It is possible that this newly discovered action of physostigmine might be due to inhibition of the esterase which is responsible for the destruction of acetylcholine and that in the final analysis the substance producing the ganglionic effect is acetylcholine. This interpretation of the effect on the sympathetic synapse seems to be in harmony with the view that the transmission through the sympathetic synapse is cholinergic (Feldberg and Gaddum[4]).

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Procedure for Quantitative Extraction of Sex Hormones from Urine.

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The excretion of sex hormones in the urine has been the subject of a voluminous literature. Attempts have been made to relate the amount of these substances excreted to various pathological conditions with the hope that new information of clinical value would be made available. Unfortunately most of this effort is valueless because certain fundamental facts are completely ignored. Thus it is known from the researches of Zondek, Cohen and Marrian,[1, 2, 3] and others that some fraction of the estrogenic substance in urine is conjugated so that it is either not extractable, biologically inert, or both. Conversion to the active form, therefore, must be an essential feature of any method for assaying the urine for total content of female hormone. Further it is immediately evident that a unit expressed solely in terms of a biological response is almost useless for the purpose under discussion. The only unit acceptable is a definite quantity of substance. Such a standard for the female hormone has been available for several years and recently a standard has been designated for the male hormone. Unfortunately, few workers have used these or indeed any standards. Only by the use of such standards can the values obtained by a given individual and by different laboratories be accurately compared, for without a standard it is impossible to detect change in sensitivity of the test

[4] Feldberg and Gaddum, *J. Physiol.*, 1934, **81**, 305.

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[1] Zondek, Bernhard, *Nature*, 1934, **133**, 209.

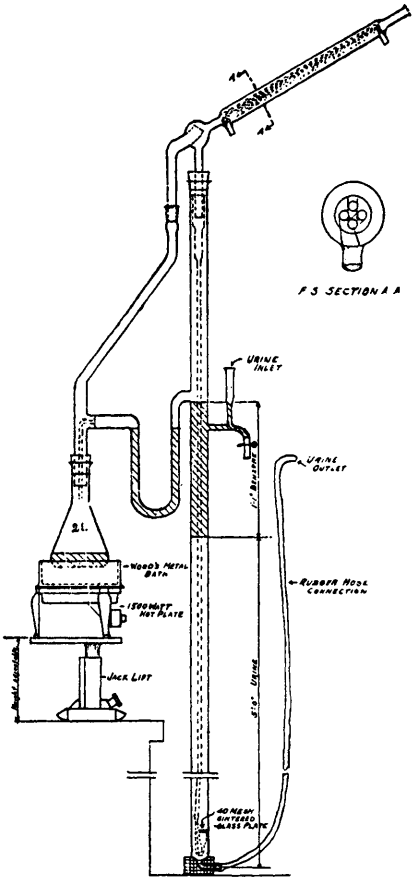
[2] Zondek, Bernhard, *Ark. Kemi., Min. o. Geol.*, 1934, part 3, paper 24.

[3] Cohen, S. L., and Marrian, Guy F., *Biochem. J.*, 1934, **28**, 1603.

animal, no matter what the cause. This assertion applies equally well for sex hormones or any biologically active substance. Finally, in order to assay normal urines it is necessary to extract the active substances. In order to accomplish this, one must be assured that the extraction is complete.

In the opinion of the authors, the only procedure fulfilling the necessary conditions is that of Cohen and Marrian. Unfortunately their technique is suitable only for urines of pregnancy, and as yet they have not extended their investigations to the male hormone. We, therefore, present the results of our investigations upon extraction and assay of the male and female components of normal urines.

Figure 1 shows the extraction apparatus we have used. By means of this device we are able to make as nearly complete contact as is



CONTINUOUS EXTRACTOR FOR SEX HORMONES FROM URINE

FIG. 1.

possible of the urine with an organic solvent and at the same time measure the rate of distillation without interrupting the extraction process. The rate of distillation achieved with benzene varies between 6 and 8 liters per hour. Thus, as we shall show, a 24-hour sample may be extracted in at most 2 hours. After some study, benzene was chosen as the most efficient solvent since it is most effective in removing both hormones with the minimum of extraneous substances. Toluene is equally as good but the higher boiling point is a disadvantage. Ethyl acetate, which has been used to some extent in this country, is relatively poor compared with benzene. It has, in addition, the disadvantage of dissolving a large amount of inert material which eventually may be toxic to the test animal.

All assays, both on spayed females and on capons, were conducted by assaying a standard preparation at the same time as the unknown. Ten rats and 7 capons were used on each assay reported here. For the estrogenic substance, the International Standard of the League of Nations' Committee was used. For the male hormone, we have used for 3 years a standard preparation which is exactly equal to 100γ of androsterone. We were fortunate inasmuch as this quantity has since been chosen as the international standard by the League of Nations' Committee.

In order to prepare the extract for assay the benzene was distilled, the residue dissolved in ethyl ether and the ethereal solution shaken with saturated aqueous sodium bicarbonate until reaction ceased. The bicarbonate removed only inert acids and was discarded. The ether solution was then worked through in either of 2 fashions. If sufficient urine had been extracted to allow both male and female assays to be carried out, the ether solution was simply washed with water and dissolved in the requisite volume of oil.

If, however, it was desired to separate the male and female activity, the ether solution was shaken with 10 separate portions of 10% aqueous sodium hydroxide. In this partition, the volume used was ether 75 cc. and alkali 50 cc. The alkali treatment removes about 95% of the estrogenic activity and no detectable amount of male hormone. The ether solution, after washing free from traces of alkali, was made to volume and assayed upon capons. The alkali extracts were combined and after acidification were shaken with 3 portions of ether, washed and made to volume for assay upon spayed female rats.

The next point was, how long must the extraction continue for quantitative values? After testing at various intervals, it was found that if the urine were extracted with 10 times its volume of fresh

benzene, no further activity, either male or female, could be extracted. (Table I.) The complete absence of male or female hormone in the re-extracted urine was demonstrated by the more rigorous technique of adding the second extract to the first in order to detect smaller amounts than might otherwise be assayed. This experiment and others similar to it convinced us that extracting the urine with 10 volumes of benzene in such an apparatus as we have used results in complete removal of both male and female hormones.

TABLE I.
Extraction of Sex Hormones from Acidified Male Urine by Benzene.

Treatment	Sample	International units/liter Male hormone	Estrogenic
Boiled 2 hr.*	4	25	80
" 2 "	"	21	85
" 2 "	"	18	100
Not boiled	"	22	25
Boiled 2 hr.	5	53	95
" 2 "	"	46	110
" 2 "	"	84	95
Same urine re-extracted		0	0

*In this case the urine was introduced into the extraction by a pump delivering the urine at one-tenth the rate at which benzene was distilled.

We had known for some time that acid hydrolysis beyond making urine acid enough to extract without emulsification was not necessary to obtain maximum yields of male hormone. This is illustrated in Table I and is confirmed by our later works upon adsorption. However, since it was equally certain that the estrogenic activity was markedly increased by boiling with acid, it was necessary to determine whether the hydrolysis would destroy the male-hormone activity. The urine was acidified by adding 100 cc. of commercial hydrochloric acid per liter and then under reflux was boiled for 2 hours. This seemed the optimum time from the work of Cohen and Marrian and our preliminary results confirmed them. No difference could be found between 50 cc. of HCl and 100 cc. HCl per liter of urine. We preferred, however, to use the higher acid concentration since we did not autoclave the urine as did Cohen and Marrian. As can be seen from Table I, no loss of male hormone nor any increase is observed subsequent to acid hydrolysis. The effect upon the estrogenic substance, however, is quite marked and is equal to an increase of 300 to 400%.

If male hormone assays alone are desired, an alternative procedure may be used. We have found that the male hormone may be almost quantitatively adsorbed by the diatomaceous earth sold under the

name of Dicalite. The untreated earth called Superaid is the best adsorbent thus far studied. The adsorption may be carried out immediately after acidification or after boiling the urine by shaking the urine with Dicalite, using 100 gm. per liter of urine. The degree of acidity seems to make no difference in the adsorption but from an alkaline urine no adsorption occurs. The earth is filtered and may be kept at least a year without deterioration and probably for a longer time.

In order to release the activity we have extracted the earth 3 times with fresh portions of *hot* 95% ethanol using each time 1 liter for 500 gm. of original earth. The alcoholic washings are combined, distilled until all alcohol is removed, extracted with ether and the ether solution then shaken with sodium bicarbonate, washed, and made to volume, as is the case with the benzene extracts. This treatment fails to remove any appreciable estrogenic substance from the urine. Table II illustrates the results obtained from a typical batch of urine. The agreement between adsorption and extraction indicates the adequacy of either method.

TABLE II.

Comparative Yields of Male Sex Hormone by Adsorption and Extraction Methods.

Treatment of Urine	Adsorbent	Internat. Units
Acidified by H_2SO_4 (not boiled)	Dicalite	25
Boiled with HCl	"	20
Routine quantitative extraction with benzene		23

Various other adsorbents have been studied and the results will be reported elsewhere. This report summarizes only the most advantageous process for routine use in the study of male-hormone excretion.

Summary and Conclusions. 1. A method for the extraction and assay of male and female hormones from normal urines has been described. 2. This procedure consists in 2-hour acid hydrolysis of the urine, extraction with ten times the volume of benzene using the extractor described, and separation into male and female fractions by alkali. 3. The assay is conducted with standards in parallel and results expressed in international units. 4. An alternative procedure for male hormone alone using an adsorption process is likewise described.