later passage virus used in each of the neutralization tests were closely similar.

Summary. Cross-neutralization experiments *in ovo* with 1st and later chick embryo passage strains of freshly recovered influenza A viruses showed no definite difference in their antigenic composition. However, the reactivity relative to guinea pig and chicken erythrocytes underwent a rapid and profound change between the 1st and 2nd embryo passages.

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Effect of Relaxin Upon Decidual Reactions in the Rat.* (19789)

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The most important physiological actions known for relaxin are displayed in effects upon connective tissue(1-5). The range of such effects has been broadened by recent observations(6) that relaxin preparations possess the property of inhibiting the extensive modification of stromal connective tissue which accompanies the development of a deciduoma in the rat uterus. Quantitative comparison of responses to a series of relaxin preparations of widely different specific activities, experiments with relaxin from the blood of pregnant rabbits, and studies of the effects of chemical modification of relaxin all indicate that inhibition of decidual growth in rats is a characteristic property of this hormone. The data also show that some relaxin preparations contain a substance, clearly distinguishable from relaxin (and from the decidual inhibitor), which enhances the action of progesterone in the development of deciduomata.

Experimental. Quantitative study of factors affecting decidual growth requires a rigidly standardized means of producing deciduomata of uniform size. We have routinely employed 100-day-old estrous rats, averaging 200 g in weight, which are made pseudopregnant by electrical stimulation of the uterine cervix. On the fifth day of pseudopregnancy, they are ovariectomized, and the right horn of the uterus traumatized by a needle scratch along its entire length. Daily injections of progesterone (1.5 mg in 0.1 ml sesame oil) are given for 3 days, and the animals autopsied on the fourth.

In some of the earlier experiments, gross comparison of the 2 horns served as a qualitative indication of the response. Subsequently, in order to obtain data which would be susceptible of quantitative comparison, the 2

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horns were separated at the cervix and weighed separately. It has been found that the increase in weight of the traumatized horn serves as a measure of the extent of reaction. In order to minimize the effects of individual variation, the data are expressed in terms of the percentage increase relative to the weight of the untraumatized horn.

Solutions of substances to be tested for inhibition of decidual development were prepared in physiological saline, the concentration being adjusted so that the total desired daily dose was contained in 1.5 ml. One-half ml was injected 3 times daily for 3 days following uterine traumatization.

Relaxin preparations. Eight different preparations of relaxin from sows' ovaries and one concentrate of pregnant rabbit serum were employed during the course of this investigation. Of the former, one was isolated from ovaries of non-pregnant animals (preparation 70-1), and another (67-1) from unselected ovaries, of which it was estimated that about 20% were from pregnant animals. For the others, ovaries of pregnant animals were used. The method of preparation was that described previously(7), with slight modifications in some instances. Preparations 66-4 and 66-5, which assayed 450 and 300 GPU/mg, respectively, were obtained by acetone refractionation of material of lower specific activity.

A sample of 'Releasin'‡ was also used. The absolute specific activity of this preparation was not known; assays in our own laboratory showed that the solution contained about 250 GPU/ml. Pregnant rabbit serum (PRS) was concentrated by the procedure of Albert and Money(8). The lyophilized product had an activity of 4 GPU/mg. To serve as an appropriate control, a sample of male rabbit serum (MRS) was concentrated similarly.

Results. Preliminary experiments indicated that the daily dose of relaxin required for qualitatively complete inhibition lay in the range 100-300 GPU. More complete and quantitative data (Table I) indicate that this preparation (59-7) has 2 different effects upon the development of deciduomata. At fairly high levels (1.0-1.6 mg/da) the effect is clearly inhibitory; lower doses, on the other hand, appear to enhance the action of progesterone.

TABLE I. Effect of Preparation 59-7 (90 GPU/mg) upon Decidual Development.

No. of animals	59-7 injected mg/day	59-7 injected GPU/day	% increase*
5‡	.0	0	4 ± 16†
13	.0	0	345 ± 65
3	.17	15	715 ± 185
6	.33	30	800 ± 92
3	.51	45	585 ± 246
3	.66	60	440 ± 110
3	1	90	110 ± 38
3	1.33	120	36 ± 14
3	1.66	150	63 ± 38

* $\frac{[(\text{Wt traum. horn}) - (\text{Wt control horn})]}{(\text{Wt of control horn})} \times 100$

† Avg ± stand error.

‡ These animals received no progesterone.

Data relating to the effects of a series of relaxin preparations (Table II) show that the inhibition produced by ovarian extracts is proportional to the relaxin content, rather than to the amount of material injected, over a 100-fold range of specific activity. Of particular interest are the data for preparations obtained from ovaries of non-pregnant animals (70-1) and from unselected ovaries (67-1). Only small amounts of relaxin have been obtained from the ovaries of non-pregnant sows; material from this source should thus serve effectively as a control for comparison with similar extracts from the ovaries of pregnant animals. Both of the above preparations appear to enhance the action of progesterone when tested at 2 mg/day, whereas an extract from glands selected from pregnant sows (66-6) shows good inhibition at half this level. Although preparation 70-1 at 5 mg/day affords more pronounced inhibition than would be expected on the basis of its relaxin content (when compared with other, more active relaxin preparations), the more pronounced inhibition obtained with 67-1 is correlated with its greater relaxin content. A possible explanation of the result with 5 mg 70-1 will be advanced subsequently.

It was found that a preparation of pregnant rabbit serum (PRS) displayed qualitatively the same features with respect to decidual in-

‡ Supplied through the courtesy of Dr. Robert L. Kroc, Chilcott Laboratories, Morris Plains, N. J.

TABLE II. Effects of Ovarian Extracts upon Decidual Development.

Preparation No.	Specific activity (GPU/mg)	Amt injected mg/day	Amt injected GPU/day	No. of animals	Avg % increase
70-1	5	2	10	3	505 ± 110
		5	25	4	210 ± 100
67-1	20	2	40	3	470 ± 190
		5	100	6	75 ± 85
62-2	70	1	70	5	103 ± 40
		1.55	110	6	67 ± 55
		2.1	150	5	18 ± 17
59-7	90	.33	30	6	800 ± 92
		1	90	3	110 ± 38
		1.66	150	3	63 ± 38
66-6	100	1	100	6	70 ± 18
66-4	450	.375	170	4	4 ± 18
Releasin*	?	—	100	3	34 ± 29

* Chilcott Laboratories, No. CH-31-95-SF.

TABLE III. Effects of PRS and MRS upon Decidual Development.

Preparation	Amt injected mg/day	Amt injected GPU/day	No. of animals	Avg % increase
PRS	7.5	30	3	465±210
	15	60	3	425± 51
	25	100	3	380±110
	37.5	150	3	188± 77
	50	200	4	64± 21
	75	300	3	156±145
MRS	52	0	4	306± 29

hibition as relaxin from sow ovaries (Table III). Both produce potentiation at low and inhibition at high dosage. Quantitatively, however, significant differences are noted. Considerably less potentiation is observed with PRS, and, within the inhibitory range, ovarian extracts possess approximately twice the inhibitory activity of the same amount (in GPU) of PRS. That the inhibition by PRS is quite likely due to its relaxin content is indicated by the failure to elicit a response with a similar preparation from the serum of male rabbits (MRS).

Since small amounts of estrogen will effectively inhibit decidual formation under the conditions of these experiments(9), it was necessary to eliminate the possibility that contamination with estrogen was responsible for the effects observed. Four of the crudest relaxin samples (including PRS) were assayed for estrogen content. At levels which produced good inhibition of decidual growth, no significant effects were observed upon either the weight or water content of the uteri of ovariectomized rats.

Investigation of the chemical properties of the decidual inhibitor indicate that they are similar to those which have been established for relaxin. Among the characteristic chemical properties of relaxin is the relative stability of its solutions toward heat. Thus, neutral solutions of relaxin may be exposed to 100° for 10-15 minutes without appreciable loss of activity(10). Comparison of the effects of heated§ and unheated relaxin upon decidual reactions (Table IV) shows clearly that the decidual inhibitor shares with relaxin the property of heat stability. Quantitatively, the heated preparation appears to be approximately 5 times as effective as the native material. This may be most readily explained by assuming that the substance in relaxin preparations which enhances the action of progesterone is heat labile; its destruction permits the inhibition to become apparent at lower concentrations, the effects of which are masked in unheated samples.

It should be noted that heated samples of PRS are also inhibitory at lower concentrations, and that the quantitative discrepancy between the effects of relaxin and PRS (Tables I and III) disappears when the effects of heated samples are compared (Fig. 1).

Relaxin has been shown to be sensitive to the action of a variety of reducing agents(11)

§ A heating period of 10 minutes was chosen as optimal on the basis of preliminary experiments.

TABLE IV. Effects of Native and Heated Relaxin upon Decidual Development.

Preparation	Amt injected mg/day	Amt injected GPU/day	No. of animals	Avg % increase
66-6, unheated	0	0	13	345 ± 65
	.20	20	5	485 ± 53
	.30	30	5	345 ± 210
	.40	40	5	420 ± 120
	.60	60	5	230 ± 73
	.80	80	5	95 ± 82
66-6, heated 10 min., 100°	.05	5	5	300 ± 150
	.10	10	5	225 ± 89
	.20	20	5	90 ± 62
	.40	40	6	42 ± 44
	.60	60	8	60 ± 50

and to prolonged exposure to acidified methanol, while neither deamination nor acetylation under relatively mild conditions results in appreciable loss of activity(10). Samples of relaxin were treated with sodium thioglycollate, anhydrous methanol containing 0.1 N HCl, nitrous acid, and acetic anhydride, and the reaction products tested for decidual-inhibiting activity (Table V).

No trace of either potentiation or inhibition survives treatment with thioglycollate. The effect of nitrous acid, however, appears to be qualitatively and quantitatively similar to that of heating, *i.e.*, the inhibitory property is enhanced rather than diminished by deamination. The fact that less inhibition is observed at the one milligram level is probably of no especial significance, being most likely due to experimental variation. The effect of treatment with acetic anhydride is also qualitatively analogous to that of heating, but quantitatively the analogy is less satisfactory. It is clear that the inhibition displayed by 1 mg of relaxin 66-6 daily is unaffected by this reagent. The average increase in decidual horn weight found with acetylated material at 20 GPU/da., although considerably less than that found for the unreacted sample, is greater

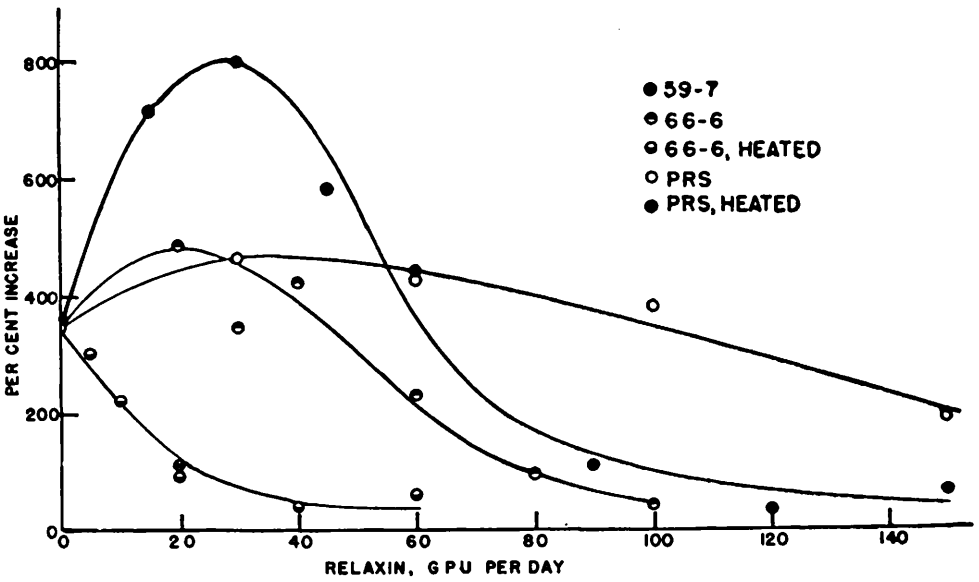

FIG. 1. Summary of effects of 2 unheated relaxin preparations (59-7 and 66-6), a sample of 66-6 which had been heated 10 min. at 100°, and PRS upon decidual development. One experiment with PRS heated 15 min. at 100° is included. Ordinate: % increase in wt of traumatized horn.

TABLE V. Effects of Chemically Modified Relaxin upon Decidual Development.

Preparation	Amt injected mg/day	Amt injected GPU/day*	No. of animals	Avg % increase
66-6	.20	20	5	485 ± 53
	1	100	6	70 ± 18
66-6, reduced†	7.50	750	2	325
66-6, deaminated‡	.20	20	5	58 ± 35
	1	100	5	95 ± 55
66-6, acetylated§	.20	20	5	203 ± 126
	1	100	5	46 ± 14
66-6, methylated‖	1	100	5	240 ± 96

* Calculated for the corresponding wt of unreacted material. † Treated with sodium thioglycollate, pH 7.1, for 15 hr. Reduction product isolated by 3 pptns. with acetone at pH 3. ‡ .1 M $NaNO_2$, pH 4, 0°, 30 min. § With 150-fold excess Ac_2O in NaOAc at 0°. ‖ Treated with MeOH containing .1 M HCl for 22 hr at 22°.

than that found with either heated or deaminated material at this level. It should be noted, however, that of the 5 animals used for this experiment, 2 displayed very considerable inhibition. The effects of acetic anhydride are assumed to be confined to destruction of the physiological activity of the potentiator. The variability observed may be due either to incomplete reaction with essential amino groups of the latter, or to some *in vivo* reactivation.

Methylation of relaxin results in 90% or greater inactivation as tested in the guinea pig. If it is assumed that the potentiating factor is completely destroyed by this reagent, retention of a small amount (10%) of decidual inhibiting activity would account for the results observed. It is also possible that the rat and the guinea pig differ somewhat in their ability to reactivate the methyl ester. An unequivocal choice between these 2 alternatives cannot be made on the basis of the available data.

Discussion. There can be no doubt that the phenomenon of decidual potentiation, especially prominent with small amounts of preparation 59-7, but observable in greater or lesser degree with most of the relaxin samples studied, is due to a substance other than relaxin and present as a contaminant. It may be distinguished from relaxin (and from the decidual inhibitor) by its sensitivity to heat, and by the fact that it contains essential amino groups, the loss of which, by either deamination or acetylation, reduces the potentiation observed. It is therefore to be expected

TABLE VI. Effect of Relaxin Preparation 66-5 (300 GPU/mg) upon Decidual Development.

Amt injected mg/day	Amt injected GPU/day	Period of heating	No. of animals	Avg % increase
.066	20	None	3	44 ± 30
.066	20	10 min.	3	18 ± 18
.330	100	None	2	31

that the magnitude of decidual potentiation would vary considerably from one relaxin preparation to another, as may indeed be observed to be the case (compare Tables I and IV). It was also to be anticipated that further purification of relaxin should lead to preparations essentially free of potentiator; these should be inhibitory at relatively low concentrations in the native state, and the effect of heating should be much less pronounced. We have been able to test this hypothesis with preparation 66-5, which assayed 300 GPU/mg (Table VI). From its occurrence in preparations which had been dialyzed during purification, and the properties described above, it may tentatively be concluded that the potentiator is a protein.

While absolute identification of relaxin with the substance responsible for decidual inhibition in the rat must await the isolation of one or the other in indisputably pure form, all of the data so far obtained support the assumption that the two are the same. The close parallel between relaxin content and inhibitory activity of preparations from sows' ovaries which differ widely in specific activity affords good evidence for their identity, as does the comparison of the effects of PRS and ovarian relaxin. As has already been mentioned, the

quantitative discrepancies between PRS and other relaxin preparations appear to be due to contamination by the potentiating factor. The presence of the latter in relatively small amounts in the ovaries of non-pregnant sows may possibly account for the unexpected activity of preparation 70-1 (Table II), since, when heated 10 minutes and tested at 2.0 mg/da., this preparation was only slightly inhibitory. Equally consistent with this assumption are the results of experiments with chemically modified relaxin.

Interaction between progesterone and relaxin shows marked species variation. Thus, while progesterone stimulates the formation of relaxin in the reproductive tract of the guinea pig(12), it (indirectly) prevents the action of relaxin upon the mouse public symphysis(13). Large amounts of relaxin are only partially effective as an inhibitor of decidual formation in mice(14), and the largest amounts tested (1000 GPU/day) were totally without effect upon decidual reactions in guinea pigs(15). In contrast to the situation in guinea pigs and mice, there is no evidence that relaxin, or an analogous substance, is involved in the adaptive mechanism of gestation in the rat; indeed, the administration of relaxin during pregnancy results in death and resorption of the fetuses.‖

The wide variations in the response of different species to relaxin serves to emphasize their common denominator. Relaxation of the pubic symphysis, deciduoma formation, and stromal changes in the mammary gland of the rat during lobulation, are all processes in which connective tissue changes predominate. It is possible, therefore, that the physiological process in which relaxin takes part may be a basic one common to certain kinds of connective tissue.

Summary. Preparations of relaxin from the ovaries of pregnant sows and from the blood of pregnant rabbits inhibit the development of a decidual reaction in the uterus of the castrate-pseudopregnant rat following traumatization and treatment with progesterone. Partially purified relaxin samples also contain a heat-labile factor which synergizes with progesterone in the production of deciduomata. On the basis of experiments with relaxin preparations covering a wide range of specific activities, and the effects of a variety of chemical reagents, it is concluded that the inhibitory property of such preparations is a specific response to relaxin.

‖ The administration of 300 GPU of relaxin on each of the first 10 days following mating resulted in an average litter size of 3.4 (1.8 live young) as compared to the normal litter size of 10.2, in 5 animals.

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