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Augmentation of Sheep Pituitary Gonadotrophin by Insoluble Metallic Hydroxides.*

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Urine extract, blood serum, yeast and yeast ash, zinc and copper salts have been shown to increase the gonadotrophic activity of pituitary gland preparations.[1-8] In addition formed elements of blood[9] and heme prepared from erythrocytes augment the gonadotrophic action of pituitary preparations,[10] but not the gonadotrophin in pregnant mare's serum or that in the urine of pregnant women.[11]

As part of a more extensive investigation directed toward finding methods for increasing the gonadotrophic activity of pituitary preparations, a study was made of the effect of aluminum, magnesium, iron and zinc hydroxides on increasing the effectiveness of pituitary gonadotrophic substances. The results obtained from this investigation provide the substance of this report.

Experimental methods: 1. Gonadotrophic extracts. Relatively crude and purified sheep pituitary extracts, a follicle-stimulating preparation, and an extract of rat pituitary glands were used in this study. The cruder of the two unpurified extracts was obtained by extracting sheep pituitary powder with water in the ratio of 1 gm powder to 10 ml water. The extraction was repeated once, the 2 extracts were combined, and the active substance was precipitated from this aqueous extract by the addition of 4.5 volumes of acetone. The precipitate was removed from the acetone by decanting and centrifuging. This insoluble material was extracted with water after which the active substance was precipitated with acetone and dried by washing with acetone. The other extract of this type was the pH 5 soluble, B fraction, previously reported.[12]

The purified preparation which was used with aluminum hydroxide was of low solid content and contained the major part of the activity of the original pituitary powder.[12] The follicle stimulating preparation was obtained by tryptic digestion.[13] An aqueous extract of air dried rat pituitary glands was made and lyophilized, and this preparation was tested with aluminum hydroxide.

2. The preparation of hydroxides. The aluminum hydroxide was prepared essentially by the method reported by Johnson and Bradley.[14] Two hundred seventy grams of ammonium alum that contained 12 molecules of water of crystallization were dissolved in 1 liter of distilled water by heating. Seventy-five milliliters of concentrated ammonium hydroxide were diluted to 2 liters with distilled water. The alum solution was added slowly to the ammonium hydroxide while a stream of

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TABLE I.
Augmentation of Sheep Pituitary Gonadotrophin by Aluminum Hydroxide.

No.	Gonadotrophic extract, mg*	Aluminum hydroxide, mg	No. of rats	Wt of ovaries, avg, mg
25B	100	—	3	52
"	100	100	3	354
"	50	—	3	25
"	50	50	3	229
"	25	—	3	13
"	25	25	3	98
"	12.5	—	3	13
"	12.5	12.5	3	24

* Indicates mg equivalents of dry pituitary powder used in making the preparation.

steam was passed into the mixture. The steam treatment was continued for 15 minutes after the final addition of the alum or until the mixture boiled. The equivalent of the alum used was in excess of that of ammonium hydroxide.

The zinc, iron and magnesium hydroxides were prepared by the same general procedure using zinc sulphate and ferric ammonium alum with ammonium hydroxide, and magnesium sulphate with dilute sodium hydroxide. Each of the preparations was kept in the incubator at 75°C for 12 hours after which the supernatant liquid was decanted and distilled water was added while stirring. A number of decantations were made over several days until the supernatant solution was relatively free of the various salts after which the hydroxide was suspended in a definite volume of water. The amount of hydroxide required was measured from these stock suspensions, centrifuged, decanted and mixed with the extract to form a suspension.

3. Assays. The combination of the different gonadotrophic preparations and the hydroxides were made by mixing *in vitro* a given quantity of the gonadotrophin with a definite amount of the various hydroxides. The gonadotrophic preparations were tested alone in the same quantities as was used with the hydroxides. The proper amounts of these preparations were injected subcutaneously into 21-day-old female rats. The rats were injected with 0.5 ml during the afternoon of the first day, and twice daily with this amount on each of the 4 succeeding days. The animals were killed during the morning of the sixth day, the ovaries were removed, examined before a strong light for the purpose of determining the presence of, and relative numbers of follicles and corpora lutea, and weighed. The increase in the weight of the ovaries obtained with the hydroxide-gonadotrophic complex over that obtained with the extract alone was used as a measure of the amount of augmentation obtained.

Results and Discussion: The data presented in Tables I and II are a summary of the results obtained with aluminum, iron, zinc and magnesium hydroxides. The crude extract, 25B, obtained by the acetone precipitation method was tested using decreasing amounts of the extract with decreasing amounts of the aluminum hydroxide. As the data show there was a corresponding decrease in the activity under these conditions. When 100 mg† of the extract was given with 100 mg of aluminum hydroxide the ovaries obtained had an average weight of 354 mg. Intermediate values were obtained when 50 and 25 mg of the gonadotrophin and the hydroxide were given. The smallest amount of each of the substances, 12.5 mg, produced ovaries which had an average weight of 24 mg. It is of interest to note that 12.5 mg and 25 mg of the gonadotrophin alone did not stimulate the ovaries.

The other relatively crude extract that was used was 28B. Fifty milligrams of this extract was given with varying amounts of aluminum hydroxide and the data are

† Indicates in all cases mg equivalents of pituitary powder used as beginning material in making the various preparations.

TABLE II.
Augmentation of Pituitary Gonadotrophins by Insoluble Metallic Hydroxides.

Kind	Gonadotrophic preparation, amt, mg*	Augmenter, $Al(OH)_3$ mg	No. of rats	Wt of ovaries, avg, mg
Sheep pituitary—				
28B	50	—	9	51
"	—	100	3	15
"	50	6.25	3	100
"	50	12.50	3	113
"	50	25	6	177
"	50	50	9	187
"	50	100	3	195
F115	100	—	12	77
"	100	25	3	118
FSH333	1000	—	6	58
"	1000	25	3	84
Rat pituitary—				
RAP126	1	—	10	58
"	1	25	6	44
Sheep pituitary—		$Zn(OH)_2$		
28B	50	—	9	51
"	50	25	6	187
		$Mg(OH)_2$		
"	50	25	6	122
		$Fe(OH)_3$		
"	50	25	6	215

* Indicates mg equivalents of dry pituitary powder used in making the preparation.

recorded in Table II. These results show that 25 mg is almost as effective as 50 or 100 mg of aluminum hydroxide when given with 50 mg of a gonadotrophic preparation of this type.

The purified sheep pituitary gonadotrophic preparation (F115, Table II) when given in a dose of 100 mg produced ovaries that had an average weight of 77 mg and it was augmented but little by 25 mg of aluminum hydroxide as the ovaries obtained with this mixture had an average weight of 118 mg. Likewise, the follicle stimulating hormone preparation was not greatly augmented by aluminum hydroxide. The extract of dried rat pituitary glands was not augmented and the results suggest that there was inhibition.

The relatively crude sheep pituitary preparation 28B was effectively augmented by the hydroxides of magnesium, zinc, and iron; iron being somewhat more effective than magnesium or zinc.

The mechanism by which sheep pituitary gonadotrophin is made more effective by addition of insoluble hydroxides is not definitely known. However, a reasonable explanation can be based upon the well known fact that the hydroxides used in this work are known to adsorb most proteins from aqueous solution. In this connection we have evidence which shows that a suspension of aluminum hydroxide removes sheep pituitary gonadotrophin from solution. These facts make it seem probable that the mechanism consists of adsorption of the gonadotrophin from solution by the hydroxide and that on injecting the hydroxide-gonadotrophic complex into the rat, the gonadotrophin is released slowly but constantly from the hydroxide so that the ovary is stimulated continuously by the low concentration of the hormone in the blood. This explanation is the same as that given for the great augmenting effect produced by heme.[10,11] We believe that substances of this type simulate the way in which the pituitary gland constantly secretes small amounts of gonadotrophin into the blood.

The present methods of administering pituitary gonadotrophins result in ineffective utilization. That is, the maximum potential of an extract is not realized when injections are made at infrequent intervals because there is a relatively high concentration of the gonadotrophin in the blood for a short time, but dur-

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.

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