose promptly with time which was reflected by parallel changes in urine osmolality and plasma osmolality.


SYNTHETIC AVP IN D.I. PATIENT
ADH
pg/ml
70
60
50
40
30
20
10
0
ADH
PITRESSIN
INJECTED
U_{Na}
U_{osm}
0
1
2
3
WATER
LOADING
URINE
mOsm
Na mEq/L
300
250
200
150
100
50
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0

3. Correlation between bioassay or RIA. Bioassays were performed by the method of Dekanski (1956). The assays performed on standard AVP gave excellent correlation between bioactivity and immune activity. However, bioassays were not performed on plasma samples because the sensitivity of the bioassay was not sufficient enough to measure the physiological concentration of ADH in plasma.

4. Measurement of half life of endogenously stimulated ADH. This was examined in three humans by asking them to smoke a cigarette to stimulate the endogenous release of ADH. Blood was subsequently withdrawn every 15 minutes for measurement of ADH concentration. The estimated half life was 20 min which agrees well with the recently determined half life of 24 min as published by Baumann and Dingman (1976).

The following drugs were studied: l-noradrenaline bitartarate (Sigman); MCN-A-343-11 (McNeil Laboratories); acetylcholine chloride, nicotine (Nutritional Biochemical Corporation); angiotensin (hypertension) (CIBA Pharmaceutical Company); isoproterenol (Sigma); mecamylamine (Merck Institute); pentolinium (Wyeth Laboratories); phentolamine (Regitine, CIBA Pharmaceutical Company); propranolol (Inderal, Ayerst Laboratories).

All stock solutions were prepared in normal saline and diluted before use and kept at $4^{o}C$.

Statistical Tests

Profile analysis and analysis of variance were calculated as described by Winer (1971).

Students t-test was calculated as described by Ostill (1963).