Biological Demonstration of Estrogenic Substances Excreted in Human Saliva. (21192)

STANLEY GREEN. (Introduced by R. Hertz.)

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A biochemical technic for prenatal sex determination was described by Richardson(1, 2). He postulated that salivary glands in women selectively screen out certain female associated hormones but allow male associated hormones to pass into the saliva. If hormones actually are liberated in human saliva and such selectivity does exist, then saliva may represent a new medium for hormone assay in health and disease.

The saliva test for prenatal sex determination has not received adequate clinical verification(3) and no attempt was made in its development to biologically confirm the chemical tests. The presence of hormone-like substances in the saliva has not been established (4).

A survey of the various hormones excreted in saliva in physiological and pathological states has been undertaken. It is our purpose to present biological evidence of salivary excretion of estrogenic substances following intravenous infusion of large quantities of stilbestrol potassium sulfate.

Methods. Two patients were selected for saliva collections, who received, therapeutically, massive doses of stilbestrol, Table I. Saliva specimens were collected following paraffin stimulation, the exact quantity and time of collection noted. These samples were frozen for storage and defrosted prior to hydrolysis. In the first case, the saliva was collected 1) prior to the intravenous infusion of 1000 mg stilbestrol potassium sulfate (given over 9 hr 15 min. period); 2) at the completion of the infusion; 3) 12 hours after completion of infusion. The samples were boiled in 10 cc concentrated HCl for 10 minutes and then extracted with 10 cc of boiling carbon tetrachloride 3 times, the solvent layer having been removed each time and collected. The samples were cooled between each step. Residual water and extraneous material were removed by filtration through anhydrous sodium sulfate. The filtrate was then dissolved quantitatively in a corn oil vehicle and the carbon tetrachloride evaporated. In the *second case,* the saliva collected after one intravenous infusion of 1000 mg stilbestrol potassium sulfate (given over a 16 hr period) was divided into equal portions. One was treated as described above. The other was allowed to incubate with beta glucuronidase (5000 units/cc), 0.75 cc/30 cc volume, and crystalline penicillin G (10000 u/cc), 0.25 cc/3000 volume for 24 hr at 37°C. The estrogens were then extracted 3 times with 10 cc carbon tetrachloride as described above and similar procedure followed. The exact total volume of the corn oil sample was noted and 0.5 cc of this was given subcutaneously to the test animals on each of 3 days. The technic for estrogen assay was a modification of the method described by Lauson *et al.*(5). Twenty-three-day-old castrated female Holtzman rats were used. A standard curve (Fig. 1) was determined employing crystalline stilbestrol potassium sulfate (Table II) for calculating the estrogenic content of the unknown samples.

Results. Table I indicates that the saliva, collected following the intravenous infusion of 1000 mg of stilbestrol potassium sulfate,

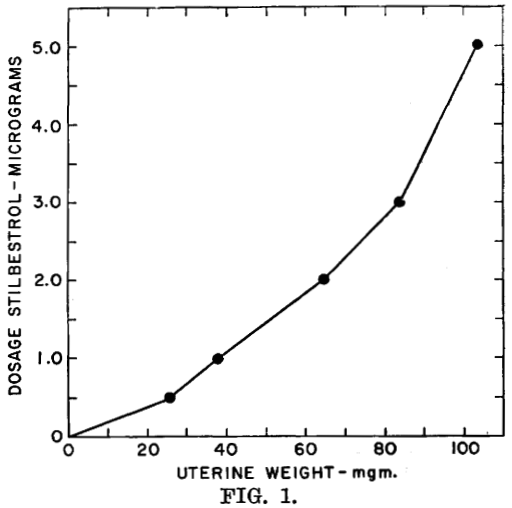

FIG. 1.

TABLE I.

Patient	Age	Sex	Specimen	Preparation	Treated uterine wt (mg)	Control uterine wt (mg)	Vol of corn oil preparation (cc)	Estrogen present in 1.5 cc of corn oil preparation (μg)	Estrogen in saliva (γ %)
B.G.	69	♂	(1) 101 cc saliva (65 min.) prior to therapy	Acid hydrolysis	16 ± 2		6.0		
Prostate carcinoma			(2) 67 cc saliva (60 min.) following 1000 mg K stilbestrol sulfate IV given 9 hr 15 min.	"	96 ± 6	15 ± 3	6.5	3.8	24.4
			(3) 92 cc saliva (75 min.) obtained after completion of above infusion	"	17 ± 6		6.7		
P.M.	76	♀	55 cc saliva (55 min.) (divided in half and prepared by 2 methods) (following 1000 mg potassium stilbestrol sulfate IV given over 16 hr period)	"	29 ± 4	18 ± 1	7.7	.63	5.8
Breast carcinoma				Glucuronidase hydrolysis	28 ± 4		7.9	.58	5.5

(patient B.G.) contained approximately 24.4 μg % of estrogenic substance. This is in sharp contrast to specimens taken prior to and 12 hours after completion of the infusion. Blood was taken simultaneously with the saliva collection, and contained 55 μg/cc serum.

In patient P.M., approximately 5.8 and 5.5 μg % estrogenic substance was present in the saliva collection following the stilbestrol infusion.

Discussion. It appears that the saliva, so readily collected, might offer a suitable media for the study of other organic and inorganic constituents. Claims of a clinical test for prenatal sex determination, even though theoretical and unverified, do raise the question of whether hormones actually are secreted in the saliva.

This report indicates that following intravenous infusion of large amounts of stilbestrol in either males or females estrogenic substances can be found in the saliva. These limited data warrant no further generalization at this time. Numerous factors, including salivary membrane thresholds for hormone excretion and steroid preparation differences must be investigated before the bioassay of hormones in the saliva assumes any clinical significance.

The correlation of salivary hormone levels with those in the blood and urine may throw additional light on the metabolic pathways of these substances. A study of the qualitative nature of the salivary excreted hormones as compared with those in the urine may reveal differences of diagnostic value. In addition, certain interfering substances found in the urine may be absent in the saliva, thereby eliminating technical difficulties. The saliva of several other patients receiving large amounts of testosterone has been tested bio-

TABLE II.

Dose stilbestrol potassium-sulfate, μg	Uterine wt, mg	Control uterine wt, mg
.5	26 ± 6	
1	38 ± 12	
2	65 ± 16	20 ± 2
3	84 ± 6	
5	104 ± 8	

logically. Preliminary work suggests that androgens may also be excreted in the saliva.

Summary. Estrogenic substance has been biologically demonstrated in the saliva of a male and female patient receiving 1000 mg of stilbestrol potassium sulfate intravenously.

1. Richardson, G. C., *Am. J. Obst. and Gyn.*, 1951, v61, 1317.

2. Rapp, G. W., Richardson, G. C., *Science*, 1952, v115, 265.

3. Reiger, J., *Obst. and Gyn.*, 1953, v2, 161.

4. Pigman, W., Reid, A. J., *J. Am. Dent. Assoc.*, 1952, v45, 325.

5. Lauson, H. D., Heller, C. G., Golden, J. B., Severinghaus, E. L., *Endocrinology*, 1939, v24, 35.

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Specificity of Cathepsin III. (21193)

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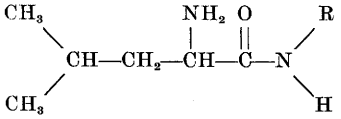
The cathepsins(10,11) have been extensively studied by Bergmann, Fruton, and their co-workers(3-5). Several distinct enzymes have been identified by their specific substrate requirements, pH optima, and their different requirements for activation by cysteine or ascorbic acid. This investigation deals with the substrate specificity of cathepsin III (leucine aminopeptidase) of beef kidneys. Several substrates which differ by the size of the group attached to the nitrogen atom of leucine have been utilized to determine the effect of chain length and of polarity upon the activity of the enzyme. The structures of the substrates used are shown by the formula below in which R represents, alternatively, –H, $-CH_2COOH$, $-CH_2CH_2COOH$, and $-CH_2CH_2CH_2COOH$.



Methods. Three different enzyme preparations were made by the procedure described by Fruton and Bergmann(3) and were stored at –10°C. Preparation B was activated prior to storage; preparations A and C were activated immediately before use. The enzyme was activated by incubating it for 2 hours at 40°C with 0.01 M cysteine at pH 5. During activation a heavy flocculent precipitate formed which was removed by centrifugation. The substrate solution was added to the buffered and activated enzyme solution and maintained at 40°C and pH 5 throughout the reaction period. Citrate buffer at a final concentration of 0.04 M was used to keep the pH constant. The initial concentration of substrate in the reaction mixture was 0.05 M. The rate of enzymic hydrolysis was followed by measurement of the liberated carboxyl groups by the micro-titration method of Grassman and Heyde(6). The enzyme concentration is expressed as milligrams of protein nitrogen per milliliter of test solution. The protein nitrogen was determined by a micro-Kjeldahl method described in the Official Methods of Analysis of the A.O.A.C.(8). Non-protein nitrogen was determined on filtrates prepared after precipitation of the protein with 5% trichloroacetic acid. The proteolytic coefficients C_0 and C_1 were calculated from the equation $C = K/E$ where K represents, respectively, the zero order and first order specific reaction rate constants and E is the enzyme concentration. The first order specific rate constant was calculated in decimal logarithms from the formula $K_1 = \frac{1}{t} \log a_0/a$, where t is the reaction time expressed in minutes. The zero order specific rate constant was calculated from the equation $k_0 = \frac{1}{t} (a_0 - a)$.

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