ing most of the time there is little or none present. This presents the problem of finding a method by which the maximum potential activity of gonadotrophic extracts can be made effective. As has been shown this can be done by increasing the number of injections from two to twenty-four times per day,[15] or by the use of heme,[10,11] but these methods are not practical for general purposes. The results obtained with the insoluble metallic hydroxides suggest that they might be utilized to make the hormones in pituitary preparations more effective without increasing the number of injections.

Summary: Aluminum, iron and zinc hydroxides were shown to be effective in increasing the effectiveness of sheep pituitary gonadotrophin while magnesium hydroxide was less effective. The effect of these hydroxides is explained on the basis of adsorption of the hormone by the hydroxide with slow release of the active substance from the site of injection.

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Further Studies on Pituitary Responses to an Oxidative Inactivation Product of Estrone.

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It has been found that Westerfeld's[1] lactone, an oxidative inactivation product of crystalline estrone, causes adrenal and pituitary hypertrophy in mature male rats.[2] The adrenal response was shown to be mediated by the pituitary and transplantation of the hypophyses indicated increased release of gonadotropic as well as adrenotropic hormone. Control experiments with estrone gave evidence that these effects are not referable to the slight estrogenic activity of the lactone and that estrone elicits them only through the formation of some inactivation product, possibly the lactone or a related compound.

The present communication deals with investigation of the lactone in relation to 2 other pituitary responses to the estrogens which might conceivably be accountable to inactivation products rather than to estrogens *per se*. The first is the release of pituitary luteinizing hormone (L.H.) and the second the suppression, by prolonged administration, of pituitary follicle-stimulating hormone (F.S.H.).

Three different batches of the lactone were used. The first was an aliquot of Westerfeld's original preparation upon which he published the results establishing its chemical identity.[1] The other two were prepared by Dr. Alan Mather of Clark University, Worcester, Mass., following Dr. Westerfeld's general scheme of oxidation with H_2O_2 and purification. The biological properties of these 3 preparations were entirely similar. Dissolved in 0.005 N NaOH, their estrogenic potency approximated one-fifteenth that of estrone in the same medium, and was increased 3 to 5 fold by hydrolysis in the presence of the Zn and HCl.[2] In the open ring form (*i.e.*, in 0.005 N NaOH) they also showed the previously described pituitary-stimulating properties as well as the effect upon pituitary L.H. to be reported below. According to Dr. Mather's experience, one cannot be sure of acquiring this compound by this method. Three other preparations supplied by him, although derived from acetates whose melting points agreed quite closely with Westerfeld's, were completely non-estrogenic even after Zn-HCl hydrolysis, and failed to exhibit any pituitary-stimulating properties.

1 Westerfeld, W. W., *J. Biol. Chem.*, 1942, **143**, 177.

2 Smith, O. W., *Endocrinol.*, 1944, **35**, 146.

TABLE I.
Enhancement of F.S.H. with the Lactone as Compared with Effect of Estrone in Corresponding Amounts of Estrogenic Activity.

Injections to immature female rats	No. of rats	Effect on ovaries: Avg wt, mg	Effect on ovaries: Avg increase in wt over uninjected, littermate, %	Effect on ovaries: No. of ovaries containing corpora lutea*
None	46	7.4		0
F.S.H. only	31	12.2	70	5 of 62 (8.1%)
F.S.H. plus the lactone, 10-20 γ, 1-3 R.U.	26	31.2	303	48 of 52 (92%)
F.S.H. plus estrone, 2-4 γ, 3-6 R.U.	27	12.6	68	4 of 54 (7.4%)

* Not all ovaries were sectioned. These figures refer to grossly viable discrete corpora lutea.

According to our own experience there is also considerable difficulty involved in eliminating contamination of the final product with unchanged estrone. Our studies of the biological properties of the lactone suggest that it may be of considerable therapeutic value and a more economical procedure for its preparation would be a real contribution. An even more practical aspect that should be investigated is the possibility that non-estrogenic compounds related to diethyl-stilbestrol may have these same biological properties.[3]

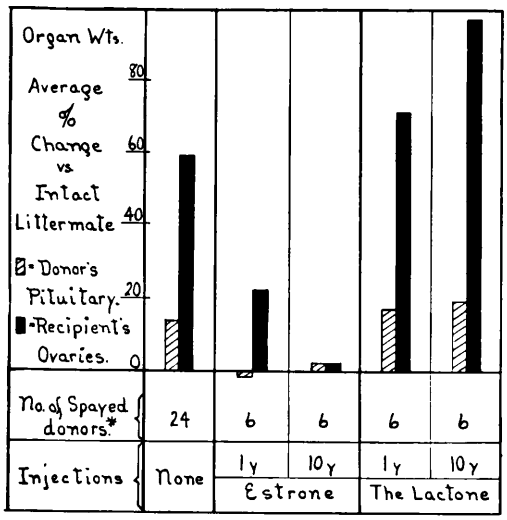
It has been shown that estrone stimulates the release of L.H. from the pituitaries of immature female rats, thereby augmenting the response to F.S.H. so that ovulation and luteinization take place.[4] In determining whether or not the lactone has this property, we experienced some difficulty in obtaining a source of F.S.H. with which Hisaw's experiments with estrone could be repeated. A partially purified powder prepared from alcohol precipitation of menopausal urine was found satisfactory and was used in most of the experiments to be reported. Only one of 3 extracts of pituitary glands was augmented by estrone, this being a Schering product prepared from sheep's pituitaries and non-luteinizing when given alone in twice the amount used for test. With both preparations the smallest amount which, when given in 6 injections over 3 days to 19- to 21-day-old, female rats, caused estrous smears on the fifth and sixth days, was considered 1 unit, and was used in testing for augmentation. Experiments were run on batches of 19- to 21-day-old littermates so that the ovaries of a rat that received no injections were compared with those of a littermate that received F.S.H. only and of another that received the same amount of F.S.H. plus the lactone. The F.S.H. was given in 6 injections over 3 days, the lactone in 6 injections on days 3, 4 and 5, and all animals autopsied on the seventh day, their ovaries weighed on a torsion balance and examined for corpora lutea. In order to be certain that enhancement with the lactone was not referable to its slight estrogenic activity, control experiments were run substituting estrone, in corresponding amounts of estrogenic potency, for the lactone. The results are summarized in Table I and demonstrate that 10 to 20 γ of the lactone augmented the ovarian response to the follicle-stimulating hormone and resulted in marked luteinization (Fig. 1), this effect being unassociated with the slight estrogenic activity of the oxidation product. Even when 10 to 20 γ of estrone were given (8 experiments, not included in Table I), the average increase in ovarian weight over that of control littermates was only 200% and only 50% of the ovaries contained corpora lutea. Since enhancement of F.S.H. with the lactone was not demonstrable in hypophysectomized animals* (6 experiments), we con-

[3] Smith, O. W., and Smith, G. V., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 198.

[4] Hisaw, F. L., Fevold, H. L., and Greep, R. O., *Proc. Assn. Res. Nerv. and Ment. Dis.*, 1936, **17**, 247.

* We are indebted to Dr. R. Tilsowitz, now of Schering Corporation, for hypophysectomizing for us the 28-day-old females used. In each experi-

TABLE II.
Effect of Estrone and the Lactone on Castration Changes of Pituitaries of Female Rats.



* For each injected spayed donor there was one intact and one spayed control littermate.

clude that its effect is due to release of L.H. from the hypophysis. The fact that estrone is less active than the lactone in eliciting this response suggests that it exhibits this property indirectly, through the formation of the lactone or some similar inactivation product.

Castration results in hypertrophy of the pituitary and an increase in its transplantable gonadotropic activity associated with cytological changes indicating increased storage in the basophiles. These effects of castration are generally accepted as evidence of increased secretion of F.S.H. and may be prevented by prolonged administration of estrone. It seemed possible that inhibition of pituitary F.S.H., like the other pituitary effects of the estrogens, might be duplicated with the lactone on the supposition that this response also was accountable to breakdown products rather than to estrogens *per se*.

Twenty-four experiments, summarized in Table II, were run upon batches of 3 littermate, 25-day-old, female rats. In each experiment, 2 were spayed and the third left intact. One of the spayed animals served as uninjected control and the other received either estrone (a total dose of 1 or 10 γ) or the same amount of the lactone in 2 daily injections during the following 16 days. All were autopsied on the forty-second day of life, their pituitaries weighed on a torsion balance, ground in saline and transplanted in 6 injections over 3 days into littermate 19- to 21-day-old, female rats whose ovaries were weighed on the fifth day.

Castration caused a 2 to 17% increase in the weight of the pituitaries and a 42 to 93% increase in transplantable gonadotropic activity as measured by the weight of the ovaries of recipients. One gamma of estrone caused a partial and 10 γ a complete suppression of these castration changes, the results of pituitary transplant experiments being definitely significant, whereas the average weights of the pituitaries were indicative only. Representative experiments were run in which the pituitaries, instead of being transplanted, were sectioned and examined for us through the courtesy of Dr. R. O. Greep of the Harvard Dental School. Dr. Greep could find no cytological evidence for the influence of one gamma of estrone, but 10 γ completely prevented the histological changes following castration. In the experiments in which the lactone was given, on the other hand, there was certainly no suppression of castration changes and some evidence, from the results of pituitary transplant experiments, of an actual enhancement of gonadotropic secretion. The basophilia in the pituitaries of spayed rats that received 1 or 10 γ of the lactone was as marked, according to Dr. Greep, as in untreated spayed littermates. The suppression of F.S.H. by prolonged administration of estrone, therefore, may well be a direct effect. It is certainly not mediated by the formation of this particular oxidative inactivation product.

In this connection a single experiment may be cited for its confirmatory value. Two mature female rats having regular 4- to 5-day cycles were injected twice daily for 27 days, one receiving a total dose of 1 mg of estrone and the other a total dose of 1 mg of the lactone. The rat that received estrone was in

ment F.S.H. alone was given to one and F.S.H. plus the lactone (10 γ) to the other of 2 littermates, injections being started the day after removal of the glands and the same procedure adhered to as in the experiments on intact animals.

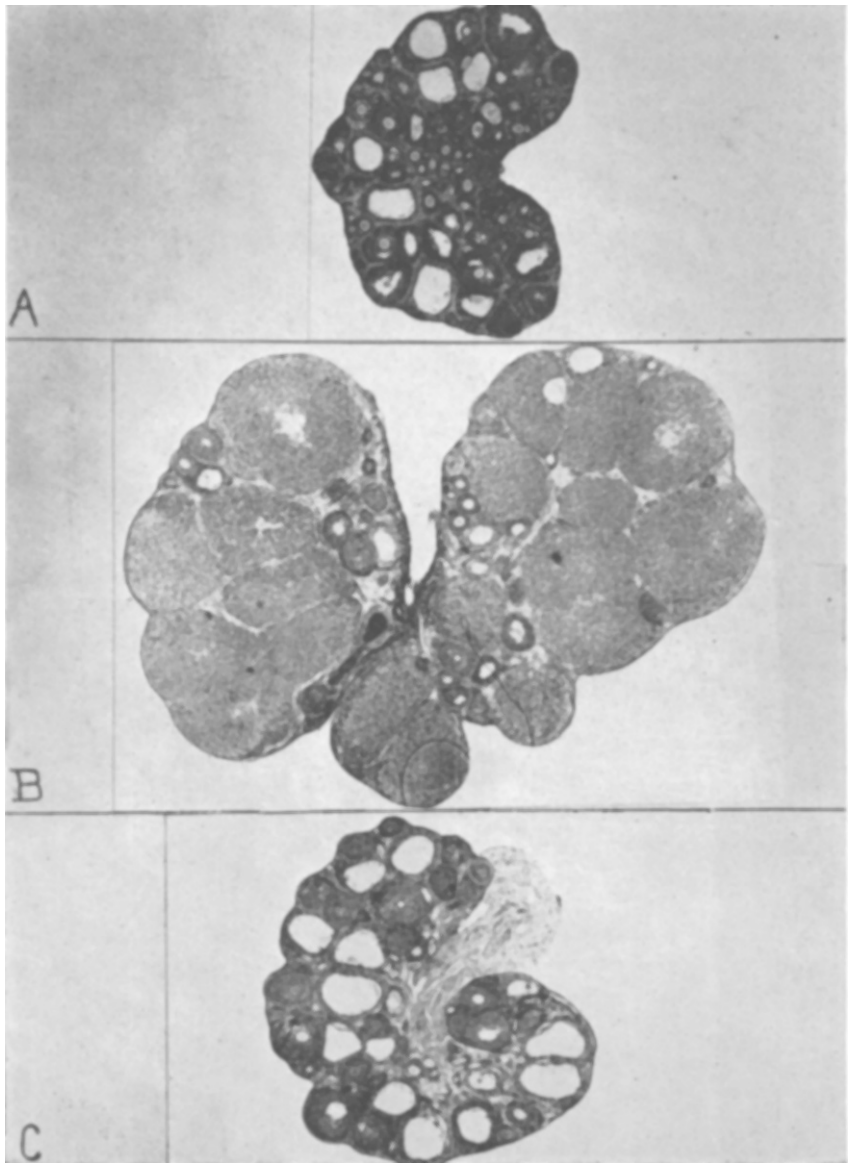


FIG. 1.

Luteinization of ovary of immature female rat with F.S.H. plus the lactone. One ovary from each of 3 littermates. A received F.S.H. (extract of menopausal urine) only. B received the same F.S.H. in the same amount plus 20 γ (2 R.U. of estrogenic potency) of the lactone. C received the same F.S.H. plus 2.0 γ (3 R.U.) of crystalline estrone.

constant estrus during injections. At autopsy on the 28th day its ovaries were atrophied (16.5 mg) and showed no evidence of secretory activity. The animal that received the lactone, on the other hand, continued to have 4- to 5-day cycles, although during the last 10 days all smears contained many nucleated and squamous cells. At autopsy on the twenty-eighth day the ovaries were normal in size (39.5 mg) and loaded with small, red corpora lutea.

Discussion. From the aspect of the thera-

peutic possibilities of the lactone, these and the previously reported observations[2] are of considerable interest. Hypophyseal extracts of animal origin have not proved very satisfactory as a means of establishing normal ovarian activity in women with functional disturbances. In certain situations estrogens have been apparently beneficial, presumably because of their pituitary-stimulating effect. As a pituitary stimulant, the lactone should be very much more useful than the estrogens, first, because it is practically non-estrogenic and would not be expected to cause such undesirable side effects as excess cervical secretion, breast changes and endometrial hyperplasia. Secondly, since, unlike estrone, it acts directly on the pituitary and is not influenced by the steroids (*viz.*, progesterone and testosterone[2]), which depress estrogen inactivation, the response to it would presumably be uninhibited by the patient's own secretion of steroid hormones. Finally, according to the above experiments, its prolonged administration should have no depressant effects upon pituitary or ovarian activity. As an inhibitor of F.S.H., on the other hand, it would presumably be ineffective.

Summary. Westerfeld's lactone, an oxidative inactivation product of crystalline estrone, stimulates the release of luteinizing hormone from the pituitaries of immature female rats, eliciting this response in smaller dosages than does estrone. Unlike estrone, it does not, upon prolonged administration, prevent the secretory or cytological changes in the pituitary which follow removal of the ovaries. Therapeutic implications are discussed.

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Effect of Fatty Acids on Acetylcholine Synthesis.*

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More acetylcholine is synthesized by the frog brain *in vitro* in the presence of serum than in its absence.[1,2] Performance of muscle work modifies the ability of serum to increase the acetylcholine synthesis.[3] Since muscle may utilize fats during performance of work[4-19] the

* This investigation was aided by a grant from the John and Mary R. Markle Foundation.

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