17144. Adsorption of Urinary Gonadotrophins on Kaolin.

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Zondek[1] described a method of obtaining gonadotrophic hormones from urine by precipitation with 4 volumes of alcohol. With minor modifications, such as the substitution of acetone for alcohol, this has remained the basic method of preparing urinary gonadotrophic hormones for assay.[2-5] Other procedures have been less commonly employed. Katzman and Doisy[6] recovered the hormone from urine by precipitation with acetone saturated with benzoic acid. Since the hormone is a protein, precipitating agents such as tannic acid[7] and ammonium sulphate[8] have been used, and ultrafiltration[9,10] has also proved successful. Katzman, *et al.*,[11] demonstrated that chorionic hormone could be adsorbed from urine by passing it through a column of permutit. Scott[12] and later Landgrebe and Samson[13] used kaolin as the adsorbing agent for chorionic hormone.

Since chorionic hormone could be adsorbed on permutit or kaolin it seemed probable that the pituitary gonadotrophin could also be recovered from urine by an adsorption technic. This report describes a procedure by which either chorionic or pituitary gonadotrophins can be recovered from urine. The main advantages are, one, a great saving in alcohol over that which has been used previously and, two, a shorter time for preparation since dialysis is not necessary.

Materials and method. Kaolin (N.F. powder), that has been washed with normal hydrochloric acid and then with water until neutral, is made up as a 20% suspension in water. This is a stable stock preparation that should be shaken thoroughly before using.

1. Use a fresh 12 or 24 hour specimen of urine. Acidify to pH 4.5 with acetic (or hydrochloric) acid and then run through a coarse filter to remove mucus or particulate matter.

2. Add 5 volumes percent of the kaolin suspension to the acidified urine and stir thoroughly. Allow the kaolin to settle out. (One hour is usually sufficient but the mixture may be set in the refrigerator overnight).

3. Decant the supernatant and collect the sediment in a large centrifuge flask. Centrifuge, and discard the supernatant.

4. Add water and resuspend the kaolin by vigorous stirring or shaking. Centrifuge or allow to settle, decant and wash again with water until the kaolin is free of urine. This washing replaces the dialysis of the alcohol method.

5. Elute the hormone from kaolin by adding a volume of normal ammonium hydroxide equal to that of the kaolin suspension originally added to the urine. Stir thoroughly and allow to stand 20 to 30 minutes. Centrifuge and save the supernatant. The kaolin may be washed again, the second ammonia eluate added to the first and the kaolin discarded.

6. Add glacial acetic acid to the combined eluates until pH 8.5 is reached. If a flocculent precipitate forms, centrifuge and discard.

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TABLE I.
Ovarian Weights in Immature Rats Injected with Extracts of Urine from Postmenopausal Patients. Aliquots of Pooled Specimens Were Adjusted to Different Degrees of Acidity Before Adsorption on Kaolin.

Exp. No.	Control Alcohol ppt.	Adsorbed on Kaolin at pH 7.0	6.0	5.5	5.0	4.5	4.0	3.5	3.0	Eluted with
1	69				59		19		46	3N NH_4OH
	47				58		15		35	
2	—				99		57		17	"
	—				76		73		9	
3	136				112		101		81	2N NH_4OH
	118				82		145		107	
4	—			16	87	72	58	65		"
	—			19	62	87	66	71		
5	—	18	18		32		39		20	"
	—	15	17		46		34		18	

Exp. 1, 2, and 3, urine acidified with hydrochloric acid.
Exp. 4 and 5, urine acidified with acetic acid.

Bring the clarified supernatant to pH 5.5 with glacial acetic acid.

7. Add 4 volumes of 95% ethanol, slowly and with constant stirring to precipitate the hormone. Set in refrigerator until thoroughly chilled, centrifuge and dry the precipitate in a gentle stream of air.

8. Dissolve the precipitate in a volume of water or phosphate buffer (pH 8.0) suitable for assay.

In our experience, urines from postmenopausal women or from patients with other hypergonadotrophic conditions give suitable assay dose ranges in infantile female rats if the final extract is made up so that 1 cc is equivalent to one hour's output of urine. Six injections of 0.5 cc each make the total dose equal to a 3 hour urine volume. The examples given below were made up in this way and show the ovarian weights at autopsy 72 hours after the first injection. If mice are used for assay the total dose can be reduced to the equivalent of about one hour's urine output. Assays to determine the quantities of hormone present in the urine of normal men or women require the use of larger quantities per test mouse or rat so the total dose should be equivalent to a 6 or 12 hour volume.

Proof of method. In the course of developing and testing the above method of adsorbing urinary gonadotrophins on kaolin, the optimal conditions and quantities to be used in each step were determined. Usually the yield of hormone was compared to that obtained from an aliquot precipitated with alcohol as a standard of reference.

It has been customary to use fresh urine specimens to avoid partial or total destruction of the hormone through bacterial or chemical degradation. In three experiments the urine was allowed to stand several days and become very alkaline through the breakdown of urea to ammonia. When precipitated directly with alcohol these specimens yielded such highly toxic extracts that assays were not possible. After acidification and adsorption on kaolin, washing with water definitely reduced the toxicity of the extracts and hormone was recovered. In order to learn whether urease would affect the yield of hormone, that enzyme was added to urine and allowed to react for 2 hours with formation of ammonia. Subsequent acidification and adsorption on kaolin resulted in quantitative recovery of the hormone. The alkaline change due to urease activity is apparently not immediately destructive of the hormone. Collection of urine under toluene tends to preserve the natural acidity and the toluene is easily separated before kaolin is added.

The optimal pH for adsorption on kaolin was determined in several trials in which ali-

TABLE II.

Ovarian Weights in Immature Rats Injected with Extracts of Urine from Postmenopausal Women. The hormone was adsorbed on kaolin and then the aliquots of the kaolin were eluted with different concentrations of ammonium hydroxide as noted.

		Eluted from kaolin with NH_4OH Normality					
Exp. No.	Control Alcohol ppt.	3.0	2.5	2.0	1.5	1.0	No. of elutions
6	139	68		117		45	1
	128	41		113		15	
	148	106		92		16	
7	47	66		101	83	24	2
	43	82		71	90	19	
8		147	169	145	177	139	2
		151	183	146	140	165	(After water wash)
		121	151	119	128	115	

quots of urine were adjusted to different degrees of acidity. In 3 experiments the pH was adjusted with hydrochloric acid (Table I) and recovery of the hormone was uniformly good at pH 5, but was poor in one instance at pH 4 and once at pH 3. In 2 other experiments using acetic acid the results were satisfactory from pH 3.5 to 5 but since the hormone was not adsorbed above or below this range, pH 4.5 has been selected as an optimal acidity for adsorption. Sulfuric acid was used in 2 experiments without success.

Having adsorbed the hormone onto kaolin, the next problem was to determine the optimal conditions for elution and recovery. Ammonium hydroxide was used in the elution process in concentrations up to 3 N without appreciable destruction or loss of hormone, (Table II). In Experiments 6 and 7 the recovery with 1 N NH_4OH was incomplete as compared to higher concentrations of ammonia but in Experiment 8 it was satisfactory. Experiments 6 and 7 were done before it was found that the kaolin can be washed with water and that the residual acid in the kaolin probably partially neutralizes the ammonia and reduces its efficiency. We have failed repeatedly to recover the hormone from kaolin with NaOH in concentrations from 0.1 N up to 3 N. Since elution with ammonia after using NaOH has also failed to recover the hormone, it appears that the pituitary gonadotrophin is easily and rapidly destroyed by sodium hydroxide. By contrast, chorionic gonadotrophin is apparently eluted quantitatively from kaolin by either 0.1 N NaOH or N NH_4OH.

Katzman, *et al.*, used 10% ammonium acetate in 40% ethanol to elute the chorionic hormone from permutit. This mixture is also satisfactory for eluting gonadotrophin from kaolin and is superior to ammonium hydroxide in alcohol, but, in our experience is not superior to aqueous ammonia. There are no experiments in which the dry weights of the final precipitate can be compared to give an indication of which eluting mixture may give the higher potency per mg of precipitate. The relative effectiveness of ammonium hydroxide in water and ammonium acetate in alcohol suggested that ethanolamine might be an effective elutant; this was proved in two experiments but seemed to offer no advantage over either of the others.

Other adsorbents were tried only casually. In three experiments the hormone was adsorbed on Filtercel and then recovered with ammonia. The coarser particles of permutit adsorbed the hormone more effectively when packed in a column and the urine allowed to percolate rather slowly. Charcoal (Norit) adsorbed the hormone but no eluting agent was found.

The quantity of kaolin necessary to provide a surface area adequate for complete adsorption and still have a minimal volume for elution procedures was determined in several experiments (Table III). In experiment 9, 1 volume percent (15 cc in 1500 cc of urine) of kaolin suspension gave as complete recovery of hormone as 10 volumes percent. However, in experiment 10, the smaller quantities of kaolin were less effective. On

TABLE III.

Ovarian Weights of Immature Rats Injected with Extracts of Urine from Postmenopausal Patients. The amount of kaolin added to the urine was varied as indicated to determine the minimal amount of kaolin that would adsorb the hormone.

Exp. No.	Control Alcohol ppt.	Quantity of kaolin suspension Volumes % 10	3	1
9	—	130	107	140
		112	68	153
10	55	91	53	18
	64	63	49	18

the basis of these observations it seems that 5 to 10 volumes percent of kaolin suspension is best for routine use.

This method has been found equally applicable to recovery of chorionic hormone or of the pituitary hormone from the urine of postmenopausal women. In 2 experiments, these hormones were adsorbed simultaneously and the expected augmentation was demonstrated by assay of the final extract. One-half of a postmenopausal urine specimen was adsorbed on kaolin (a), while 100 cc of pregnancy urine was added to the other half before addition of the kaolin (a plus b). A second 100 cc portion of the pregnancy urine was also treated with kaolin (b). In the subsequent assays, the pituitary gonadotrophin (a) induced rat ovarian weights of 52 and 58 mg, whereas, those for the pregnancy urine extract (b) were 34 and 36 mg (with corpora lutea), and those of the combined urine eluate (a plus b) were 180 and 176 mg. This represented a definite augmentation reaction resulting from the simultaneous adsorption of the pituitary and chorionic gonadotrophins. Another example may be cited: a 19-year-old boy with infantilism associated with testicular agenesis had high urinary levels of pituitary gonadotrophins (kaolin extract of 6 hour urine volume induced ovarian weights of 52 and 58 mg). He was given chorionic gonadotrophin, and on the third day of treatment, excretion of the administered hormone together with the pituitary gonadotrophin was determined by assay. The same urinary equivalent of kaolin extract (6 hours output per test rat) produced ovarian weights of 257 and 260 mg.

This study was primarily a series of spot checks to determine the limits within which a routine procedure could be developed. Small numbers of rats were used in each test since quantitative precision was not attempted. In 12 tests the extracts of alcohol precipitates were compared with those of kaolin adsorbates. In 3 instances material obtained by the alcohol method produced significantly greater increases in ovarian weights, in 6 trials the kaolin method seemed superior, and there was no significant difference in the other three. The sum of the ovarian weights for the 26 rats receiving extracts of alcohol precipitate was 2081 mg, as compared to 2073 mg for the 26 rats receiving kaolin extracts. Thus, there is no significant difference in the recovery of hormone by the two methods.

The main advantage of the kaolin method is the marked saving of ethanol. For example, the hormone from 1000 cc of urine can be adsorbed upon 50 cc of kaolin suspension, then eluted in two 50 cc portions of N ammonium hydroxide, which on subsequent acidification will not exceed 120 cc in volume and from which the hormone can be precipitated with 500 cc of alcohol. The original urine specimen would have required 4000 to 5000 cc of alcohol plus an additional quantity used in the second precipitation (after dialysis). A second advantage is that washing the kaolin with water accomplishes the same detoxification as dialysis but much more rapidly and avoids the transfer of solution to, and from, the dialysing bag.

Another advantage is that the same urine specimen can be used for both gonadotrophic and steroid determinations if the supernatant urine and the water washings from the kaolin are saved. A series of tests has shown that 17

ketosteroid estimations after the use of kaolin are the same as those obtained from untreated aliquots of urine.

Summary. A method is described for the adsorption of gonadotrophic hormones from urine onto kaolin and their subsequent elution with ammonium hydroxide. The relatively small volume of the eluate reduces the amount of alcohol needed to precipitate the hormone from solution, the final extracts are less toxic and can be prepared in a shorter time. The hormone assays are comparable to those obtained with the usual alcohol precipitation method.

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17145. Streptomycin-Producing Capacity of Different Strains of *Streptomyces griseus.**†

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Since the first announcement[1] that certain strains of *Streptomyces griseus* produce the antibiotic streptomycin, pertinent questions have been raised in regard to the ability of this organism to produce the particular antibiotic: 1. Is this a characteristic property of all strains of the species *S. griseus?* 2. How widely distributed is the capacity for production of streptomycin among actinomycetes? 3. Did the original strain of *S. griseus,* first isolated in this country in 1915 by Waksman and Curtis,[2] also possess the capacity for producing streptomycin? (The last question is of considerable theoretical and historical interest, since it has a bearing upon the identity of the streptomycin-producing strains isolated in 1943 with the original 1915 isolate, as well as upon the fact that had the subject of antibiotics been recognized at that time, it would not have taken that long to recognize the actinomycetes as highly important producers of antimicrobial agents.)

The first two questions were soon answered. It was established[3] that only certain few strains of *S. griseus* are capable of producing streptomycin, whereas most of the other strains that can easily be isolated from natural substrates are either unable to produce any antibiotics at all or may give rise to antibiotics other than streptomycin, such as grisein,[4] and that certain actinomycetes, other than *S. griseus,* also possess the capacity of forming streptomycin.[5]

In an attempt to answer the third question, the original 1915 culture, which has been kept alive by frequent transfers since its isolation in the collection of the New Jersey laboratories under the ATCC number 3326, was tested for its antibiotic-producing capacity. The same culture was deposited in 1920 in the Baarn collection in Holland; it recently was obtained from that collection and given the number 3326a. Repeated tests for their antibiotic-producing capacity revealed the fact that the culture kept in New Jersey was totally unable to produce either streptomycin or any other antibiotic substance, whereas the corresponding Baarn culture was capable of forming an antibiotic

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