of lipopolysaccharide no inhibitory effect was seen.

It must be concluded from these observations that the inhibitory effect of nitrogen mustard upon the ability of rabbits to react to endotoxins with the peculiar syndrome of generalized Shwartzman reaction, cannot only be due to granulocytopenia. The extent of granulocytopenia 4 and 5 days after nitrogen mustard is the same as on day 3, whereas the incidence of the generalized Shwartzman reaction differs significantly. These findings in part correspond to the unimpaired ability of nitrogen mustard treated leukopenic rabbits to produce an equal fever response to typhoid vaccine as obtained more than a week earlier in the same rabbits(9).

In rabbits made granulocytopenic with nitrogen mustard no granulocyte replacement is necessary to make the animals ready to react with the generalized Shwartzman reaction to two appropriately spaced injections of enterobacterial lipopolysaccharide. Only if the preparative dose of lipopolysaccharide was given 3 days after nitrogen mustard is there some inhibition.

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Effect of C_{21}-Methyl Steroids on the *Musculus complexus* and Hatching of The Chick.* (32127)

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The *Musculus complexus,* termed "hatching muscle" by Fisher(1), has been implicated in the hatching process of birds by several writers. The muscle is paired, originates on cervical vertebrae 3 to 5 and on the fascia covering underlying cervical muscles, and inserts broadly on the parietal bones at the back of the skull. In the chick it increases rather abruptly in size and weight, beginning 3 or 4 days before hatching, then decreases again to a somewhat normal appearance in about the same number of days after hatching. The increase is attributed to an influx of fluid which is perhaps in part lymph, since the lateral portions of the muscle are enclosed by 2 large lymph glands. When it is infiltrated with fluid the *M. complexus* appears very non-muscular, being quite mucoid and yellowish in color, and it is apparently unable to contract appreciably. Smail(2) has suggested a mechanism whereby pipping (the initial breaking of the egg shell) is brought about when the enlarging muscle pushes the back of the head away from the shell of the egg, forcing the beak tip against the opposite side until the pressure there breaks the shell. No muscular movement is necessary, the work by the *M.*

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complexus being done hydrostatically. Pipping is prerequisite to successful completion of hatching, since it gives the chick a weakened portion of the shell from which to begin, and gives more room in which to work. Further breakage of the shell, to complete hatching, is accomplished by directed movements of the chick, employing muscles other than the *M. complexus*.

Hypophysectomy by partial decapitation of young embryos usually results in hatching failure(3), and treatment with thiourea delays or precludes development of the *M. complexus* and hatching(4), indicating that normal endocrine function is necessary. Since various steroids are known to influence water relationships in different tissues, and since hypophysectomy has a negative effect on hatching, it was reasoned that perhaps adrenal or gonadal steroids were involved directly in the fluid uptake by the *M. complexus* and thus in hatching. In this report evidence is presented that specific steroids can bring about earlier development of the *M. complexus* and can retard its posthatching decline as well.

Materials and methods. Six groups of 12 viable white leghorn eggs each were injected at day 18 of incubation with 100 μg of one of the 3 progesterones: progesterone, 16-hydroxyprogesterone, and 17-hydroxyprogesterone; or with 100 μg of testosterone; or with 10 μg of estradiol-17β; or with an ethanol-polyethylene glycol:0.9 saline (15:15:70) vehicle. A total of 0.2 ml of solution was injected into each egg at the small end, in or near the allantoic sac. Chicks hatched on days 20 and 21. A few did not hatch. Beginning on day 20 all chicks, hatched or unhatched, received daily intraperitoneal injections of the same dosages, except that the group receiving 10 μg of estradiol was now given a decreased dose of 1 μg/day. The unhatched chicks were injected in the abdomen through the egg shell. On the fifth day after the first chicks hatched all were sacrificed and weighed immediately. The entire *M. complexus* (a piece of shank muscle serving as an internal control) were dissected out and weighed as quickly as possible, then were dried at 55°C for 48 hours and weighed again.

Eggs in a second experiment were implanted in the small end at day 17 of incubation with pellets consisting of 10 mg of talc powder and 1 mg of a steroid(5). Five groups of 14 eggs each received either progesterone, 16-hydroxyprogesterone, testosterone, corticosterone, or talc alone. Chicks no. 6 and 7 in hatching order from each group (except the group receiving corticosterone, where too few hatched) were sacrificed soon after hatching and were handled as before to determine the condition of the *M. complexus* at hatching. No later than 6 hours after hatching each remaining chick was implanted subcutaneously between the scapulae with a pellet containing 5 mg of steroid and 15 mg of talc, or with 20 mg of talc alone in the controls. The chicks were sacrificed 3 days later. The pellets were removed, dried, and weighed to determine roughly the amount of steroid taken up by each chick. Pellets in the eggs had been too disrupted by the movement of the chicks to be recovered.

Results. In the first experiment (Table I) the difference between the per cent water in the *M. complexus* and that in the shank muscle (last column) is considered to be the value of major interest. With respect to these values, the 3 progesterones retarded the posthatching loss of fluid from the *M. complexus,* while estradiol and testosterone did not affect fluid loss. Chicks receiving the progesterones hatched earlier, whereas chicks receiving estradiol and testosterone hatched later than the controls. Since the difference in age at the time of sacrifice was as much as plus or minus one day from the controls, the values of fluid retention in chicks treated with the progesterones were probably actually greater than indicated in Table I, while the values of fluid retention for chicks injected with estradiol or testosterone were probably greater than they would have been had these birds hatched at the same time as the controls.

The second experiment was conducted to corroborate further the results of the first experiment and to test whether the major adrenocortical product of adult birds,

corticosterone, had any effect on fluid uptake and retention. The results for the 2 chicks from each group sacrificed at hatching showed that the *M. complexus* in the progesterone and 16-hydroxyprogesterone treated chicks was very large in comparison to that in the testosterone treated and control birds (Table II). The difference in size was evident from the gross appearance of the muscles as well as from the weight measurements. The smaller differences in the per cent water in the *M. complexus* between groups is due to the fact that apparently a considerable portion of the weight gain in the muscles included lipid and other dissolved lymph solids. These, of course, were not removed by desiccation of the tissue at 55°C. It is recognized that percentage of water is not a sufficiently good measure of the increase in tissue mass and that further studies should include quantitative measurements of other cell components. The comparisons of fresh and dry weights and of percentage of water in the *M. complexus* and shank muscle demonstrate that differences do exist, however, and therefore are of value in these first determinations of steroid effects.

TABLE I. Data from Steroid Injected Chicks. (MC = *musculus complexus*, SM = shank muscle.) Means ± SE. Dosages: all except estradiol, 100 μg steroid/day; estradiol, initial injection 10μg, thereafter 1 μg/day.

Treatment	n	Fresh body wt (g)	MC fresh wt (mg)	MC % of body wt	MC dry wt (mg)	MC water %	SM water %	MC-SM △water %
16-Hydroxy-progesterone	10	46.0 ± 1.0	137 ± 7.6	.30 ± .02	27 ± 1.2	80.0 ± 2.2	76.6 ± 2.3	3.4 ± .4*
17-Hydroxy-progesterone	10	48.7 ± 1.5	140 ± 4.8	.29 ± .01	29 ± 0.7	79.4 ± 1.4	76.7 ± 0.6	2.7 ± .4
Progesterone	10	46.3 ± 1.3	143 ± 8.0	.31 ± .02	29 ± 1.4	79.7 ± 1.3	76.7 ± 0.7	3.0 ± .5
Testosterone	8	46.5 ± 1.7	132 ± 5.4	.28 ± .00	28 ± 1.3	79.0 ± 1.0	77.3 ± 0.8	1.7 ± .3
Estradiol	11	47.8 ± 0.9	134 ± 6.1	.28 ± .01	28 ± 1.3	79.3 ± 1.3	77.4 ± 0.7	1.9 ± .5
Control	9	48.5 ± 1.6	120 ± 7.1	.26 ± .01	25 ± 1.4	78.9 ± 0.8	77.3 ± 0.8	1.6 ± .3

* Underlined values indicate significant difference from control ($P < .05$) using a standard F test.

The pellets of progesterone, 16-hydroxyprogesterone, testosterone, and corticosterone which were implanted for 3 days between the scapulae of the hatched chicks gave inconclusive results due to different amounts of steroid being absorbed. Except for corticosterone very little absorption of steroid occurred, and there was no appreciable effect on the *M. complexus* or shank muscle. Corticosterone was absorbed in appreciable amounts. However, a general toxic effect with corticosterone pellets was produced, since the body weights were low and the shank muscle as well as the *M. complexus* percentages of water were altered. Corticosterone, in addition, delayed hatching the eggs and lowered the percentage hatching to 50% of controls.

The order and percentage hatching of eggs in this experiment indicated also that 16-hydroxyprogesterone and progesterone were effective. No explanation can be advanced for the fact that the steroids were absorbed more readily from the egg than the chick implants. The percentage hatched and an index of the time of hatching in each group is given in Table III. The index was calculated by dividing the total 2-day hatching period into 5 equal periods of time, giving each period a numerical value of 1 (first period), 2, 3, 4, or 5 (last period). The percentage of chicks hatching, for example in the progesterone treated group, in

TABLE II. Data from Newly Hatched Chicks After Steroid Pellets (1 mg Steroid/Pellet) Were Implanted in Eggs. (MC = *Musculus complexus*, SM = shank muscle.) Means of 2 birds.

Treatment	Fresh body wt (g)	MC fresh wt (mg)	MC % body wt	MC dry wt (mg)	MC water %	SM water %	MC-SM water %
16-Hydroxy-progesterone	41.0	714	1.74	76	89.3	82.2	7.2
Progesterone	39.6	717	1.81	72	90.0	83.0	7.0
Testosterone	40.0	490	1.22	62	87.4	81.7	5.7
Control	38.0	418	1.09	59	85.2	82.8	2.4

each particular period was multiplied by the numerical value assigned to that period. These figures were summed for the 5 periods and then were divided by the total number of eggs hatched in that group to give the index value. The earliest hatching groups thus have the lowest index. These values indicate that 16-hydroxyprogesterone and progesterone, in that order, both accelerated hatching and increased hatchability, that testosterone delayed hatching, and that corticosterone delayed hatching and decreased hatchability quite markedly.

Discussion. On the basis of steroid administration it is concluded that the progesterones have a positive effect on the infiltration of fluid into the *M. complexus* of the chick, and thus an effect on hatching. A number of other derivatives related to C_{21}-methyl compounds have yet to be tested for activity in this system. The C_{21}-hydroxylated adrenal steroids are not active on the *M. complexus*. On the basis of the data in Tables II and III, and considering the fact that corticosterone can be inhibiting the process, the major products of adrenal secretion at the time of hatching may be progesterone or related hydroxylated derivatives.

TABLE III. Percent Hatching and Index of Time of Hatching for Eggs Implanted with Steroid Pellets (1 mg Steroid/Pellet). Fourteen eggs per treatment group.

Pellet treatment	% hatching	Index* of hatching time
16-Hydroxy-progesterone	93	1.2
Progesterone	93	1.5
Control (talc)	86	2.0
Testosterone	79	3.1
Corticosterone	43	5.3

* See text for explanation of Index.

The inhibitory action of administered corticosterone on the *M. complexus* and on hatching could be explained by decreased ACTH secretion, which in turn would result in decreased amounts of C_{21}-methyl steroids necessary for the increase in size of the *M. complexus*.

In recent years there has been a major effort in endocrine research to gain information about the mechanism of action of the hormones including estrogens, androgens, and the adrenocortical steroids. Of the different types of steroid hormones, the least success has been achieved for the substance progesterone and closely related steroid derivatives (6,7). The absence of comparable basic studies on progesterone action is no doubt due to the inherent difficulties in investigating target tissues such as the uterus or mammary gland, which are drastically altered by the prior stimulation of estrogen. A study of the mode of action of a single hormone is difficult enough without the added complication caused by the interactions of two. In the growth of the *M. complexus* of the chick during the hatching process, we have a tissue which can respond directly to progesterone of C_{21}-methyl steroids without the apparent necessity for a priming action of estrogen. Although such an action prior to hatching could not be disproven here, it was shown that estrogen administration was without effect on the size of increase of the muscle, at least during the time of hatching. The *M. complexus* is a unique and convenient organ for study of hormonal regulation because of its pronounced response to specific steroid compounds of the progesterone type, because of the relative assessibility of this tissue, and because of its ease of handling.

The fact that 16-hydroxyprogesterone and

17-hydroxyprogesterone have an activity similar to progesterone in the *M. complexus* and in hatching is of considerable interest since progestational activity of the progesterone molecule in mammalian tissues is essentially lost and no new major biological activity has been demonstrated for these hydroxylated products. It is possible that the progesterone administered here was converted to a hydroxylated metabolite which then had an effect on the *M. complexus*. The 17-hydroxyl position plays a key role in the biogenetic pathway leading to the formation of a number of steroid hormones, but no such intermediate role or specific biological action has been ascribed to the 16-hydroxyprogesterone compound. The 16-hydroxylated steroids have been reported(8) in a number of animal species. Of particular interest is the increased activity of 16-hydroxylation in fetal adrenal(9) and in human neonatal tissues(10,11,12).

While it has not been possible on the basis of these preliminary experiments to obtain direct evidence for a specific biological role for 16-hydroxyl or 17-hydroxyl derivatives of C_{21}-methyl steroids in the hatching of eggs, it is perhaps not too speculative to consider that one of the well known hydroxylated forms of progesterone can serve as a specific hatching hormone or birth hormone. This would be in keeping with our present concepts of a common steroid biogenetic pathway and with the demonstrated changes that occur in steroid metabolism and in the fetal and neonatal tissues.

Summary. The unique uptake of fluid into the *Musculus complexus* ("hatching muscle") of the chick beginning 3 or 4 days prior to hatching, apparently a necessity for successful hatching, was influenced positively by progesterone, 16-hydroxyprogesterone, and 17-hydroxyprogesterone; but negatively or not at all by cortisone, estradiol-17β, and testosterone. The possibility of the formation of a specific hatching hormone by hydroxylation of progesterone is suggested.

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