Phosphaturic Activity of Urine Extracts Not Associated with Parathormone* (32617)

H. C. Stoerk, T. Budzilovich, and R. M. Aceto
Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City 10032

Parathormone (PTH) activity in urine of normal individuals and its increase in urine of patients with hyperparathyroidism has been reported from several laboratories (1–4). The values obtained in fair agreement even though various methods of extraction were employed. The PTH activity was bio-assayed by the hypercalcemic as well as by the hyperphosphaturic response.

In previous experiments (4) we employed the solvent system, introduced for the isolation of PTH by Aurbach (5) for the extraction of the hormone from serum and urine. In the present experiments we have attempted to somewhat purify the active material in urine by means of counter current distribution (CCD).

During introductory studies dealing with the quantitative recovery from urine of Bovine Parathyroid USP (PTE) it was found that human and rat urine contains a material which, although it separates at a different point from known PTH, gave rise to hyperphosphaturia (HPU) in parathyroidectomized (PTX) rats.

Materials and Methods. Human urine was frozen immediately after voiding and rat urine was collected in containers submerged in dry ice and alcohol. After thawing, large pools of urine were divided into 100-ml samples and immediately extracted. Bovine Parathyroid USP[1] was mixed into some of the samples. Glacial acetic acid was added to all of them to give a final concentration of 20%. The majority of the urines were taken to dryness in a flash evaporator at 40°C and the residues taken up in 10 ml of 20% acetic acid containing 3% of NaCl. They were then extracted in a repetitive fashion between equal volumes of 10 ml of 20% acetic acid and *n*-butanol in a 10-tube Craig apparatus. After 10 transfers, the CCD was continued without further addition of upper phase and the effluent series of 10 tubes was combined as indicated in Table I and evaporated. All residues were dissolved in acidified saline (pH 3.4) and 0.12 *M* cysteine and bio-assayed in 6 parathyroidectomized (PTX) rats per group either by the Munson procedure (6) or by a modified "phosphaturic-^{32}P" method (4). In addition, urine collections of rats, PTX 60 days earlier, and of intact rats, fed a Ca-deficient diet, as well as urine collections of a hypoparathyroid, and two hyperparathyroid patients, were extracted and bio-assayed as indicated in Table II.

Results. The distribution coefficient of highly purified PTH, in the present solvent system, was known to be $K = 0.87$ (5). The HPU-activity from bovine PTE, after 60 transfers in 30 tubes, essentially distributes in the same fashion. Except for a small but significant peak in a fraction comprising tubes 5, 6, and 7, the calculated recovery of PTH ($K = 0.87$) and the recovered HPU-activity from 200 USP units (U) of PTE estimated by the Q-32P(4) were in good agreement. Table I illustrates the selective transport of HPU-activity from PTE alone and in admixture with human and rat urine in a 10-tube train. Previously, normal human urine, extracted by various procedures, has been estimated to contain some 20 U in about 1,000 ml (24-hours collections). Thus the 100-ml quantities of urine extracted, were expected to contain only negligible amounts of PTH-activity. However, it can be seen from Table I that much higher PTH-activity is extracted by the present procedure than had been anticipated from previous estimates. The proportions of recovery of bovine PTE following 20 transfers in a 10-tube train were calculated to be the following: Of the total number of U added to the first tube of the lower phase in the train the combined first 3 effluent tubes of upper phase were expected to

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[1] Generously supplied by Eli Lilly & Co.

TABLE I. % PTH-Activity Recovered in Upper Phase.

Expt.	Effluent tube no.:	PTH-Activity (%) 1–3	4–6	7–10	Total
	Calculated	1	9	28	38
1	200 U PTE —	0	7	29	36
2	200 U PTE + human urine A	16	22	24	62
3	200 U PTE + human urine B	27	20	12	59
	Effluent tube no.:	1–5		6–10	
	Calculated	5		33	38
4	100 U PTE	2		35	37
5	100 U PTE + human urine C	24		32	56
6	Tube 1–5 (Expt. 6) redistributed	18		2	
7	Human urine C	(22 U)		(1 U)	
8	100 U PTE + rat urine A	33		28	61
9	Tube 1–5 (Expt. 9) redistributed	26		0	
10	Rat urine A	(25 U)		0	

contain 1%, the second fraction (tubes 4–6) 9%, and the third (tubes 7–10) 28%. From the first experiment in Table I it is seen that recovery of PTH-activity from bovine PTE, determined by the "phosphaturic" bio-assay, agreed well with the calculated values. However, in Exp. 2 and 3, when the residues of a mixture of PTE and of 100 ml of normal, human urine were fractionated and bio-assayed, recovery was much larger than expected. Also, high PTH-activity was found in a fraction (tubes 1–3) which should have been essentially inactive if PTH-activity had been related to the bovine PTH that was added to the urine. The excessive HPU-activity, due to a component in urine, distinct from PTE, was established with certainty since the first 3 effluent tubes contained 16 and 27 times more HPU-activity than expected. Recovery in the second fraction also appeared unduly high, especially in view of the fact that in the last 4 tubes, recovery of added PTE was not less than expected. Subsequently, extracts of urine to which no PTE was added (Expts. 7 and 10) were found to exhibit hormone activity of a similar extent, in a distribution (tubes 1–5) incompatible with that of known PTH thus explaining the excess of HPU-activity recovered. In the lower part of the table, only 2 fractions, comprising the first 5, and the second 5 effluent tubes, were subjected to bio-assay. There was again good agreement between calculated and observed values when 100 U of bovine PTH alone were distributed and then assayed in the 2 pools of fractions (Expt. 4). Expt. 5 confirms the findings in Expts. 2 and 3. However, this time the counter current extraction was done in duplicate, so that the contents of the first fraction (tubes 1–5) containing the unexpected excess of HPU-activity could be collected, evaporated and redistributed through the train (Expt. 6). From this experiment and from an identical one carried out with rat urine (Expts. 8 and 9) it became evident that the

TABLE II. HPU-Activity in Urine of Normal and PTX Rats, and of Hypoparathyroid and Hyperparathyroid Patients.

	Experiment	HPU-Activity (units of PTH/100 ml) effluent tubes, nos. 1–10 av ± SE	No. of samples
	Rat Urine		
1	Control	38 ± 6	10
2	PTX (60 days)	27 ± 18	3
3	Control	24 ± 9	3
4	Low Ca diet (5 weeks)	24 ± 19	4
5	Control	16 ± 9	4
	Human Urine		
6	Control	39 ± 14	3
7	PTX (2 years)	27	1
8	Control	34	1
9	Hyperparathyroid	45	2
10	Control	28	2

HPU-activity which attended urine per se was almost fully recovered in the first five tubes. This was further borne out by the fact that extracts of human (Expt. 7) or of rat urine (Expt. 10), to which no PTE was added, contained HPU-activity in the first fraction only. These findings indicated that normal human urine contains much higher HPU activity than has previously been extracted and that this activity was associated with a material which differs from known PTH in terms of its partitioning properties. The distribution coefficient of HPU activity in normal human urine was then determined from a single extraction of acidified normal human urine with the same volume of butanol. HPU-activity assayed in triplicate, averaged 28 U (20–37) in the lower, and 2.4 U (2.1–2.7) in the upper phases. This finding demonstrated that the HPU-activity in urine extracts rested with a material whose distribution coefficient was about 10 times lower than that of PTH. It was seen that two urine samples, one containing 24, the other 40 U/100 ml, after ashing (600°C for 24 hours), became devoid of HPU-activity. However, boiling of the extracts for 10 min did not lead to any loss of activity.

The relation of the secretory state of the parathyroid gland to the extent of the HPU activity in urine were examined in the following experiments. Table II summarizes bioassays of extracts of urine to which no PTE was added. The extracts were obtained from a pool of all 10 effluent tubes. Parathyroid insufficiency of long duration in rats (Expt. 2) and man (Expt. 9) did not lead to any significant decrease in PTH-like activity in urine. Conversely, spontaneous, primary (Expt. 11) or experimental, secondary hyperparathyroidism (Expt. 4) was not associated with any measurable increase in HPU activity.

Discussion. Since neither excessive nor deficient parathyroid activity was found to alter the extent of the HPU-effect of urine extracts, it appears that the material in question is not related to the secretory activity of the parathyroids. In the present experiments, dealing with extracts of only 100 ml of urine, fractions expected to contain PTH in its known form, were devoid of PTH activity. It appears possible, however, that similar fractions from extracts of larger quantities of urine may contain measurable amounts of PTH. Findings that seem to negate even this possibility are the following: repeated attempts (six trials in 20 rats each) to recover from 24 hour urine collections, exogenous hormone (30–300 U PTE per 100 gm body weight injected intravenously or intraperitoneally) have failed in all instances. Also, others were unable to detect PTH by means of a highly sensitive radio-immuno assay in urine extracts containing high HPU activity by bio-assay[2].

Preliminary experiments suggested that the HPU activity of urine extracts is not due to a chemical artefact produced by the procedure of extraction. Unextracted urine, injected in 4-ml quantities into PTX rats, exhibited similar activity which was within the reach of the sensitivity of the "phosphaturic" assay. A characteristic of HPU activity of urine extracts, other than the low solubility in butanol, became evident when some of the extracts were administered orally to PTX rats. While bovine PTE given by stomach tube lost more that 95% of its activity, the urine extracts appeared equally active by the oral or parenteral route. Since vitamin D is known to raise the blood Ca and to cause phosphaturia in PTX rats it was considered that its HPU action may account for the active moiety in urine. However, it became evident that up to 2000 U of vitamin D2, injected subcutaneously, failed to change the ^{32}P output of the assay animal during the 3-hour period of the experiment.

Finally, experiments, to be published in detail elsewhere, have shown the following: When butanol extracts of 10 samples of rat urine were examined not only for HPU activity, but also for hypercalcemic (HCE) activity, it was found that 100 ml of urine contained a material equipotent to 40 ± 6 U of bovine PTH by the Munson assay and to 38 ± 7 U of PTU when assayed from its HPU effect. Thus, butanol extracts of human and of rat urine exert both biological actions of PTH to an amazingly similar extent. However, that these two PTH-like effects rested with at least two substances distinct from each

[2] Personal communication from A. G. Aurbach.

other and from PTH became evident from that fact that the HPU activity in urine was dialyzable while HCE activity was not. Also, HCE activity in urine, on CCD, separated at a point different from both HPU activity and bovine PTH.

Summary. Butanol extracts of normal human and rat urine contained two types of substances, distinct from each other and unrelated to PTH, which interfered with the bio-assay of PTH in urine. One caused hypercalcemia, the other hyperphosphaturia in PTX rats. The latter material was dialyzable and equilibrated between 20% acetic and *n*-butanol with a distribution coefficient (*K*) about 10 times lower than that of PTH. The "hypercalcemic" material was nondialyzable and its *K* about 5 times lower than that of PTH. Thus, both materials could be separated by CCD from PTH added to the urine before extraction. From 100 ml of normal (human or rat) urine, no PTH activity could be detected in fractions expected to contain PTH in its known form.

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Canine Tracheobronchitis: Isolation and Characterization of the Agent with Experimental Reproduction of the Disease (32618)

ABRAHAM KARPAS, NORVAL W. KING, FELIX G. GARCIA, FERNANDO CALVO AND REX E. CROSS (Introduced by J. F. Enders)
The Animal Research Center, Harvard Medical School, Boston 02115

In a preliminary paper (1), the isolation of a herpes virus from a clinical case of infectious canine tracheobronchitis was described. This represented the first confirmed report of herpes virus isolation from natural cases of canine respiratory disease. In the present paper the properties of the virus and preliminary results of experimental infection of dogs with the agent are presented. The virus was found to be identical with herpescanis virus. The pathogenicity of this agent will be evaluated on the basis of our studies and those of previous workers.

Material and Methods. Tissue culture. Monolayer cultures of whole dog embryo, fetal and newborn dog kidney (DK) cells were prepared according to standard techniques and incubated at 37°C. The growth medium consisted of "basal" Eagle's plus 10% calf serum, and the maintenance medium (MM) of Eagle's plus 2% calf serum. The medium contained 200 units/ml of penicillin and 200 μg/ml of streptomycin.

Viruses. The virus to be characterized in this paper was isolated from a dog with a clinical case of tracheobronchitis. It was repassed on DK cell cultures and clarified by centrifugation at 2000 rpm for 20 min. Herpescanis virus for comparative studies was provided by Dr. Carmichael of Cornell University. This virus was handled in a similar manner.

Virus isolations. Three naturally infected dogs with advanced clinical tracheobronchitis were used in the initial study. After taking blood samples from each of them, they were sacrificed and postmortem examination was performed. Trachea and bronchi were washed with Eagle's medium containing 2000 units of penicillin, 2000 μg of streptomycin and 15 μg of Fungizon, per ml. Representative tissue samples of tracheal, laryngeal and bronchial mucosae, retropharyngeal lymph nodes, lungs, livers, kidneys, and spleens were processed for viral isolation. Subsequently nasal and throat swabs were taken from a random group