Hirschfelder[3] reported that after 20-40 g of magnesium sulfate were administered to a human subject, no increase occurred in the magnesium content of the blood, but 40% was excreted in the urine in 24 hours. How much of the sulfate was absorbed was not stated.

Verzar and McDougal[4] pointed out that the rate of absorption of the sulfate ion is about the same as that of substances of equal weight and compares favorably with that of pentoses and certain hexoses. The data we are presenting show a parallelism between the rates of absorption of the magnesium and the sulfate ions. So far as we are aware, this has not been previously emphasized. Magnesium sulfate is widely employed by both practitioner and laymen and is probably one of the most commonly used household cathartics. A number of cases of magnesium poisoning[5,6] have rather recently been reported, some of which have proved fatal.

Summary. The rate of absorption of magnesium sulfate was studied in a series of barbitalized dogs each of which had suffered a hemorrhage equal to 3% of its body weight. The control animals absorbed 14% of the magnesium and 13% of the sulfate, whereas the experimental animals absorbed 17% and 16% respectively. The differences were not statistically significant. A fact not sufficiently appreciated is that the intestine is only relatively impermeable to this salt.

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15701

Estimation of Steroid Estrogens by Fluorimetry.*

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Estrogens are still generally determined by biological methods. As these present all the difficulties and uncertainties of the biological technic, considerable interest attaches to the development of an adequate chemical assay for the estrogens. During more than a decade repeated attempts have been made to elaborate a colorimetric procedure suitable to this purpose.[1-10] Most of this work has been based on the Kober reaction. The available colorimetric methods are, however, difficult in practice, of imperfect specificity, and often insufficient in sensitivity. A search in the field of fluorimetry for an adequate solution of this analytical problem would seem to be indicated. The proven high sensitivity of fluorimetric methods in vitamin assay encourages the hope that methods of this type could find usefulness in determination of hormones at biological level.

* This paper was partly aided by a grant from the Max London Foundation administered by Dr. M. Bloom.

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The present paper describes a sensitive fluorimetric method for assay of the steroid estrogenic hormones on the basis of the fluorescence afforded when these substances are heated with phosphoric acid.

Method for estrone, estradiol and estriol in pure solutions. It has been known for some time that several natural estrogens—estrone, estradiol, estriol—produce on warming in concentrated sulfuric acid orange-colored solutions with green to blue fluorescence.[11-13] The fluorescence reaction has been employed widely as a qualitative test for estrogen in preparative work, and diagnostically as a qualitative test for pregnancy in the mare.[14,15] As sulfuric acid produces color under the same conditions, and in some cases fluorescence with a large range of substances, it seems not to have been considered worthwhile hitherto to attempt quantitative assay of estrogens on the basis of this reaction. In the method described below, this difficulty is avoided. By the use of phosphoric rather than sulfuric acid in the reaction solution, the interference of nonspecific response is largely eliminated.[16] Thus a quantitative test possessing a desired degree of specificity is afforded.

Reagents. 1. Phosphoric acid, s. g. 1.75, purest. 2. Crystalline estrogens.† 3. Ethanol, pure, redistilled from an all-glass still.

Special Apparatus. 1. Test tubes provided with ground-glass stoppers (capacity 15 cc, Pyrex). 2. Fluorimeter provided with a micro-cuvette (capacity 2 cc).

Procedure. A volume not exceeding 2 cc of the pure alcoholic solution of estrogen is evaporated from a test-tube over a water bath and oven-dried for one hour at 110°C. To the dry residue, 3.0 cc phosphoric acid is added from a burette. The test-tube, closed with ground-glass stopper, is transferred to a boiling water bath (98°) and heated in the dark for 30 minutes, the mixture being shaken during the first 2 min. of heating to insure the solution of the estrogen. Reaction is interrupted by cooling in tap water. The fluorescence is measured within an hour. The concentration of the estrogen solution is read against a concurrently prepared standard reference curve.

Prolonged exposure of estrogen in acid to light is avoided as light interferes with the development of the fluorescence and also causes gradual fading of developed fluorescence. In performing the reaction, care is taken to prevent accidental entry of water (spray or vapour) into the reaction mixture, as water sharply depresses the reaction and exerts a marked quenching effect. The reaction vessels should be sealed with close-fitting ground-glass stoppers, rubber and cork being unsatisfactory.

Experimental. Pure crystalline estrogens were used for the experiments. The fluorescence was measured in a 2 cc micro-cuvette with a Lumetron photofluorimeter‡ using a Uviarc mercury vapor lamp equipped with primary filter as for riboflavin assay, and secondary orange filter which transmits freely above 5500 Å. The instrument was calibrated against fluorescein solution of known concentration.

Effect of heating time. The effect of heating time on the intensity of the fluorescent response of estrone and estradiol was determined for the time interval 5-30 minutes. The results in Table I demonstrate that the reaction proceeds regularly with time, rising rapidly in the first 10 minutes, and then less rapidly to a maximum at 20 minutes and is maintained at 30 minutes. Prolonged heating (60 minutes) led to loss of fluorescence. A heating time of 30 minutes seems therefore to be suitable and is employed in all following experiments.

11 Wieland, H., Straub, W., and Dorfmuller, T., *Z. physiol. Chem.*, 1929, **186**, 97.

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15 Sala, S. L., cited in *Sex and Internal Secretion*, p. 892, Williams & Wilkins Co., 1939.

16 Hestrin, S., and Mager, J., *Nature*, 1946, **158**, 95.

† Estrone and estradiol were kindly supplied by Dr. B. J. Brent, Roche Organon, Inc.; estriol was obtained through the courtesy of Dr. O. Kamm, Parke Davis Co.

‡ Thanks are due to Prof. I. S. Friedenwald for his kind gift of a Lumetron apparatus.

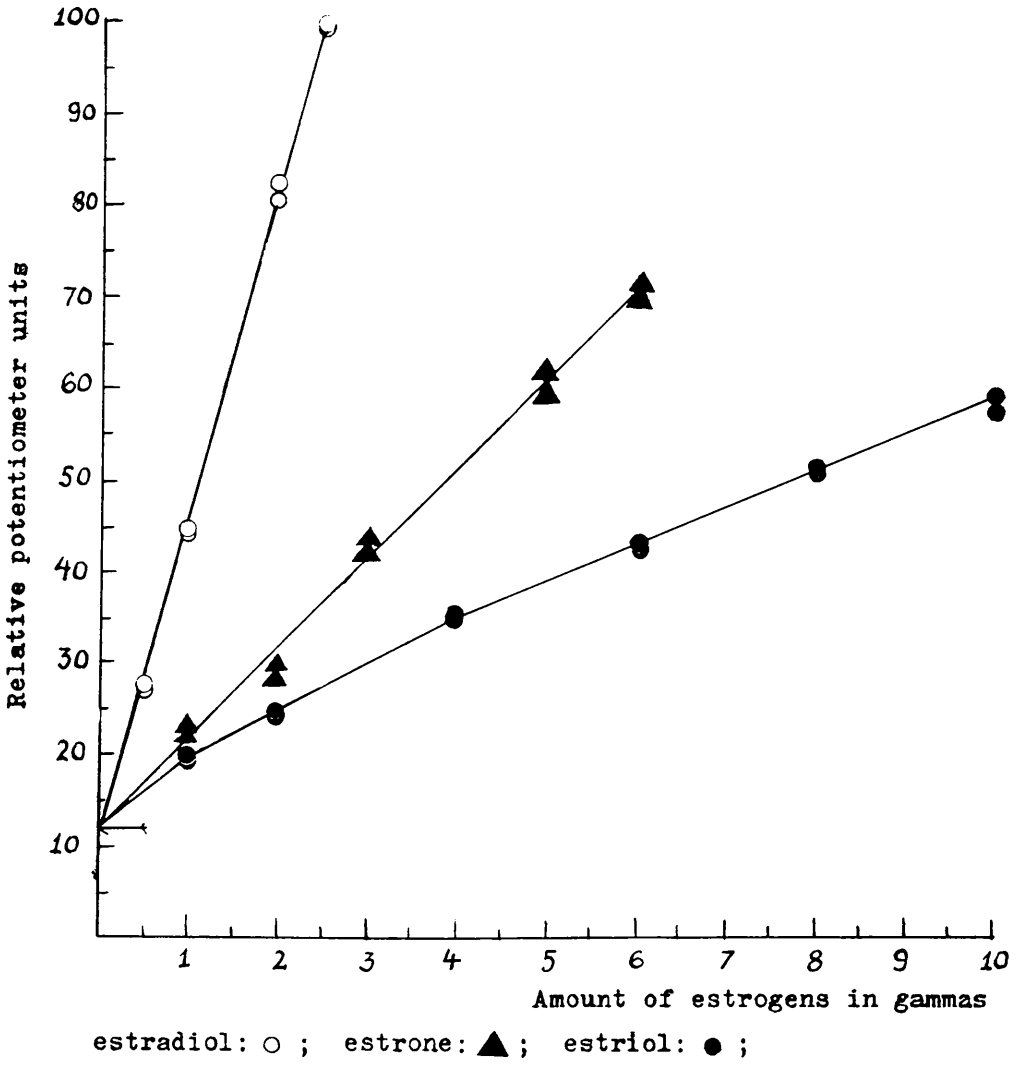


Chart 1.

Influence of estrogen concentration on fluorescence response.

The ordinate intersect represents an intrinsic instrument factor.

TABLE I.

Effect of Heating Time in Phosphoric Acid on Fluorescence Reaction of Estrone and Estradiol.

Substances	Fluorescence measurement in relative galvanometric units at different times			
	5′	10′	20′	30′
Estrone, 3 γ	27.5	35.5	56.0	57.5
Estradiol, 1 γ	17.0	31.0	40.0	42.5

TABLE II.
Analyses of Estrogen Solutions by the Fluorimetric Method.

Estrogen	Conc. per cc in γ as given	Conc. per cc in γ as read	Error of estimation in γ
Estrone	0.00	0.0	0.0
	6.50	6.5	0.0
	8.50	8.3	—0.2
	9.00	8.9	—0.1
	9.30	8.95	—0.35
	10.00	9.75	—0.25
	10.00	9.80	—0.20
Estradiol	1.50	1.46	—0.04
	2.20	2.15	—0.05
	2.40	2.40	—0.00
	4.30	4.22	—0.08
Estriol	1.50	1.5	0.0
	1.50	1.65	+0.15
	4.00	3.8	—0.2
	4.00	4.0	0.0

Effect of estrogen concentration. Typical concentration response curves are shown in Chart 1. It can be seen that the intensity of fluorescence is a linear function of estrogen concentration to at least 2.5 γ of estradiol, and 5 γ of estrone. The curve for estriol seems to be slightly convex to about 2 γ, then straightens out and follows a linear course from 3 γ to at least 10 γ. The estriol solutions have a faint pink color; the estrone and estradiol solutions are virtually colorless. Weight for weight, estradiol is 3.6 times and estrone about 1.3 times as active in production of the fluorescence as estriol. The sensitivity of the method therefore varies with the estrogen determined.

Results obtained on solutions of pure estrogen in concentrations unknown to the authors at the time of the analyses are shown in Table II. In optimum ranges of estrogen concentration and when each test was performed in duplicate on 2 dilutions of the test solution, the mean error of estimation in a series of 30 determinations proved to be ±3%.

Preparation of standard curve. The instrument was calibrated against sodium fluoresceinate so that a solution of a concentration of 0.8 γ/cc rated near 40 on the galvanometer scale and precisely 100 on the potentiometric scale.

Calibrations were made both before each series of measurements and during measurements at intervals of 10 minutes. Standard curves were prepared from parallel measurements on graded estrogen concentrations so selected to fall within the potentiometric range 10-90. The ordinary scatter of results in a parallel replicate series can be seen in Fig. 1. Standard curves obtained on different days using the same batch of phosphoric acid fluctuated within ±5%. A daily check of the standard curve is recommended for accurate work.

Specificity. Effective use of the fluorescence reaction as the basis of estrogen assay in biological materials is dependent on proof of the reaction's specificity as between different ether-soluble biological substances which tend to accompany the estrogens in usual procedures for their extraction.

Nonestrogenic mononuclear urinary phenols (tyrosine, phenol, and cresol) showed in an amount of 1 mg neither fluorescence nor color reaction under the conditions of the test.

The behaviour of 13 sterols§ listed in order of decreasing activity is recorded in Table III. It may be seen that of the examined nonestrogenic sterols only desoxycorticosterone and testosterone have sufficient activity to produce a false positive estrogen reading under the conditions of the test. As

§ The authors are indebted to Dr. F. Bergman, Daniel Sief Research Institute, Rehovot, for a kind gift of sterols.

TABLE III.
Correlation of Chemical Structure and Fluorescence Response to Heating in Phosphoric Acid.*

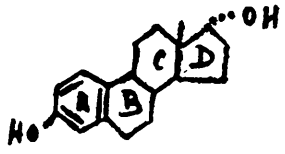
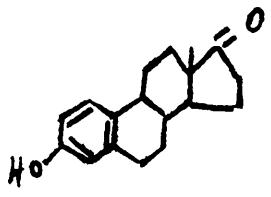
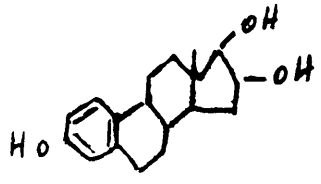
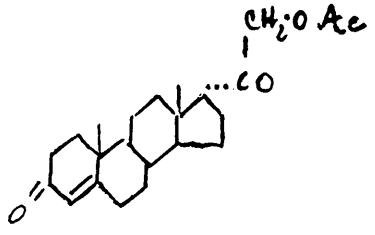
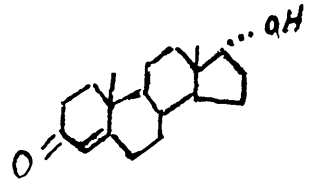
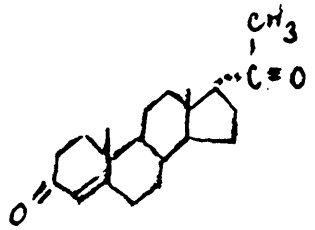
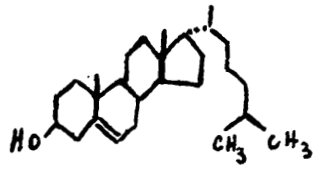
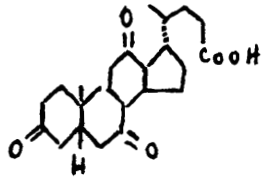
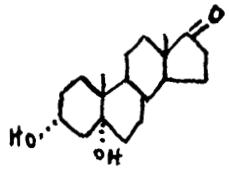
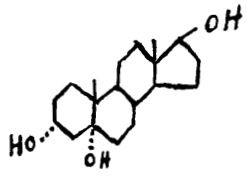
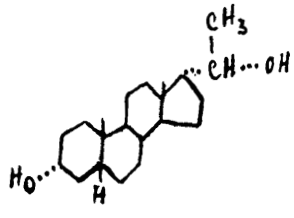
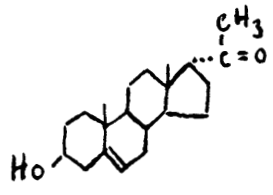
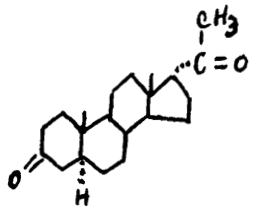
No.	Substance	Structural formula	Fluorescence activity per γ relative to estrone = 1.
1	Estradiol		2.7
2	Estrone		1.0
3	Estriol		0.75
4	Desoxycorticosterone-acetate		0.3
5	Testosterone		0.1
6	Progesterone		0.01

TABLE III. (Continued).

No.	Substance	Structural formula	Fluorescence activity per γ relative to estrone = 1
7	Cholesterol		0
8	Dehydrocholic acid		0
9	Androsterone		0
10	Androstanediol		0
11	Pregnanediol		0
12	Pregnenolone		0
13	Allo-pregnanedione		0

* The structural formulæ conform to the conventions adopted by H. Selye (*Encyclopedia of Endocrinology*, Franks Publishing Co., Montreal, 1943).

neither of these 2 possible interferants is a phenol, their separation from the urinary estrogens presents no great difficulty.

In test-tube experiments on a random collection of substances, the following results were recorded. Tryptophane and quinine showed marked fluorescence. Stilbestrol, catechol, all amino acids other than tryptophane, glucose and polyglucosides, several purines and polyhydric alcohols showed no reaction. Carbohydrates containing a non-glucose sugar constituent gave amber reactions but no fluorescence.[17]

Discussion. The fluorescence reaction described seems suitable for use as the basis of an estrogen assay. Furthermore this reaction may afford also a basis of assay for certain nonestrogenic sterols, *e.g.* testosterone and desoxycorticosterone acetate. The fluorescence response of these latter, though smaller than that of the estrogens, is readily measurable at a level of about 10 γ. On the other hand, the minimum amounts of estrogen that can be determined in terms of fluorescence reaction within an error range of $\pm 3\%$ are for estrone 1 γ or 10 I.U., for estradiol 0.5 γ or about 5 I.U., and for estriol 1 γ or about 0.01 I.U. Thus the sensitivity of the fluorescence assay in the case of estriol exceeds that of the biological test and in all the cases exceeds that of known colorimetric procedures.[1-10]

When estrogens are heated with sulfuric acid, a fluorescence exceeding in intensity that afforded by phosphoric acid is obtained. In principle, therefore, sulfuric acid might seem preferable to phosphoric acid as the reaction agent. When sulfuric acid is used, however, blank fluorescence of the reagent complicates the reading, color changes obscure the determination, the results are less reproducible, and greater technical inconvenience is encountered. For quantitative work, phosphoric acid seems decidedly to be preferable.

The experiments reported show that fluorescent response to heating in phosphoric acid is a specific characteristic of certain sterols. A consideration of the structural formulae of the investigated sterols (Table III) leads to certain generalizations concerning structural features which mediate the production of fluorescence under the conditions of this test. It is evident from the data that the investigated sterols fall into 2 groups:

(a) Substances Nos. 1-6 which show a definite fluorescent response.

(b) Substances Nos. 7-13 which show no measurable fluorescent response.

All the substances of Group (a) possess a conjugated double-bond system and have this system in ring A of their structure. Two types of conjugated double-bond systems are exemplified in this group: aromatic or polyenoid (C:C·C:C), *e.g.* substances Nos. 1-3; and catioenoid (C:C·C:O), *e.g.* substances Nos. 4-6. On the other hand, no member of Group (b) possesses a conjugated double bond, although some of the members of this group do possess one or more nonconjugated double bonds. It is suggested therefore that the presence in the sterol molecule of a conjugated double bond is a prerequisite of the fluorescent activity. From the fact that the magnitude of the response of different substances in Group (a) varies markedly, it must be concluded that the magnitude of a fluorescent response is determined not merely by the presence of a conjugated double bond but also by other characteristics of the molecule. It is evident that the presence and position of polar groups exerts an important influence on the magnitude of the fluorescent response. High activity such as the urinary estrogens exhibit appears to be associated with aromatic phenol character in ring A and presence and position of oxygen bonds in ring D (Table III).

As may be seen below, the biological activity and fluorescent response of the investigated estrogen trio are correlated, though by no means perfectly.

Relative activity	Estriol	Estrone	Estradiol
Estrogenic	0.01	1	5
Fluorimetric	0.75	1	2.7

Since the ratio of estrogenic activity to fluorimetric response per gamma is a variable, it is clear that the application of the re-

[17] Hestrin, S., and Mager, J., 1946, unpublished.

action to estrogen assay in a material which contains several estrogens must entail prior separation of the different components. Several recommended fractionation technics fortunately meet this requirement.[6,7,18-20] It may be noted here that the nonsteroid estrogen, stilbestrol, does not produce fluorescence under the conditions of the test.

The fluorimetric method is simple, the reproducibility of the result is good, and the specificity of the test promises to be as good as or better than has been obtained with the previously described colorimetric methods. Further study of the analytical possibilities of this assay seems therefore to be desirable. A survey of the effect of phosphoric acid on a larger number of sterols and related substances with a view to elucitating the relation of sterol structure to fluorescent response has accordingly been undertaken. At the same time the application of the fluorimetric method to estrogen determination in blood and urine is being examined. The results will be reported elsewhere.

Summary. 1. A method for estimation of estrone, estradiol, and estriol in pure solution is based on the observation that these substances afford products with a green fluorescence when heated in phosphoric acid.

2. The fluorescent response of the urinary estrogens is over a wide range a linear function of their concentration. The error of the fluorimetric assay in suitable ranges of estrogen concentrations (estradiol, 0.5-2.5 γ; estrone, 1.0-5.0 γ; and estriol, 1.0-10.0 γ) does not exceed ±3%.

3. Sterols which do not contain a conjugated double bond, some naturally occurring nonsteroid aromatic substances, and many common constituents of biological material give no fluorescent response under the conditions of the test.

4. Estrogen fluorimetry appears to compare favorably as to sensitivity, specificity and simplicity with previously described methods of estrogen assay.

The authors wish to express their indebtedness to Prof. B. Zondek, for his kind interest and support of this investigation.

18 Mather, A., *J. Biol. Chem.*, 1942, **144**, 617.

19 Pincus, G., and Pearlman, W. H., *Endocrinology*, 1942, **31**, 507.

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15702 P

Induction of Renal Glomerular Lesions by Urethane in Inbred Mice Susceptible to Spontaneous Glomerulonephritis.*

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Mice of the NH strain develop spontaneous glomerulonephritis. Histologically the glomeruli are similar to those of the human chronic disease.[1]

Dunn and Larsen[2] reported that urethane induced hyaline alterations in the glomeruli of mice of the Strong A strain, whereas similar changes were lacking in the glomeruli of other stocks of mice following the administration of this drug.

The work of Dunn and Larsen suggested to the authors that urethane might provoke interesting changes in the glomeruli of mice which are susceptible to the development of spontaneous glomerulonephritis. Twenty-one NH mice received 1 mg per g of body weight of urethane (anesthetic dose) in

* This investigation has been aided by a grant from the Life Insurance Medical Research Fund. The authors are indebted to Miss Marthella Frantz, Miss Elnora Stahn, and Mrs. Sonia Braun for valuable technical assistance.

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2 Dunn, T. B., and Larsen, C. D., *Fed. Proc.*, Part I, 1946.